THE ANALYSIS OF SOME SOUTH AFRICAN ESSENTIAL OILS BY COMPREHENSIVE TWO-DIMENSIONAL GAS CHROMATOGRAPHY (GC×GC)

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The analysis of some South African essential oils by comprehensive two-dimensional gas chromatography (GC×GC)

by

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Summary

By virtue of their fragrance and therapeutic nature, essential oils have gained great application in the cosmetic and pharmaceutical industries. These oils, as products consumed by the public for the general health and cosmetics, should be of high quality and unadulterated.

In this project comprehensive two-dimensional gas chromatography (GC×GC) was used for the qualitative analysis of some South African essential oils. The main purpose of the project was to evaluate GC×GC for identification of essential oil constituents and for fingerprinting the different essential oils for quality control purposes. Essential oils of the same kind but of different origin were compared and the quantitative variation between their components was studied.

Gas chromatography–time-of-flight mass spectrometry (GC-TOFMS) as well as comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry (GC×GC-TOFMS) was used to help identify component peaks separated in the GC×GC instrument.

Once certain precautions are taken and the delicate modulator is correctly optimised, the GC×GC results are reproducible and easy to interpret. Overall, the GC×GC technique utilised showed good promise for quality control of essential oils.

Opsomming

Die kosmetiese en farmaseutiese industrieë is baie afhanklik van essensiële olies vanweë hulle reuk en terapeutiese eienskappe. Synde produkte wat deur die publiek gekoop word om skoonheids- en algemene gesondheidsredes, moet hierdie olies eg (onvervals) en van 'n hoë gehalte wees.

In hierdie projek is omvattend-tweedimensionele gaschromatografie (GC×GC) gebruik vir die kwalitatiewe analise van sommige Suid-Afrikaanse essensiële olies. Die hoofdoel van die projek was om die tegniek GC×GC te evalueer vir die identifikasie van essensiële-olie-komponente en vir die vingerafdruk-kartering van verskillende essensiële olies met kwaliteitskontrole as oogmerk. Essensiële olies van dieselfde spesie maar van verskillende oorsprong is vergelyk en die kwantitatiewe variasie tussen hul komponente is bestudeer.

Gaschromatografie-vlugtydmassaspektrometrie (GC-TOFMS) sowel as GC×GC -TOFMS is gebruik vir die eenduidige identifikasie van komponent-pieke wat deur die GC×GC instrument geskei is.

Indien sekere voorsorgmaatreëls getref word en die delikate modulator korrek geoptimiseer word, is die GC×GC resultate herhaalbaar en maklik om te interpreteer. Die GC×GC tegniek hou groot belofte in vir die kwaliteitskontrole van essensiële olies.

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Abbreviations

2D	Two-dimensional
CGC	Capillary gas chromatography
cm	Centimetre
D1	First dimension
D2	Second dimension
d_f	Stationary phase film thickness
FID	Flame ionisation detector
GC	Gas chromatography
GC-GC	Heart-cutting gas chromatography
GC×GC	Comprehensive two-dimensional gas chromatography
GC×GC-TOFMS	Comprehensive gas chromatography-time-of-flight mass spectrometry
GC-MS	Gas chromatography-mass spectrometry
GC-TOFMS	Gas chromatography-time-of-flight mass spectrometry
HMW	High molecular weight
I.D.	Internal diameter
kPa	Kilo-Pascal
L	Column length
LC-GC	Liquid chromatography-gas chromatography
LMCS	Longitudinally Modulated Cryogenic System
m	Metre; number of components in a sample
µm	Micro-metre
min	Minute
ms or msec	Milli-seconds
N	Theoretical plate number
n_c	Peak capacity
p	Number of single component peaks in a chromatogram
PPG	Polypropylene glycol
PEG	Polyethylene glycol
R	Resolution
%RSD	Percentage relative standard deviation
RTL	Retention time locking
s	Number of visible component peaks in a chromatogram

sec	Second
SFC	Supercritical fluid chromatography
SFC-GC	Heart-cutting supercritical fluid chromatography-gas chromatography
SFC×GC	Comprehensive supercritical fluid chromatography-gas chromatography
SFE	Supercritical fluid extraction
STD or σ	Standard deviation
TLC	Thin layer chromatography
TDM	On-column thermal desorption modulator
t_m	Dead time
t_R	Retention time
u	Peak widths at base
w_b	Peak width at half height
w_h	Average linear gas velocity

1 INTRODUCTION

1.1 Background

Humans are and have always been at the quest of conquering nature and its harvest since the beginning of time. Starting from searching and fighting for the basic means of survival they have managed in manipulating and shaping the forces and bounties of nature to their ways and desires. With the advent of ancient civilisation, merchants and explorers have helped in transferring cultural, religious and other practices between distant civilisations. Trade between ancient civilisations flourished and surplus goods were exchanged. Among the goods traded were fragrant plants (*e.g.* Frankincense and myrrh) and spices that could be considered as old as the practice itself [1].

Herbal plants and their essential oils have been used for various purposes since antiquity. The ancient civilisations of Egypt, Rome, Greece, China, and others were known for their use of essential oils as herbal medicines, perfumes, for massaging, as additions to bathing water, and essences were used as offerings to gods and goddesses (*e.g.* burning of Frankincense in temples) [2].

In modern times, essential oils have gained numerous commercial and industrial applications. They are extensively used as ingredients in the perfumery, pharmaceutical and flavour industries. The world trade and consumption in essential oils involves huge amounts of essential oils and money. For example, in 1994 alone, US$ 9 billion worth of flavour and fragrance materials of natural origin were consumed worldwide [3]. Their pleasant fragrance and therapeutic nature have also gained essential oils a wide application in the field of aromatherapy, such as their use as massage oils to relieve different ailments and anxieties or merely as beauty accessories (skin toners). Consequently, many essential oil studies are undertaken which include plant cultivation to increase oil yield, improving oil extraction

methods, and the analyses of essential oils for component identification, pattern recognition and quality control purposes.

Essential oils are obtained from different parts of plants, most commonly from leaves, stems and flowers. The oils are very expensive to produce, some more so than others, due to the high labour and capital requirements during cultivation and extraction of the oils and due to the large amount of plant material required to produce sufficient amounts of oil. For example, about 8 million Jasmine flowers are required to produce only 1 kg of Jasmine essential oil [4].

Essential oils consist of a wide variety of compounds including terpene hydrocarbons, alcohols, esters, ketones, aldehydes and acids. The analysis of such complex mixtures calls for the use of various advanced analytical techniques. As early as 1956, gas chromatography has been used for the separation of essential oil components. This technique is able to provide information describing the qualitative and quantitative composition of the volatile and semi-volatile organic components of essential oils. Liberti and Conti were the first to present their work on the separation of monoterpenes in lemon and bergamot oils by using this technique. Later on, from about 1963, gas chromatography-mass spectrometry (GC-MS) came to be widely used as a new separation and identification technique in essential oil analysis. Formacek and Kubeczka also used ^{13}C-NMR in addition to capillary gas chromatography (CGC) to characterise different essential oils of commercial interest [5].

With the development of more analytical techniques, the techniques used to analyse essential oils have diversified over the years. From the coupled chromatographic techniques, in addition to the more dominant gas chromatography-mass spectrometry (GC-MS), supercritical fluid chromatography coupled to capillary gas chromatography has been used for the analysis of essential oils [6]. More recently, comprehensive two-dimensional gas chromatography (GC×GC) has been utilised as an effective tool for the analysis of essential oils [7, 8, 9, 10].

1.2 Approach

This work focuses on the analysis of four South African essential oils by comprehensive two-dimensional gas chromatography. The essential oils analysed are lemongrass, *Artemisia afra, Tagetes minuta*, and Bourbon Geranium. The essential oil samples were obtained from different South African commercial farmers through the Department of Agriculture, Conservation and Environment, Lowveld College of Agriculture, Nelspruit. The main aim of this project was to evaluate comprehensive two-dimensional gas chromatography (GC×GC) as a technique for the analysis of essential oils, with special emphasis on the quality control of some locally produced oils. Cost, reliability and ease of data interpretation were among the evaluation criteria.

1.3 Arrangement and Presentation

This dissertation is divided into six chapters. Chapter 1 gives a general introduction. In Chapter 2 a short overview of the origin, definition, use and methods of preparation of essential oils is given. The selected four plants and their essential oils are also separately described in this chapter. Chapter 3 discusses the principles and advantages of multidimensional chromatography and the analysis of essential oils using coupled gas chromatographic techniques. Instrumentation in comprehensive two-dimensional gas chromatography and the optimisation of experimental parameters is dealt with in Chapter 4. Chapter 5 gives the details of the experimental results and the discussions. Concluding remarks are given in the last chapter, Chapter 6. More complete sets of data and GC×GC chromatograms are presented in the appendix.

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2 THE ORIGIN, DESCRIPTION, USE, AND PREPARATION OF ESSENTIAL OILS

2.1 What are Essential Oils?

Essential oils are highly concentrated volatile substances extracted from various segments (flowers, roots, leaves, barks, stems, etc) of herbal plants, trees and grasses. The essential oils are accumulated in the oil glands of specialised plant tissues. It is believed that plants utilise essential oils to attract pollinating insects, to repel predators and to protect themselves from diseases. Although almost all plants have odour, not all plants produce commercially viable volatile oils [1]. The quality and quantity of essential oil a plant produces is affected by many factors including the climate, altitude, the fertility, the type of soil where the plants are cultivated and the maturity of the plants. Moreover, the time of the day and season of the year the plants are harvested affects the quality and abundance of the essential oil, which moves around the plant according to both a seasonal and a daily cycle. Individual plant species have their own characteristic rhythm. The quality and availability of an essential oil may also vary from year to year. The other factors that may affect essential oil chemistry are the way the oils are extracted from the plant materials [1, 2, 3, 4].

Though referred to as oils, essential oils are different from cooking or vegetable oils mainly due to their fragrance and volatile nature. Their colour differs from source to source. Their physical state ranges from light liquid having the consistency of water or alcohol (lemongrass, lavender, peppermint, rosemary) to viscous, thick and sticky (myrrh, vetiver) to semi-solid (rose otto) at room temperature. They have a complex chemistry consisting of terpene hydrocarbons, alcohols, aldehydes, acids and esters as major or minor constituents. Due to this complexity, it is impossible to exactly replicate an essential oil in the laboratory [1].

The use of essential oils ranges from the perfume to the pharmaceutical industries. In the field of aromatheraphy[1], they are used as antiseptic (limonene), antiviral (pinene), anti-inflammatory and antibacterial (chamazulene and farnesol from chamomile essence) and anti-rheumatic agents. Esters found in essential oils (linalyl acetate, geranyl acetate) are known for their fungicidal and sedative properties. Although it is true that some ketones like thujone and pulegone are known for their toxicity, there are non-toxic ketones obtained from essential oils with medicinal value. Ketones like jasmone (from jasmine) and fenchone (from sweet fennel) ease congestion and aid the flow of mucus. Alcohols like linalool and geraniol have good antiseptic and antiviral properties [1].

2.2 History of Essential Oils and Aromatherapy

Aromatherapy (aroma = smell, fragrance; therapy = treatment) is an old 'science' dating back as far as the ancient Egyptian civilisation where evidence of the use of herbal medicine to cure illnesses was found in medical papyri (from ~1555 B.C.). Burning of Frankincense was probably one of the earliest ways of using fragrant plants, which was used as an offering to ancient gods and goddesses. The Egyptians also used aromatic herbs for embalming to help preserve the flesh [5].

The Greeks and the Romans also used aromatic herbs for medicinal and cosmetic purposes. The herbal book of the Greek physician, Pedacius Dioacorides, had been used as the Western world's standard medical reference for a very long time. Many of the herbs mentioned in his book are still being used today in aromatherapy [5].

It is believed that the knowledge of fragrant oils and perfumes spread to the Far East and Arabia during the crusades. An Arabian physician called Avicenna or Ibn Sina (980 to 1037 A.D.) is believed to be the first to have used distillation to distil essence of rose. Around this time, the Arabs as well discovered how to distil alcohol, making it possible to produce perfumes without a heavy oily base [5].

[1] **Aromatherapy** is the use of volatile oils, including essential oils, for psychological and physical well being by massage or inhalation [1].

On the other hand, the ancient Chinese might have been practicing their own aroma treatment as early as the Egyptians. Shen Nung's herbal book, the oldest surviving medical book in China, dating back to about 2700 B.C., contains information on over 300 herbal plants [5].

The Indians have also their own traditional medical practice called *ayurveda*, which has been practiced for over 3000 years. In South America the *Aztecs*[2] were known for their plant remedies and the great wealth of medicinal plants at *Montezuma*'s[3] botanical gardens had impressed the Spanish invaders. In the North the American Indians also have their own herbal practice [5].

There are quite a few references to essential oils 'perfume' in the Bible too: "*Take the finest spices 6 kg myrrh, 3 kg sweet-smelling cinnamon, 3 kg sweet-smelling cane, 6 kg of cassia. Add 4 l of olive oil, and make the Sacred anointing oil. (Exodus 30:23-25).*" "*You provided no olive oil for my head, but she has covered my feet with perfume (Luke 7:46)*" etc.

European scientists began researching the effects of essential oils on bacteria and human health late in the 19th century. A French chemist, Rene Maurice Gettefosse, began his research in the medical use of essential oils after he accidentally found the healing powers of lavender oil. In 1937 he published a book about the anti-microbial effects of essential oils and coined the word '*Aromatherapy*'. Around the same time another Frenchman, Albert Couvreur, published a book on the medicinal uses of essential oils [5].

The French medical doctor, Jean Valnet, continued Gattefosse's research on essential oils. The method of applying essential oils to the skin with massage was developed by the French biochemist Margaret Maury. Michine Arcier, a former student and co-worker of both Maury and Valnet, combined all their techniques to create a form of aromatherapy presently used in the world [5].

[2] **Aztecs**: Natives of North America (Mexico).
[3] **Montezuma**: 1466-1520, Aztec emperor of Mexico killed by the Spanish conquistador Cortes.

2.2.1 Aromatherapy and Essential Oil Safety

Used properly, the physical application of essential oils has great benefits in matters of health (massage oils), beauty (in body lotions, ointments, facial toners, perfumes, etc) and hygiene (in soaps, shower gels, shampoos, etc.). Some essential oils can be steam inhaled (*e.g.* eucalyptus oil) to help relieve colds and influenza or for relaxing overworked muscles. Essential oils are applied to the skin after diluting them in carrier oils, which are pure vegetable oils like sweet almond oil, apricot kernel oil, sesame, avocado, grape-seed oil, etc., to help their absorption by the body. Additionally, essential oils are widely used in room refreshing (rose, lemon) and insect repellent (citronella, lavender, peppermint) sprays [5].

To enjoy all the benefits of essential oils and to avoid the risks involved in their use, the following is generally recommended [5]:

- Essential oils should never be used undiluted. Some oil constituents may have a negative effect on health.

- Due to possible allergic reactions, essential oils have to be used with caution. The use of some essential oils in aromatherapy may have dire consequences. Examples include onion, wormwood, pennyroyal, camphor, horseradish, wintergreen, rue and bitter almond essential oils.

- Some essential oils should be avoided during pregnancy (*e.g.* lemongrass) or by persons with asthma or epilepsy. Essential oils should never be used on open wounds or taken orally in such cases.

- Essential oils are flammable and constitute a fire hazard.

2.3 General Description and Use of the Four Essential Oils Analysed in this Project

2.3.1 Lemongrass (*Cymbopogon citratus & C. flexuosus*)

Lemongrass, from the Gramineae (aromatic grasses) family, is native to India [6]. It is a vigorous grass, which may be harvested within six months after planting and may be cut three to four times a year. Lemongrass essential oil, obtained mostly by distillation [7], has a lemony sweet smell, dark to amber-reddish colour and watery viscosity [1].

The essential oil is widely used industrially and it is found in cosmetics such as hair conditioners, facial water, lotions, deodorants and soaps [8]. Added to water or vinegar and sprayed in the air, along walls and floors, or on pets, it acts as an insect repellent and attacks fungi by discouraging mould growth. An antiseptic wash helps to improve skin infections and sores. The numbing of nerve endings, which dulls the intensity of pain that reaches the brain, caused by lemongrass essential oil makes it useful to relieve headaches, indigestion, pain, rheumatism, and nervousness. The scent reduces irritability and drowsiness [7]. Blended with other essential oils, such as lavender, basil, and jasmine essential oils, it is used in massage or diluted in bath to help cellulite, digestive problems, over exerted ligaments and as a general tonic [1]. But the oil should only be used under the guidance of a professional aromatherapist to avoid any side effects that might occur [6].

Traditionally, lemongrass is used widely for the treatment of different ailments. In Tanzania and Kenya an infusion of lemongrass is taken as tea for fevers and jaundice, to treat acne, skin infection, and ringworms. It is also used as a body wash and to perfume the body. The Zulus used the plant juice for ritual cleansing washes. The Zulus also used the sap from the cooked roots to settle the stomach [9].

Fresh lemongrass leaves have been traditionally used in Thai, Vietnamese and Caribbean cooking for many years. The herb is frequently used in curries as well as in seafood soups. Its light lemon flavour blends well with garlic, chillies and ciliantro. But due to its strong odour it is normally used in small amounts [8].

2.3.2 Bourbon Geranium (*Pelargonium capitatum x P. radens*)

Geranium, from the Geraniaceae family, constitutes a large number of flowering plants (~700), of which only about 10 varieties and some hybrids, like the one analysed in this work[4], produce viable quantities of essential oils. It is a hairy perennial shrub; stands up to about one meter high, with pointed leaves serrated at the edges. Originally, the plant was native to South Africa, Reunion, Madagascar, Egypt and Morocco before its introduction to other countries worldwide [10].

The essential oil is extracted from the leaves and stalks mostly by steam distillation. The essential oil analysed here has a slightly light green colour, watery viscosity and lemony fragrance. Geranium oil has quite a number of therapeutic properties and can be used to help treat: acne, bruises, burns, cuts, dermatitis, ulcers, diabetes, colds, flu [5], etc. It is used as massage oil and diluted in bathing water. The oil blends well with other essential oils like cedar wood, bergamot, basil, jasmine, lavender, orange and rosemary. It has also insect repellent properties. Due to its hormone balancing behaviour, the essential oil of pelargonium is not recommended for pregnant women [1].

2.3.3 *Tagetes minuta* (Kakiebos)

Tagetes minuta, from the Compositae (daisy family) [6], is an herbaceous plant/weed about 1 m tall with small yellow flowers and a very strong smell. Other names of *Tagetes minuta* include Mexican Marigold, tall Khaki weed (English), 'Kakiebos', and 'Lang-kakiebos' (Afrikaans). *Tagetes minuta*, believed to be native to Africa [5], is grown in East and South Africa, South America, and Australia. Grown in the wild it is a problematic weed of pastures and numerous crops. *Tagetes minuta* seeds have an unpleasant odour and can reduce the value of grain harvest when it is a contaminant [11].

Commercially, it is grown for its oil, which has a heavy, pungent, sweet smell and gold-yellow to reddish-amber colour. It is medium in viscosity that turns thick and even gel-like if exposed to the air for a long time. *Tagetes minuta* oil is extracted (distilled) from leaves,

[4] A cross breed between *Pelargonium capitatum* and *Pelargonium radens*.

stalks and flowers picked when the seeds are just starting to form. The oil is used in French perfumes and for flavouring numerous food products. As a food flavourant it is used in cola and alcoholic beverages, frozen dairy desserts, candy, baked goods, gelatines, puddings, condiments, and relishes [12].

Tagetes minuta is rich in many secondary compounds, including acyclic, monocyclic, and bicyclic monoterpenes, sesquiterpenes, flavonoids, thiophenes, and aromatic compounds. There is evidence that the secondary compounds in *Tagetes minuta* are the effective deterrents of numerous organisms, including fungi, bacteria, round worms in general, trematodes, nematodes and numerous insect pests through several mechanisms [12].

Traditionally, *Tagetes minuta* is best known for its skin care remedies. Ailments such as calluses, bunions, and fungal infections are treated with an infusion from the leaves and flowers made into a wash or cream applied to the area [8]. Crushed mature *Tagetes minuta* leaves and flowers can be used as general insect repellent and fungicidal agents. Ants, aphids, blowflies, caterpillars, flies, fleas, maggots, mosquitoes and termites are repelled if soil or surfaces are sprayed with a *Tagetes minuta* juice or if a freshly crushed plant part is dug into the soil[5].

Keeping in mind all its uses, *Tagetes minuta* is a very powerful oil and should be used sparingly. It should be avoided during pregnancy. It should not be used on sensitive skin as it may cause photosensitivity and some form of dermatitis [1]. *Tagetes minuta* oil should never be taken orally [5].

2.3.4 African Wormwood (*Artemisia afra*)

Artemisia afra, a well known medicinal plant in Eastern and Southern Africa, is also known by other common names like African Wormwood (English), 'Wilde Als' (Afrikaans), 'Lanyana' (Sotho, Tswana) and 'Umhlonyane' (Xhosa, Zulu). African Wormwood, which got its name from its ability to get rid of worms, is a perennial drought resistant, feathery,

[5] Mexican Marigold (*Tagetes minuta*)- author anonymous.

grey-green shrub. It is about 1 – 2 m tall with small yellow flowers and can grow almost in any soil [13]. The essential oil has a light yellow colour, watery viscosity and strong odour.

Traditionally, the infusion of *Artemisia afra* was used as a lotion to bathe hemorrhoids, and with a hot bath to bring out measles. In Tanzania a weak infusion of the leaf was used to treat colic. Warmed leaves were used as a poultice for pimples, boils, mumps, and sprains [8]. In South Africa, *Artemisia afra* is traditionally used as a remedy for chest problems, coughs, colds, influenza, loss of appetite, malaria and other ailments. Its volatile oil shows antibacterial, narcotic, analgesic and antihistamine activities [14].

2.4 Essential Oil Preparation Methods

Essential oils are very expensive to produce, some more so than others, due to the huge labour, expertise and capital investment requirements during cultivation and extraction of the oils. As already indicated, essential oils are obtained from different herbal plants and plant parts. A plant may give oil from one or more parts, but the amount of oil that might be produced may not be equally valuable both in quality and quantity. The method of extracting the essential oils from a specified plant material may also have an effect on its final composition [15].

Commercially, essential oils are obtained using a variety of methods. Distillation, Solvent Extraction, and Supercritical Fluid Extraction (CO_2 extraction) are the three main essential oil isolation methods employed in industry and will be dealt with in the sections to follow.

2.4.1 Distillation

Distillation is the oldest and most widely used extraction technique in the commercial production of essential oils due to its cost effectiveness. It is done in a variety of forms *viz.*: water distillation, steam distillation, hydro diffusion, water-steam distillation, cohobation, and rectification. The extraction of essential oils using distillation requires great care as prolonged heating may induce chemical changes that can damage the 'true' essence and nature of the essential oils [16].

(a) Extraction of Essential Oils by Water Distillation

In this process the plant material is completely immersed in water and the mixture is boiled. After some time the condensed mixture is allowed to cool and settle down, whereby the water-oil liquid mixture is separated from the solid plant residue. The essential oil floats on top of the hydrosol[6] and the two are separated for their destined use [16].

Essential oil sources rich in esters should not be extracted by this method as extended exposure to hot water will start to break down the esters to their respective alcohols and carboxylic acids, mostly acetic acid. To limit damage to heat sensitive essential oils, distillation can be performed under reduced pressure. This method is more effective for oils extracted from tough materials like roots, wood, or nuts [16].

(b) Extraction of Essential Oils Using Steam Distillation

In steam distillation, pressurised steam is forced through the plant material to help release the fragrant molecules from the pockets in which the oils are present. The molecules of these volatile oils then evaporate and escape from the plant material into the steam. The pressurised steam is kept hot enough (~ 100°C) to release the oil from the oil glands, yet not too hot to damage the plant material or the essential oil. The steam containing the oil is then passed through a cooling system, condensed, and decanted to give the oil and hydrosol [16, 17].

Some of the drawbacks of this method are [18]:

- The steam released from the bottom of the still[7] does not disperse very well, making the process less efficient.
- A long time is required to completely distil the oil, which increases the time the plant material is exposed to heat.
- It is costly as it takes lots of energy to produce the steam and for cooling the products.

[6] A **Hydrosol** or **floral water** is the water that remains after extracting an essential oil *via* steam or water distillation, which is then used in skin care and bathing products or as light colognes or body sprays.

[7] **Still**: An apparatus for distilling liquids, such as alcohols, consisting of a vessel in which the substance is vaporised by heat and a cooling device in which the vapour is condensed.

(c) Extraction of Essential Oils Using Hydro-Diffusion

Hydro-diffusion differs from steam distillation in that the steam is introduced into the plant material from the top down instead of from the bottom up as in steam distillation. The oil containing steam mixture condenses below the area in which the botanical material is held in place by a grill, a grating of metal or wood used as a barrier. Less steam requirement, shorter processing time and higher oil yields are some of the advantages of hydro diffusion [16].

(d) Extraction of Essential Oils By Water and Steam Distillation

This technique is a combination of both water and steam distillations. The plant material is immersed in a still with a heat source, and additional steam is fed into the mixture [16]. This method is best suitable for leafy material, but has little use for tough materials such as woods, roots and seeds [19].

(e) Extraction of Essential Oils by Cohobation

Cohobation is a method employed to distil and re-introduce water-soluble oil components into the essential oil after the oil has been extracted by water distillation. For example, when Rose oil is extracted using water distillation, one of its components, ethyl phenyl alcohol, dissolves into the water and needs to be redistilled from the water and re-introduced to the essential oil to make the oil 'complete' [16].

(f) Extraction of Essential Oils Using Rectification

Rectification is a process of re-distillation used to refine and purify extracted essential oils that have water, resinous matter or solvents as impurities. It is done either in steam or under vacuum. For example, Eucalyptus oil is re-distilled to create standard quality oil [16].

2.4.1.1 Problems Associated with Distillation

The length of the distillation period affects the composition of most essential oils. This is especially true for oils that are obtained from seeds, barks, or any other plant parts with a

hard resistive barrier between the oil glands and the surface in contact with the boiling water. At the first stage of distillation higher boiling oxygenates are liberated more easily than lower boiling hydrocarbons; in the process of distillation the boiling water breaks into the plant tissue, dissolves the water-soluble oil components and carries them to the outer surface to be vaporised. This process called hydro-diffusion continues until all the essential oil is removed. The hydrocarbons remain associated with the plant material longer than the oxygenated oil constituents because of the latter's lower water solubility. As a consequence of the process of hydro-diffusion, essential oil components are distilled in the order of their water solubility and not in the order of their boiling point [15].

Non-volatile fats that might be present in essential oil materials have the power of retaining essential oil components, preventing complete distillation of the oil. This is more the case with hydrocarbons that show a greater affinity for the fatty component than the oxygenated compounds [15].

The process of distillation also introduces new constituents due to chemical changes that may occur during the extraction procedure, including isomerisation, saponification, polymerisation or degradation. One of the main effects observed during essential oil extraction by distillation is the effect of temperature and pH, which makes some oil constituents prone to undergo rearrangement or hydrolysis reactions. Increased temperature and decreased pH (increased acidity), for example, transform labile components like linalyl and α-terpinyl acetate to their corresponding alcohols: linalool and α-terpinol [15]. Linalool, which is present in almost all essential oils, rearranges to terpineol during steam distillation [20], as shown in figure (2.1). The presence of trace metals in plants has also been observed to cause rearrangement of some compounds to their isomers. For example, *cis*-dihydrocarvone isomerises to its *trans* isomer during distillation [15].

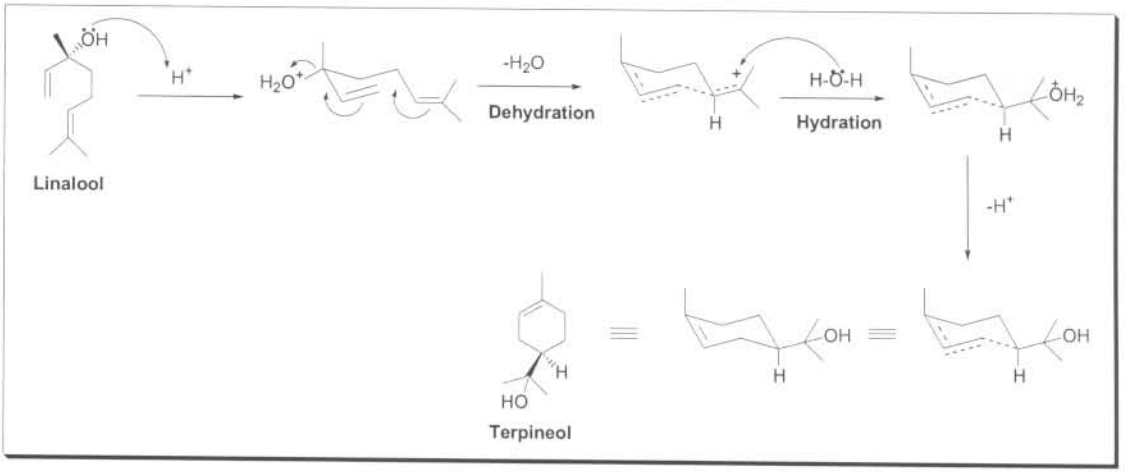

Figure 2.1 The re-arrangement of linalool to terpineol during steam distillation [20].

By contrast, the oil enclosed in glandular hairs is easily liberated because the only barrier, the outer membrane or the cuticle, simply bursts open when the distillation water reaches boiling point. Therefore, the above-mentioned problems of distillation are theoretically least expected to act in cases where the oil is extracted from leaves, flowers or any other soft plant material [15].

2.4.2 Solvent Extraction of Essential Oils

Essential oils can also be extracted by solvent extraction. Solvent extraction, which is most suitable for delicate and heat sensitive fragrant oils [16], is done by either a Soxhlet extraction or by mixing the plant material and solvent in an Erlenmeyer flask [15]. Different solvents including hexane, acetone, petroleum ether, ethanol, and methanol [16] are used for this extraction method. During solvent extraction the solvent dissolves a range of substances from the plant including non-aromatic waxes, pigments, and highly volatile aromatic molecules. The solution containing both solvent and soluble plant material is filtered and the filtrate is subjected to low-pressure distillation to remove and recover the solvent for further use. The remaining waxy mass called the *concrete* contains the volatile essential oils [19].

To remove the waxy materials, the concrete is warmed and stirred with alcohol (usually ethanol). The heating and stirring process breaks the concrete into minute globules. The alcohol soluble oil components separate out efficiently with a certain amount of alcohol-

soluble waxes. Agitating and cooling the solution at low temperatures helps to precipitate out the waxes. Finally, the solution is cold filtered to get the pure essential oil called the *absolute* [19].

The use of low boiling solvents for the extraction of oils has an advantage over distillation, as the temperature remains relatively low during most of the process. Solvent extracted essential oils have more of a natural appearance, free of distillation-induced artefacts. However, besides the need for further purification to remove undesired waxes and pigments, solvent extraction may also introduce artefacts. Artefacts produced from solvent (acetone, petroleum ether) and essential oil component (non-terpenoids) interactions may be observed in the volatile extracts. Alcohol extraction might also give rise to the formation of artefacts by esterification, etherification, and acetal formation [15].

2.4.3 Supercritical Fluid Extraction (SFE) of Essential Oils

Supercritical fluid extraction employs the unique properties of some substances (water, CO_2, butane, etc.), which give a supercritical fluid with gaseous and liquid properties at certain temperatures and pressures called the critical points. Supercritical fluids have good solvent properties similar to those of liquids as well as the transporting properties of gases. Carbon dioxide, due to its low cost, low critical pressure and temperature and low toxicity is the most widely used supercritical fluid. It forms a supercritical fluid at about a pressure of 73.8 bars and 31.1°C. In CO_2 extraction, the plant is placed in a stainless steel tank and CO_2 is injected into the tank. Under the appropriate pressure and temperature the CO_2 is liquefied and acts as a solvent to extract the essential oils from the plant materials [16]. The extraction capability and capacity (dissolving power) of the inert CO_2 fluid can be manipulated by regulating the system's temperature and pressure (fluid density) [21].

The advantage of CO_2 extraction over solvent extraction is that it doesn't chemically interact with the essential oils [18] and no solvent residue remains. At normal pressure and temperature the CO_2 reverts to the gas phase and evaporates leaving the pure essential oil behind. Compared to distillation, it is gentler and thermally labile compounds can be extracted easily [21] as high temperatures are not employed. Supercritical CO_2 extraction

helps not only to extract oils which do not usually yield to other methods of extraction (*e.g.* Rose Hip Seed, Calendula), but also provides more intensely scented essential oils since more of the fragrant chemicals are released through this process [19].

As already stated, the process of supercritical extraction requires heavy-duty stainless steel equipment and hence high capital investment is needed in using this extraction method [16]. The other aspect that needs consideration in this extraction process is the relative acidity of CO_2 (~ 4.5), which might change the composition of some oils. Therefore, oils with a high content of acid sensitive compounds, such as terpenes, are not suitable for supercritical CO_2 extraction [22].

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3 MULTIDIMENSIONAL CHROMATOGRAPHIC TECHNIQUES AND THE ANALYSIS OF ESSENTIAL OILS

3.1 Multidimensional Chromatographic Techniques

A Multidimensional chromatographic technique is a technique that employs two or more methods of separation in series to effect the separation of components, which are otherwise difficult to achieve using one-dimensional systems. According to J.C. Giddings [1], a multidimensional separation is characterised by two conditions. Firstly, a sample is analysed by two or more analysis steps of different characteristics and secondly, components separated in the previous step generally remain separated until the end of the analysis process. Because of these two conditions, multiple parameters (elution times, fragmentation pattern, etc) are required to distinctively describe the final position of a component after a multidimensional separation. Therefore two components will be successfully separated and have different final positions, if at least they have a difference in one of the separation parameters [1].

The first attempt at creating a multidimensional chromatographic separation was performed along the axes of a paper sheet (TLC×TLC) in 1944. This was followed by gas chromatography coupled to electrophoresis in 1948 and a two-dimensional electrophoresis (electrophoresis-electrophoresis) system in 1951 [2]. In 1968 Deans demonstrated the possibility of heart-cutting two-dimensional gas chromatography *via* the *Deans switch* valve to transfer part of a GC chromatogram into a second column with different polarity [3]. This was followed by the coupling of isoelectric focusing with electrophoresis (1975-1982) to achieve a two dimensional system for the analysis of proteins [2]. In 1980 Majors also reported the coupling of liquid chromatography to capillary gas chromatography [3].

The early 1990's witnessed the development of new techniques in multidimensional column chromatography and comprehensive two-dimensional column chromatographic techniques were born. In 1990 Bushey and Jorgenson described the first comprehensive 'all-inclusive' multidimensional column chromatography. They coupled an ion exchange first dimension column with a size exclusion liquid chromatographic second dimension column. The two dimensions were interfaced by means of two sample loops mounted on an eight–port valve to effect the subsequent separation of all injected solutes in the serially coupled columns [4]. In 1991 Phillips and Liu [3, 5] broke new ground for a comprehensive two-dimensional gas chromatographic (GC×GC) system. In this technique the authors utilised a thermal desorption modulator to trap and focus sample components eluting from the first dimension column before being introduced into the second dimension column as sharp solute pulses [5].

The drive for the use of multidimensional techniques has its roots in the inability of linear systems to handle complex samples. One-dimensional techniques lack the power of clearly and efficiently separating complex samples, *i.e.*, they have insufficient peak capacities to adequately resolve complex samples and they end-up giving overlapping component peaks. Peak capacity is the maximum number of peaks that can be separated by a given separation mechanism with satisfactory resolution between neighbouring components [1]. To efficiently separate a certain number of components in a sample a peak capacity (n_c) of greater or equal to the number of components (m) in the sample is required. The use of multidimensional systems greatly enhances the n_c from that of a single column, to be between the sum ($n_{c1} + n_{c2}\ldots + n_{cn}$) and the product ($n_{c1} \times n_{c2}\ldots \times n_{cn}$) of the number of columns or techniques serially coupled [1, 2, 6]. The peak capacity is related to the theoretical plate number N [1], which signifies the column efficiency by the following equation:

$$n_c = \theta\sqrt{N} \approx \frac{1}{2}\sqrt{N} \qquad (3.1)$$

The constant θ, which depends on the retention time range, is approximately 0.5 [1].

According to the above equation increasing n_c by a factor of two requires quadrupling the theoretical plate number of the column. In a one-dimensional system this implies increasing

the column length four times, which substantially increases the analysis time, respective cost and the technical difficulty of working with such columns. On the other hand, the theoretical plate number and n_c can be improved more easily without substantially increasing the column length by using multidimensional techniques and exploiting the multiplicative increase in n_c they create [1, 6].

The amenability of analytical samples to a separation or an analysis system does not entirely depend on sample complexity, *i.e.*, the number of components (m) in a given sample. There is another factor that affects the effective separation of samples by a certain analysis system called *sample dimensionality* or *sample variability*. Giddings defined sample dimensionality as: the number of independent variables that must be specified to identify the components of a sample. This parameter measures the make-up of a sample in terms of the differences that exist between components and the parameters required to separate them from each other [7]. For example, if a sample entirely consists of straight chain-hydrocarbons it has one dimension, boiling point difference. Therefore, it is theoretically possible to fully separate such components using a one-dimensional separation system with high enough n_c utilising a volatility-based separation. The use of a multidimensional system to separate such samples gives no actual increase in separation power, as there will be strong correlation between the successive parts of the system(s) used [7].

On the other hand, if a sample contains compounds from a number of homologous series, such as hydrocarbons, alcohols, esters or aldehydes, the sample dimensionality or variability is not single and simple any longer. The use of only volatility based separation will not effect good separation as there will be overlapping of components of similar boiling points. Aromatic hydrocarbons with almost the same boiling points as non-aromatic compounds can serve as an example. The separation of such samples requires a system which is capable of using more than one variable to effect separation. The use of a multidimensional separation method with higher n_c and extra separation parameters is not only useful but a necessity. Consequently, the success of multidimensional techniques in the analysis of complex samples in terms of defining variables needed to identify them, depends entirely on the availability and effectiveness of the required parameters in the system utilised to separate and

identify the individual components *viz.* boiling point, polarity, size, etc. [7]. The analysis of multivariable samples using multidimensional systems not only is effective in terms of its separation power but it also provides very ordered chromatograms which have the potential advantage of being much more interpretable. In ordered chromatograms, patterns of peaks define members of a homologous series (aliphatic, mono-aromatic, di-aromatic, etc) and help to recognise chemical classes easily.

3.1.1 Peak Capacity and the Statistical Component Overlap Theory

To separate a number, m, of components in a certain sample with a unit resolution between two adjacent peaks, one needs a system with at least equal or more peak capacity, n_c. As n_c represents the maximum attainable number of resolved peaks in a chromatogram, the above statement is theoretically correct. However, this is only true if the component peaks of a sample are evenly spaced during the separation process. In reality, that is far from the case. In most complex samples peaks are randomly spaced and sometimes overlap with each other even when the separation space has much greater n_c than theoretically needed for complete resolution of the analytes at hand. This overlap of component peaks greatly reduces the amount of sample components that can be successfully resolved by a system of given n_c [1].

To explain and understand the component overlap theory it is necessary to look into some of the variables that explain the make-up of a chromatographic plot. The number of visible peaks (p) of a sample of m components that can be resolved by a given n_c ($m = n_c$) is given by equation (3.2) [1, 8]:

$$p \approx me^{-\frac{m}{n_c}} \approx 37\% \qquad (3.2)$$

From equation (3.2) it is clear that a system with peak capacity of n_c is only able to resolve less than 50% (≈ 37%) of the number of sample components presented to it due to the extensive overlap of peaks during separation. Furthermore, the numbers of single component peaks (s) that can be expected are much less than the number of p, as can be referred from equation (3.3) [1, 8].

$$s \approx me^{-\frac{2m}{n_c}} \approx 18\% \qquad (3.3)$$

Although the use of linear systems with n_c in excess of the number of components in the sample ($n_c >> m$) could address this problem, the attempt to design or use such systems has its own complications in terms of length of analysis time and excessive cost. The true remedy, as already suggested above, comes from the use of multidimensional methods, which have n_c values much higher compared to any linear chromatographic techniques [1].

The n_c of a truly orthogonal comprehensive two-dimensional chromatographic system, with uncorrelated separations and complete transfer of sample components between the two chromatographic dimensions without loss of resolution, is a product of the n_c of the individual columns [5, 9, 10]. This enhanced n_c makes a comprehensive two-dimensional chromatographic system more powerful to resolve complex mixtures than its one-dimensional counterpart. However, the multiplicative rule is only an estimation of the actual n_c that can be theoretically reached in two-dimensional separations. Correlations of solute retentions, *i.e.* lack of complete orthogonality between the two-dimensions reduce the available separation space to a restricted region [10]. If there is too much communication or cross-information between the two dimensions much of the separation space is unoccupied or even completely inaccessible and sample components tend to cluster along a diagonal. Reducing cross-information between successive columns increases the efficiency of information generation from multidimensional separations [1].

Mathematically, the n_c of an orthogonal two-dimensional separation system is given by equation (3.4) [1, 6]:

$$n_{cT} \approx n_{c1} \times n_{c2} \qquad (3.4)$$

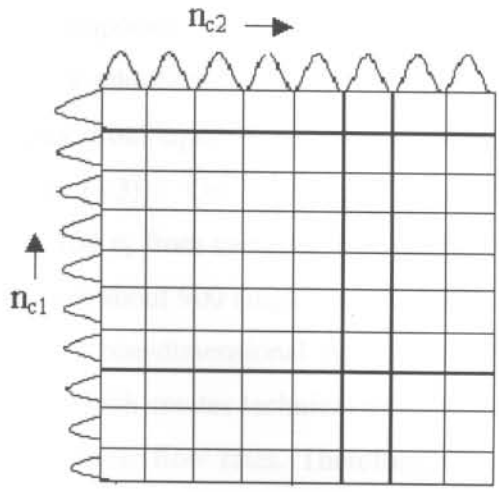

Figure 3.1 Orthogonal n_c of a 2D chromatograph, square boxes represent resolution units in the 2D space. The area of the plane represents the total theoretical peak capacity (n_{cT}) of a perfectly orthogonal system. But the available n_c is determined by the retention correlations between the two dimensions, which may be reduced to a one-dimension peak capacity if the two systems are highly correlated *i.e.*, with the same separation mode in the two dimensions. Therefore, the actual available n_c is usually smaller than the theoretical n_c [6, 10].

The use of two-dimensional chromatography does not directly reduce peak overlap. In the true sense, peak overlap is even worse in two-dimensional systems due to the overlap of peaks occurring in both the first and second dimensions. As a result the amount of p that can be expected is further reduced to about 16% and the number of s to about 9% as can be inferred from equations (3.5) & (3.6) [8]:

$$p \approx me^{-\frac{2m}{n_c}} \approx 16\% \qquad (3.5)$$

$$s \approx me^{-\frac{4m}{n_c}} \approx 9\% \qquad (3.6)$$

Fortunately, the enormous n_c and extra space that can be obtained from multidimensional systems substantially reduces peak saturation, given by the ratio of the number of components divided by n_c, m/n_c. And the resulting statistical-overlap of component peaks from complex samples is reduced as component peaks are allowed to spread out across additional coordinates [7]. This large n_c gives multidimensional techniques the upper hand in

resolving large numbers of components that cannot be practically achieved by a single column system. For example, a one-dimensional chromatographic system with n_c of 1000 (with 4×10^6 theoretical plates; from equation (3.1)) can resolve approximately 180 single-component peaks (equation (3.3)). On the other hand, a two dimensional gas chromatographic system with 100 n_c from each column will have an n_{cT} of 10,000 (equation (3.4)) and can effectively resolve about 900 single-component peaks (equation (3.6)) [8]. To produce the same results from a one-dimensional system calls for the use of an extremely long and narrow bore column, which creates technical difficulties of working with very high pressures and high carrier gas linear flow rates. Therefore, the use of comprehensive two-dimensional systems for the analysis of complex samples does not only provide high separation power but also relieves one of the technical difficulties of working with one-dimensional systems [1].

3.2 Comprehensive Two-Dimensional Gas Chromatography (GC×GC)

Comprehensive two-dimensional gas chromatography (GC×GC) is a multidimensional technique in which two capillary gas chromatographic columns of different selectivity are serially coupled to provide better and efficient chromatographic resolution. In its basic form, all sample components eluting from the first column are completely trapped and focused by a modulating interface to be re-injected in the form of distinctive pulses into the second dimension column. The second dimension column with different separating characteristics, shorter length and narrower bore than that of the first column further analyses and separates co-eluting first dimension solutes to give sharp peaks [5, 9].

In GC×GC the second dimension column must be operated fast enough to preserve the information contained in the first dimension separation step [9, 11]. Should a second dimension column be too slow the information obtained from the first column will be degraded, as there will be not sufficient number of cuts across each first dimension peak to preserve its shape. Peaks already separated in the first dimension will overlap. Also, if the second dimension column is too fast relative to the first dimension column, poor use of the analysis time results in lower second dimension resolutions [11, 12].

Solutes injected into the second dimension column at a particular time are rather few and of similar volatility and separation is a function of chemical class rather than vapour pressure differences. For this reason the second dimension column does not need large n_c. Although the total analysis in GC×GC is usually a temperature programmed run, the individual second dimension sub-sample injections are so short in time that they can in practice be considered as isothermal runs ($\Delta T \sim 0.1^{\circ}C$/chromatogram) [12].

Overall, during a GC×GC run there must be no loss of information obtained from the first dimension during transfer to the second dimension column or in the process of solute separation in the second dimension column [12]. A complete set of secondary chromatograms is then generated in real time as the primary chromatogram develops. This is only possible if the two dimensional system is in an orthogonal arrangement in which the mode of separation in the first dimension column is independent of the analytical separation in the second dimension [9, 13].

The success and orthogonality of a GC×GC system depends on the proper choice of the two columns, the interfacing modulator and the proper operation of their combination. The type of sample analysed may also give a false signal about the orthogonality and efficiency of the system. A sample containing components of similar properties, for example, only *n*-alkanes, may not show the true orthogonality of a GC×GC system. To test the orthogonality of a system the test sample itself must contain substances distributed over the whole range of properties relevant to the first and second dimension columns [14]. In addition, the temperature range *i.e.* volatility of the entire sample has to match the temperature limits of the columns selected for use. Special notice has to be given to the temperature limits of the second dimension column, as its upper temperature limit is usually lower than that of the first dimension column. The maximum allowable temperature of the polar second dimension column determines the upper limit of boiling point of samples that can be analysed by a GC×GC system. This is especially the case when the two columns are housed in the same oven. If the temperature exceeds the higher temperature limit of the second dimension

column, the column starts to bleed and gives a distorted base line interfering with trace component detection in the sample [11].

In an orthogonal GC×GC system the retention times on the two columns depend on different solute characteristics. In the first dimension solutes are separated according to their vapour pressure differences. The retention times of the polar second dimension column are only determined by chemical class membership. Polarity and molecular shape differences in components of a sample determine retention in the second dimension, making secondary column retention a measure of these molecular properties. That is, the second dimension separates sample components on the basis of their homologous series independent of molecular size or volatility providing a type of functional group analysis. Components of the same homologous series have approximately the same second dimension retention times [13, 14].

The two columns in comprehensive two-dimensional systems may be housed in either of two ways. The two columns can be operated in a one main oven system. Alternatively, an auxiliary oven is used inside the main oven to house the second dimension column. Generally, comprehensive two-dimensional gas chromatographic analyses are performed in a temperature programmed mode. In the one oven system the two columns are operated under the same temperature-programming rate. If a two oven system is utilised it is possible to independently temperature program the two columns. The later case has some advantages [14]:

- The two columns can be operated independently at different temperatures and/or temperature programming rates.
- Columns of high temperature phases can be coupled with columns of limited thermal stability. The overall temperature limit of the system is no longer determined by the phase with the lower temperature limit.
- Optimisation of stationary phase selectivity by temperature adjustment of two sequentially coupled columns, coated with different stationary phases, is easily accomplished.

3.2.1 Advantages and Shortcomings of Comprehensive Two-Dimensional Gas Chromatography

Comprehensive two-dimensional gas chromatography, compared to linear gas chromatography, has some distinct advantages. The main advantages can be summarised as follows:

1. By virtue of the multiplicative effect of separation space available, comprehensive two-dimensional GC provides very high n_c. This high n_c is directly reflected in the ability of the system to separate complex mixtures effectively. It allows a complex sample to be separated into individual peaks. The peaks are classified into groups creating an easy group type analysis [10, 12, 15].
2. Due to the orthogonal nature of the two dimensions, GC×GC offers two totally independent retention times. These independent retention times increase the certainty of qualitative identification of components [10, 12].
3. It provides increased sensitivity and superior resolution relative to conventional GC. The pulsing of components from the first dimension to the second dimension creates narrower bands which are much taller in magnitude and this translates to a much more sensitive analysis due to the increase in peak height. If a narrow band can be pulsed into a short column leading to the detector, the response can be significantly increased. This allows easy detection and determination of trace components in a complex mixture. A short second dimension column with different stationary phase helps to resolve peaks unresolved in the first column. A fast second dimension column, eluting peaks at a rate faster than the first dimension peak widths, generates a sequence of second-dimension chromatographic separations that increases the overall resolution power of the system [15, 16].
4. Analysis times are comparable with one-dimensional GC, but GC×GC runs contain more information and have superior resolution [8, 12].

Comprehensive two-dimensional gas chromatography also has a few shortcomings [12]:

1. Limited availability of fast detectors. The only readily usable detectors presently are the mass flow sensitive detectors like the flame ionisation detector. Conventional mass spectrometers cannot be used as GC×GC detectors, as they are too slow to record a spectrum during the elution of very narrow second dimension peaks. The first generation commercial instruments with fast time-of-flight mass spectrometers have only recently become available.
2. GC×GC systems are sometimes inadequate for the separation of high boiling components, often exceeding the temperature limits of the second column. This is especially the case in one-oven systems.
3. The lack of easily accessible data handling software for the automated interpretation and easy processing of the large amount of data produced. The first automated peak integration software package has only recently come onto the market.

3.3 Coupled Gas Chromatographic Techniques for the Analysis of Essential Oils

The analysis and research in herbal plants and their essential oils is becoming increasingly important. This is due to their tremendous applicability not only in traditional herbal preparations but also due to the great demand for essential oils as raw materials in the pharmaceutical and cosmetic industries. The analyses of essential oils aim at one or all of the following: component identification, component comparison between different oils, or for authenticity and quality control purposes. To achieve any of these goals one needs a suitable method or combination of methods, which is able to give the best result in a reasonable time and cost. Two-dimensional methods for essential oil analysis include: capillary gas chromatography-mass spectrometry (GC-MS), supercritical fluid chromatography-gas chromatography (SFC-GC), and comprehensive two-dimensional gas chromatography (GC×GC).

3.3.1 Gas chromatography-Mass Spectrometry (GC-MS)

GC-MS is a coupled technique combining two independent systems. The first dimension, the gas chromatograph, disperses solutes according to their vapour pressure differences. The second dimension in GC-MS, the mass spectrometer, acts as a detector but with added independent resolving power. It ionises and fragments sample components dispersing them along a secondary mass-to-charge ratio axis [13].

Essential oils have a complex chemistry consisting of terpene hydrocarbons, alcohols, aldehydes, and esters [17]. The components range from highly volatile to semi-volatile and from non-polar to highly polar organic components. The use of gas chromatography as a separation tool is therefore ideal for essential oils. The use of mass spectrometry for the characterisation and detection of the separated solutes follows logically. This offers the reason why most essential oil analyses are done with this coupled technique.

The complete identification of essential oil components is possible with the use of known standards, use of literature retention indices or by comparison with mass spectrometric databases. The use of authentic standards is mostly useful when the analyst has an idea about the composition of the sample at hand. On the other hand, the use of retention times and Kovat's indices requires the use of the same analytical conditions, including same temperature programming rate, column dimensions, stationary phase and flow rates, as the reference data. The use and success of spectrometric databases depends on the quality and amount of data stored. Problems arise when analysed samples contain components not included in the database.

Almost all the studies dealing with essential oils employing GC-MS techniques in the scientific literature focus mainly on the identification of the oil components. Only few publications deal with the factors affecting oil yield and concentration variation of some oil components. Most of these studies were done with similar columns and analytical conditions. Amongst the many studies on essential oils using GC-MS, there are quite a few references that deal with the analysis of the essential oils studied in this project. The GC-MS studies of

Tagetes minuta indicated that the essential oil contains 22 - 35 identified components [18, 19, 20, 21, 22, and 23,]. The chemical composition of lemongrass consists of more than twenty (20) identified components [24, 25, 26 and 27]. The essential oil of Bourbon Geranium has about thirty (30) identified components [27]. In the case of *Artemisia afra* (African Wormwood) forty-eight (48) components have been identified [28].

3.3.2 Supercritical Fluid Chromatography-Gas Chromatography

The coupling of supercritical fluid chromatography with gas chromatography is one of the alternatives that can be employed to enhance n_c of a chromatographic system and to significantly improve the resolution of individual components in a sample. One of the attractive features of supercritical fluid chromatography (SFC), which makes it ideal for coupling to a fast gas chromatographic system, is its ability to handle low volatility and thermally labile organic components. Supercritical fluid chromatography's compatibility with non-volatile and thermally labile compounds comes from the solvation characteristics of the mobile-phase, supercritical fluid CO_2, at low temperatures. The solvent strength of the supercritical fluid CO_2 is used to pre-separate classes of solutes (hydrocarbons, alcohols, esters and aldehydes) that can be selectively or comprehensively introduced into a high-resolution capillary GC for detailed fingerprinting or quantitative analysis. Therefore, in the first dimension the sample components are subjected to a group-type separation in the polar silica gel SFC column, while in the second dimension they are further separated according to their boiling point by the fast GC column. At the SFC-GC interface the mobile phase is decompressed into CO_2 gas, allowing easy replacement with better GC mobile phases (hydrogen, helium). As compared to the fundamentally similar normal phase LC-GC coupling, no solvent evaporation techniques are required in SFC-GC, as the CO_2 lacks detector response in the flame ionisation detector of the GC.

The use of an in-house built [29] comprehensive two-dimensional SFC and fast temperature programmed gas chromatograph (SFC×GC) for the analysis of the four South African essential oils studied in this project has been reported [30]. The system comprised of an SFC system coupled to a fast GC by a flow modulator that samples every peak from the SFC and re-focuses it into the GC injector using pressure drop focussing. There is a T- junction

(splitter) after the stop-flow modulator, which splits the SFC flow into the FID of the SFC and directs the rest of the flow into the fast GC injector. The CO_2 from the SFC is replaced by hydrogen as a GC carrier gas. The GC column is resistively heated and rapidly cooled by liquid CO_2 in a one-minute cycle [30].

Comprehensive SFC×GC analysis of essential oils has only been done in our laboratory [30]. Other reports on this coupled technique deal with heart-cutting methods instead. T. Yarita *et al.* [31] reported the use of a heart-cut SFC-GC system for the analysis of some citrus essential oils, *viz.* lemon, grapefruit and orange essential oils. To interface the SFC to the GC, a switching valve was placed between the SFC UV detector and a restrictor to split the CO_2. Then part of the flow was introduced into the GC injector *via* a capillary tube. Helium was used as a GC mobile phase and the components were cryo-focused just above the GC column using liquid CO_2. These authors have demonstrated the capability of the system to efficiently separate the essential oil components into their respective classes, first according to their polarity in the SFC and then their boiling points in the GC. A very similar set-up reported by P. Manninen and H. Kallio [32] was used for the analysis of volatiles in the edible oil of cloudberry seed oil. Here the authors utilised the power of the SFC to separate the volatile components of the oil, which were introduced into the GC after being cold-trapped and re-focused. The low volatility compounds that eluted later were led directly to the FID of the SFC and not introduced into the GC. The authors concluded that this method is very successful in the characterisation of edible oil volatiles [32].

3.3.3 Comprehensive Two-dimensional Gas Chromatography (GC×GC) and the Analysis of Essential Oils

Compared to one-dimensional chromatographic techniques, comprehensive two-dimensional gas chromatography (GC×GC) gives an unmatched separation capability and larger n_c. As in SFC×GC, this technique offers an orthogonal separation of sample components by the use of two serially coupled columns. The solutes are separated according to their volatility in the first dimension and the second polar or chiral column is used for polarity or chirality separation, respectively (the experimental principles and instrumentation of GC×GC will be

dealt with later on). This option makes GC×GC a much sought after tool for the analysis of complex samples, including essential oils.

Since its inception more than a decade ago, comprehensive GC×GC has been widely used for the analysis of complex petroleum products. Its applicability to other samples, especially essential oils, seems to be gaining momentum in the last three to four years [33, 34, 35, and 36]. The analysts aim at the identification of sample components of different essential oils, separation of enantiomers and more complete fingerprinting of essential oils of commercial and industrial value.

In this study, comprehensive two-dimensional gas chromatography was used to analyse the four South African essential oils described in Chapter 2. GC×GC analysis of these essential oils has not been reported before.

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4 INSTRUMENTATION AND OPTIMISATION OF EXPERIMENTAL CONDITIONS

Part I: GC×GC INSTRUMENTATION AND MODULATOR TYPES

4.1 GC×GC Instrumentation

Since the first publication in 1952 by James and Martin [1], gas chromatography has developed tremendously in its application, specialisation and productivity. This was largely due to developments in the general gas chromatographic system, improved column production techniques, variation in stationary phases and their coating techniques. The introduction of coupled chromatographic techniques such as GC-MS, heart-cutting GC (GC-GC), SFC-GC and comprehensive two-dimensional GC (GC×GC) has also added new dimensions to gas chromatography. Developments in data handling software and hardware to cope with the new techniques have been part of the overall progress.

The instrumentation in comprehensive two-dimensional gas chromatography is not fundamentally different from that in linear gas chromatography. However, GC×GC includes some additional hardware. The basic components of our GC×GC system are a 6890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA) equipped with a split/splitless sample inlet and a flame ionisation detector, two serially coupled columns, a modulating interface with all its accessories (Zoex, Lincoln, NE, USA) and a computer for instrument control and data handling.

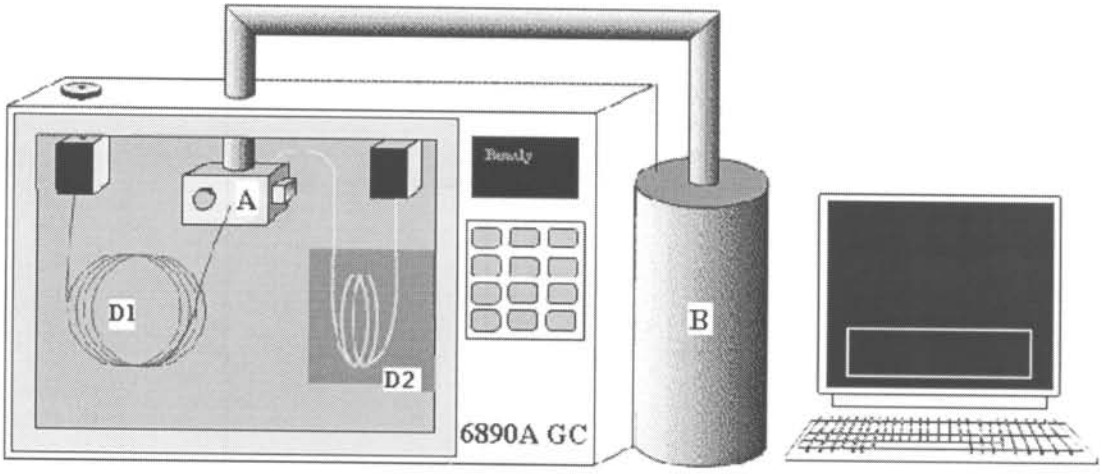

Figure 4.1 The GC×GC system with the jet-cooled cryogenic modulator. (**A**) The jet modulator with no moving parts, (**B**) Auto-fill liquid nitrogen cooling unit (dewar), (**D1**) and (**D2**) are the first and second dimension columns, respectively.

4.1.1 The Sample Inlet

The sample inlet, as the part of the gas chromatograph responsible for the proper introduction of samples to the column, plays a major role in the overall quality of a gas chromatogram. The 6890A gas chromatograph used in this project is equipped with a split/splitless injector and all experiments were performed in the split mode.

In split injection a syringe is used to manually inject liquid samples into a heated chamber called the inlet liner. Then the injected sample is swept by a relatively large flow of gas and vaporised. The gaseous sample-carrier gas mixture is driven into the head of the analytical column and is split into two parts. The larger part of the mixture is vented out of the gas chromatograph *via* the split outlet and a small part is introduced into the analytical column. The split ratio, the flow through the column divided by the flow discharged through the vent, depends on the amount of sample the analyst wants to introduce to the system. Split ratios ranging from 1:5 to 1:1000 are possible [2].

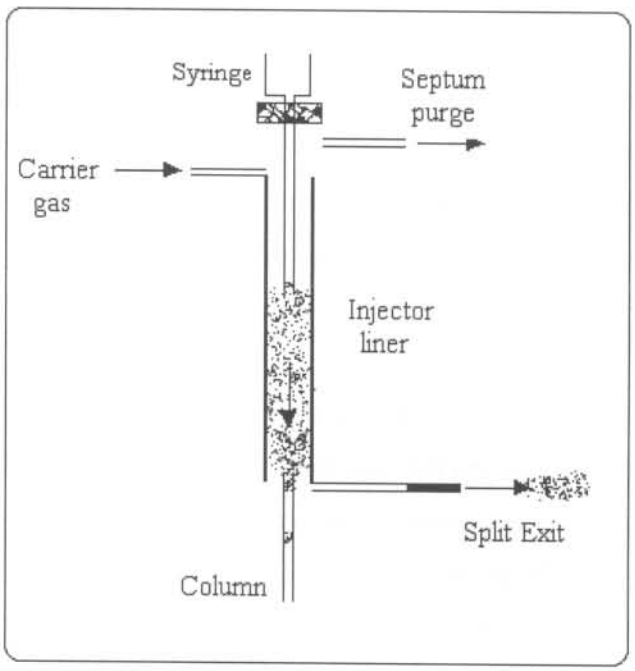

Figure 4.2 The split injector: a small portion of the injected sample enters the capillary column and a major portion is vented through the split exit [2].

The split system is suitable for introducing samples in the micro and sub-microlitre range, and it is mostly used for relatively concentrated samples. The success of the split injection depends on some instrumental and personal factors. The split injector must vaporise the sample rapidly and completely and should mix it with the gas stream thoroughly [3]. Imperfections in injection, either from the instrument or the analyst, result in inaccuracies in the amount of sample introduced and distortions to the peak shapes. During manual injection into a hot injector liner the sample may start to evaporate inside the needle. This problem creates discrepancies between solutes injected due to losses on the needle wall. High boiling components, especially, are discriminated against. The success of manual injection depends on the analyst's good command of syringe techniques in rapidly executing the injection. To avoid such problems, either high boiling solvents or fast auto-samplers should be used. Properly executed split injections provide sharp initial bands [2].

4.1.2 The Analytical Columns

The analytical column, one of the main parts of a gas chromatographic system, is where all the sample interactions and separations take place. Fused silica capillary columns are produced in a wide variety of lengths and internal diameters. There are also a wide variety of stationary phases used with these columns, ranging from non-polar to highly polar substances.

The mechanism of separation in gas chromatographic columns depends on the type of stationary phase used. Depending on their chemical and physical properties (molecular mass, vapour pressure, polarity, solubility, stereochemistry, etc.) sample components are temporarily adsorbed on or dissolved in the stationary phase. The extent of stationary phase interactions affects the retention time of particular components. Effective separation is possible if sample components show different selectivity and interaction with the stationary phase [4].

Effective gas chromatographic analysis of samples of any type is influenced by parameters such as column length, column internal diameter, stationary phase film thickness, temperature programming rate, carrier gas linear velocity, etc. Good separation is possible when the above-mentioned parameters are successfully optimised and work in harmony according to the demands and requirements of the samples to be analysed.

This research was done under specific column parameters including column length, internal diameter, film thickness, and type of stationary phases. The GC×GC system consisted of two column sets. The first set consisted of a ~ 28.6 m length × 250 μm internal diameter (I.D.) × 0.250 μm film thickness (d_f) non-polar, 100% dimethylpolysiloxane stationary phase (HP-1, Hewlett Packard Corp., USA) first-dimension column and a 1 m × 100 μm I.D. × 0.100 μm d_f medium polar, 14% cyanopropylphenyl 86% dimethylpolysiloxane stationary phase (Rtx-1701, Restek International, USA) second dimension column. The second set consisted of the same first dimension column and a polar carbowax stationary phase (Rtx-Wax, Restek International, USA) second dimension column with the same dimensions as above. The use of serially coupled columns of different dimensions and stationary phases is the main theme

exploited in comprehensive two-dimensional GC to effect superior sample resolution, as has already been dealt with in the previous chapter.

4.1.3 The Detector

The detector monitors analytes after the separation process depending on the different physical and chemical properties, which the eluted substances show towards it. In gas chromatography, the detector generates electrical signals, which are continuously recorded as a chromatogram. The intensity of this signal changes during the elution of a solute depending on the solute concentration or mass flow reaching the detector. When only the carrier gas reaches the detector, a 'straight' baseline is observed, but with increase in temperature, column bleed material is also measured, resulting in a continuous rise in the baseline [4].

There are different types of detectors in gas chromatography, amongst others the thermal conductive detector (TCD), which works on the principle of changes in the thermal conductivity of the carrier gas caused by the presence of a solute. Another type of detector used in GC is the electron capture detector (ECD). This detector's measuring mechanism depends on the electronegativity of the eluted components, *i.e.* their ability to form negative ions by capturing electrons. A third type of GC detector is the photoionisation detector (PID), which measures the amount of ionisation current produced when the solutes are irradiated with vacuum UV photons. Other types of detectors include the nitrogen-phosphorous detector (NPD), the sulphur selective detectors and the flame ionisation detector (FID) [5] that has been used throughout this study.

4.1.3.1 The Flame Ionisation Detector (FID)

The FID, which was introduced in 1958 is the most common mass flow type detector used for the detection of organic compounds [5]. The signal of the FID is generated by the ionisation of molecules of the eluted compounds in a hydrogen-oxygen (air) flame. When trace amounts of hydrocarbons or any carbonaceous material, including stationary phase bleed, enter the flame zone, the detector response increases due to the ionisation of these materials. The FID response is the sum of all the ionisation processes taking place in the

flame during the elution of organic substances. The response is highest for hydrocarbons, being proportional to the number of carbon atoms. The detection entirely depends on the mass flow of the solute into the detector and increase in carrier or make-up gas flow has no effect on detector signal. The area of the peak recorded during elution of a separated solute, is proportional to the amount of the relevant solute [4, 5].

The FID is a very sensitive detector, which has an ionisation efficiency of about 10^{-1} C/mole *i.e.*, a change of 10^{-6} mole per second solute in the detector leads to a change of 10^{-7} A in the current [5]. It has also a very broad dynamic range, the range of sample concentration for which the detector provides reproducible quantitation, covering a range of 10^{7} between the highest and lowest detectable mass flow.

4.2 Interfacing and Modulator Types in Comprehensive Two-Dimensional Gas Chromatography (GC×GC)

The proper performance of any multi-dimensional technique mainly depends on the quality and proper method of interfacing used. In comprehensive two-dimensional gas chromatography, the modulator plays this crucial role. In order to preserve the separation obtained and to further separate overlapping peaks from the first dimension column, the modulator collects peaks eluting from the first dimension column and re-injects each fraction into the second dimension column as sharply focused pulses. The modulator is the heart of a comprehensive two-dimensional GC system. The main functions of a GC×GC modulating interface include the following [6, 7]:

- Trapping and concentration of the effluents from the primary column during the trapping time, avoiding the possibility of remixing already separated components in the second dimension column, while the first dimension separation proceeds,
- Focusing of the trapped solutes into very narrow pulses, and
- Injecting the trapped solute plugs onto the head of the second column quantitatively, in as narrow a band as possible, for further separation and elution before the next batch of solutes is injected.

To perform these three functions properly for the whole chromatographic run, the modulator:

- Must start trapping again as soon as possible after each re-injection to prevent breakthrough of analytes eluting from the primary column at this time [6, 7],
- Should not only effectively retain substances diverted into it, but also should be of low thermal mass for rapid heating to allow rapid re-introduction of the trapped fraction into the second column [8].
- Should concentrate and re-inject solutes reproducibly and without discrimination across a range of anlaytes [7].

There are some factors that affect the above-mentioned interface (modulator) operations, including [9]:

1. The carrier gas velocity, which affects the width of the pulse leaving the modulator. High column head pressure with high carrier gas velocity produces sharper peaks mainly due to the shorter retention times in the second dimension column.
2. The heating and cooling efficiency of the modulator,
3. The thickness of the stationary phase. The modulated part of the column should not be exposed to higher temperatures, which may decompose its stationary phase. Hence, the use of thin film providing less analyte retentions is called for.
4. Heat capacity of the modulated column part. Insufficient heating of the modulated column part may result in irregular peak shapes.

4.2.1 Types of GC×GC Modulators

Since the publication of the first paper on comprehensive two-dimensional gas chromatography by Liu and Philips in 1991 [10] numerous authors on GC×GC have published work on new or modified modulators. Generally, all the modulators used so far can be considered to be of two types: thermal and valve flow modulators. The thermal modulators are the most widely used and constitute various designs and modes of operations. The first of the thermal modulators is the on-column thermal desorption modulator described by Liu and Philips [10], which was followed by the longitudinally modulated cryogenic

system (LMCS) used by Marriott *et al.* [11]. Later, a mechanical thermal sweeper modulator was described by Blomberg *et al.* [12] and recently jet cooled modulators were described by Ledford *et al.* [13] and then by Beens *et al.* [14]. For the purpose and convenience of this dissertation, GC×GC modulators are divided and described into five types, *viz.*:

1. The on-column thermal desorption modulator
2. The mechanical thermal sweeper modulator
3. The longitudinally modulated cryogenic system
4. The differential flow or switching valve modulator
5. The jet cooled non-moving cryogenic modulator

1. On-Column Thermal Desorption Modulator (TDM)

An on-column thermal desorption modulator is a simple trapping and focusing arrangement which constitutes part of the head of the second dimension column. It is prepared by spraying an electrically conductive paint onto the modulator portion of the second dimension column to obtain a uniform film by multiple coatings. The paint-coated part of the column is dried in the oven after each coating and its resistance measured until the target resistance is reached [6, 10]. The total length of the modulator part of the column depends on the length of the second dimension column (15 - 20 cm). The modulator part is divided into two stages and requires no moving parts. The two stages have different lengths, which help to improve the chromatograms. Making the first stage, the accumulator stage, longer prevents sample breakthrough. A shorter, focussing second stage accelerates the concentrated pulses to the velocity of the carrier gas in the rest of the second dimension column [6, 15, 16].

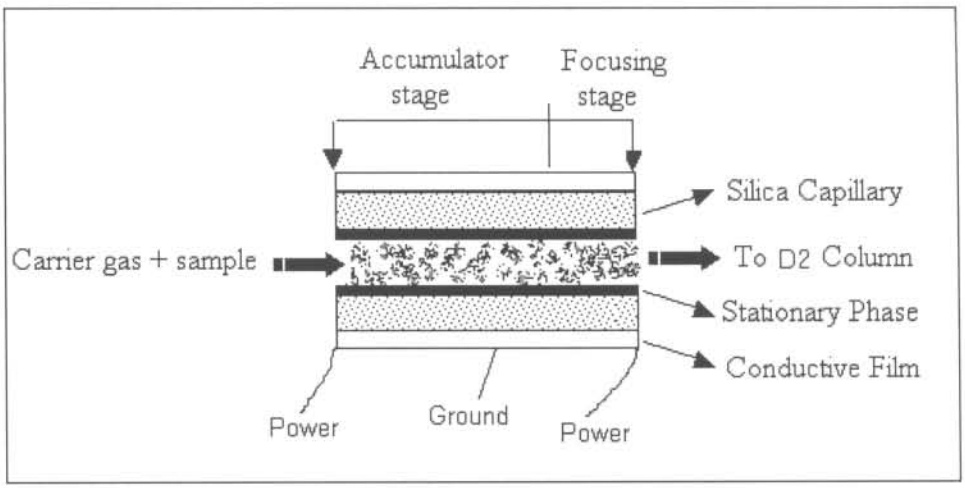

Figure 4.3 An On-Column Thermal Desorption Modulator [17, 18]

After the first dimension column separation, the analytes are trapped for a certain period while the modulator is at room temperature in a separate temperature programmable box outside the GC oven. Next, they are quickly transferred to the second dimension column by applying a short current pulse to the two modulator stages, one after the other. The heat pulse releases components trapped in the stationary phase to be carried away by the mobile phase to the second dimension column. After each cycle the modulator is cooled by ambient air and the trapping and releasing process is repeated in the same manner. Such computer controlled current pulses are generated repetitively until the end of the run [6, 10, 16].

The initial modulator temperature should be low enough to retain solutes, the final temperature high enough to release them and the ramping rate must be fast enough so that the column rather than the modulator limits peak broadening. In this case, the fast second dimension column produces sharp secondary chromatograms while separation is in progress in the first dimension column. A comprehensive two-dimensional gas chromatogram is obtained by plotting all the second dimension chromatograms [15, 17].

The low thermal mass of fused silica columns and their ability to change temperatures rapidly are the two key features that affect the success of the TDM. The thin film of conductive paint affords superior thermal contact while inducing insignificant change in mass [16]. The TDM should not be heated for longer than the time required for the trapped

analytes to be pulsed out of it. The linear gas velocity and the length of the TDM determine the minimum heating required to release all trapped analytes from the TDM [19].

The most problematic aspect of this type of modulator is the application and curing of the conductive film, attaching electrical leads of low thermal mass and avoiding cold spots. Moreover, thin film modulators may burn out at unpredictable times, limiting the modulator's life span [15]. In broad terms, the TDM lacks robustness for practical use [12].

2. Mechanically Driven Thermal Sweeper Modulator

A thermal sweeper modulator is a device that controls the temperature of a short length of a gas chromatographic column as a function of position and time. This modulator has two main components, *viz*. the modulator tube or modulator capillary used to serially connect the two capillary columns and the rotating slotted heater. The function of the slotted heater is to periodically sweep over the thick-film stationary phase section of the modulator tube to focus the solutes into very narrow bands and force them to evaporate from the stationary phase into the mobile phase. The thick film of the modulator tube helps to retard the solutes before they are swept away by the modulator. The narrow bands are then carried away by the higher linear velocity of the carrier gas into the second column for fast separation. The short second dimension column with high phase ratio (thin film) and small inner diameter allows all peaks to be eluted and detected before the subsequent thermal modulator injection [12, 20].

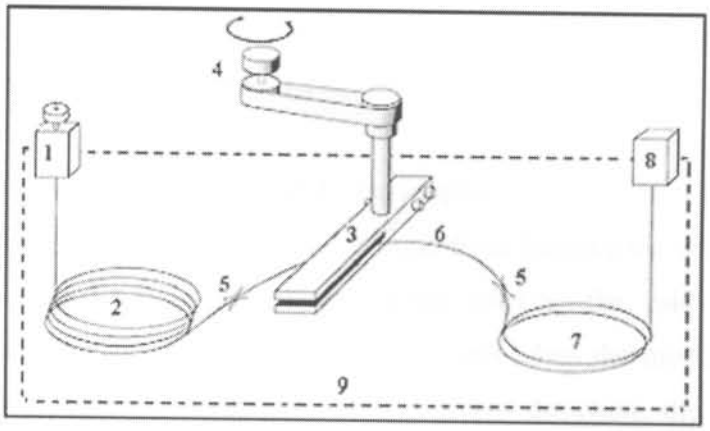

Figure 4.4 Schematic diagram of a GC×GC system with the thermal sweeper modulator. (**1**) Injector, (**2**) 1st dimension column, (**3**) Heated slot, (**4**) Stepper motor, (**5**) Press-fit, (**6**) Modulation capillary, (**7**) 2nd dimension column, (**8**) Detector & (**9**) GC oven [12].

As noted above, during the transfer of components from the first dimension column to the second dimension column their bandwidths are greatly reduced. The reduction in bandwidth enhances the peak height and hence the limit of detection of GC×GC is much lower than in one-dimensional capillary gas chromatography [21].

When using the thermal sweeper modulator, the temperature of the modulation capillary should be kept at least 100 K lower than the temperature of the slit of the sweeper so that the modulator can effectively focus and produce sharp solute bands. Exposing the modulator capillary to temperatures above its maximum produces column bleed, hindering the analysis [21].

In addition to the temperature difference required between the sweeper and the modulation capillary, the overall design of the thermal sweeper modulator is complex. It has several moving parts, occupies large oven space and the GC has to be greatly modified to accommodate the modulator [22].

3. Longitudinally Modulated Cryogenic System (LMCS)

The longitudinally modulated cryogenic trap, as detailed in the works of Marriott and Kinghorn [11, 23, 24, 25, 26, 27 and 28] is constructed from a hypodermic-steel tube with three openings on the sides. One of the openings at the centre of the cryo-trap serves for cryogenic entrance and the other two holes on left and right ends of the sides of the trap are used for venting the coolant out (see details in Figure 4.5).

When the trap is in place, liquid cryogen (CO_2) is supplied through the trap inlet and expands to cool the body of the metal-trap. To prevent the trap from freezing the column, a small flow of nitrogen is allowed through the centre. Solutes entering the cold trap are stopped, accumulated and focused into sharp bands. This happens when the modulator is in the so-called *Trap* position. Moving the trap to the up-stream direction, the *Release* position, exposes the focused band to the oven temperature. When the trap is in the release position the cryogen supply is temporarily interrupted. The oven heat forces the band to be moved into the second dimension column and then continues its migration to the detector. The GC,

through a solenoid, controls cryogen supply and the trap movement is controlled by pneumatic drive [27] or by an electrically driven solenoid [26]. The modulator movement between the trap and release positions is repeated at a predetermined frequency for the duration of the chromatographic run [27].

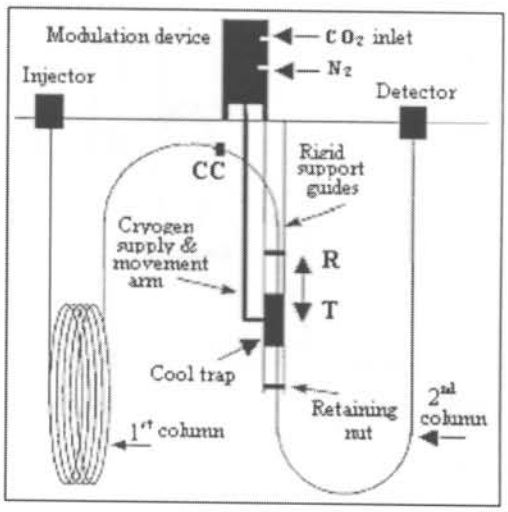

Figure 4.5 Schematic diagram of the LMCS in a GC×GC system. (**CC**) Column coupling, (**T**) Trapping and (**R**) Release positions. The capillary is held by retaining nuts to the support. The cool trap is connected to a cryogenic supply and movement arm, and moves back and forth along the guides [28, 29].

A slightly different design of a moving cryogenic modulator from that of Marriott *et al.* is the one described by Beens and co-workers [30]. This cryogenic modulator has a cooling chamber and heating coil to help accelerate the heating of the trapped solutes. But, as reported by the authors, the heating coil was later removed, as it did not effectively perform the intended process of heating the trapped solutes for remobilisation. A pneumatic ram, connected to the cooling chamber with stainless-steel tubing from the top of the GC oven, performs the up and down movements of the modulator.

In this design the cryogen (CO_2) is directly sprayed on the capillary column inside the cooling chamber, instead of cooling a stainless steel modulator tube. Air or moisture is prevented from entering the cooling chamber by the escaping gas, avoiding any problem of freezing inside the chamber [30].

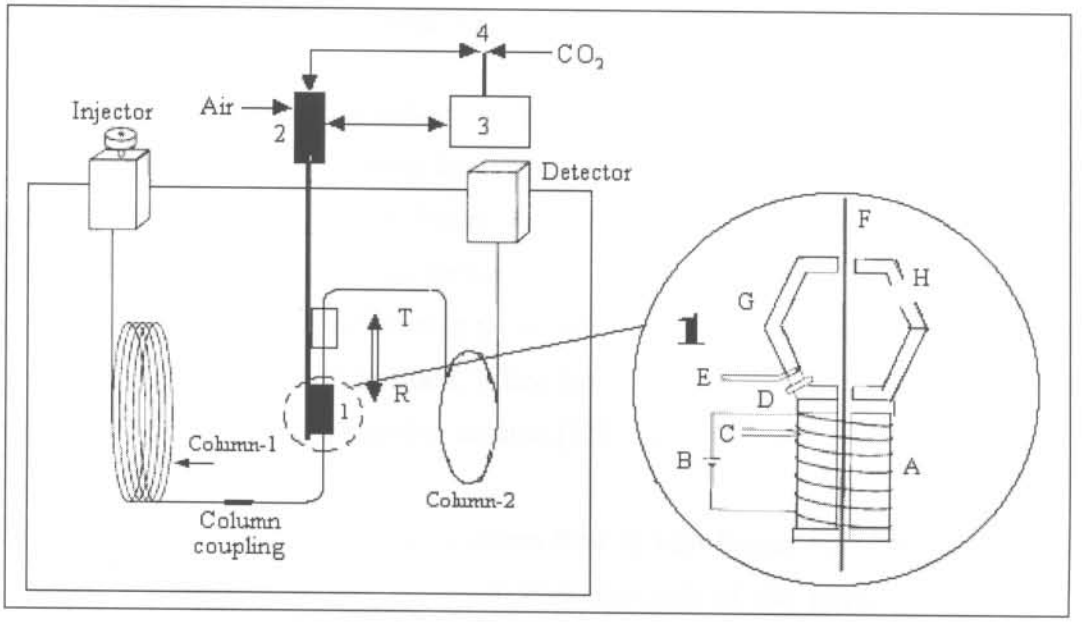

Figure 4.6 Schematic diagram of a moving cryogenic modulator set-up. (**1**) Modulation chamber, (**2**) Pneumatic ram, (**3**) Modulation controller, (**4**) Two-way CO_2 valve, (**T**) Trapping position and (**R**) Release position. *Insert*: (**A**) Heating coil, (**B**) Electric power for heating coil, (**C**) Thermocouple, (**D**) Temperature sensor, (**E**) CO_2 inlet with orifice, (**F**) capillary column, (**G**) Cooling chamber and (**H**) CO_2 outlet [30].

Compared to the thermal sweeper modulator, the longitudinally modulated cryogenic system has some practical advantages. When using the LMCS there is no need for using an extra thick film modulator capillary, only one ordinary column connector is used and there is no temperature restriction [26]. Therefore, the cryogenic modulator has a practical advantage, as heated modulators will put extra thermal stress on thermo-labile compounds during the few seconds of heating and they have limited potential for the remobilisation of high boiling compounds.

One of the problems in using the moving cryogenic modulator is the frequent breaking of the fragile second dimension column due to its contact with the moving modulator part [30].

4. Differential Flow Modulation

The differential flow modulator utilises a 6-port diaphragm valve fixed at the centre of the GC platform to collect effluents from the first dimension column and periodically inject them into the second dimension column. Fused silica unions connect the deactivated fused silica transfer tubes to the columns inside the GC oven. Solutes exiting the primary column enter the 6-port valve and they are collected in the sample loop, a deactivated stainless steel tubing, when the valve is in the collect position. When the valve is in the inject position the contents are transferred to the second dimension column [22]. (Refer to Figure 4.7b for details).

As the authors put it: "The secondary column flow is kept higher than the primary column flow. Thus, the volume of gas collected from the exit of the primary column can be transferred to the secondary column as a pulse with a width of approximately 5% of the collection time. In the absence of substantial axial mixing, the flux exiting the loop in the inject position should be a compressed mirror image of the flux that entered the loop during the previous collection cycle. The sample plug then passes through the secondary column where chromatographic retention and peak broadening occur. Assuming only moderate broadening, the peak flux exiting the secondary column is still several times higher than the flux that originally exited the primary column due to the higher secondary-flow. Detectors that have a response proportional to component flux, such as the flame ionisation detector, will have increased response when the differential flow method is used" [22].

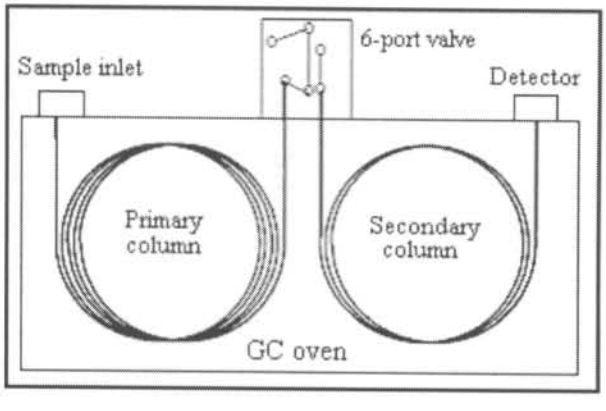

Figure 4.7a Schematic diagram of a differential flow modulated GC×GC System [22].

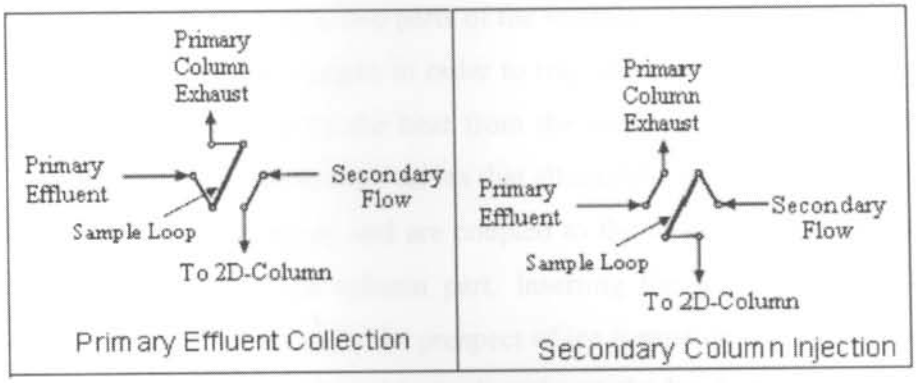

Figure 4.7b Details of the 6-port differential flow modulator [22].

The differential flow GC×GC system features a simple modulation system, which is cost effective as it can be constructed from easily available non-expensive materials, requires no great modification to commercial GCs and the system is quite durable [22]

The incomplete sample transfer from the first column to the second column in the valve based flow modulators restricts the use of these modulators to relatively concentrated samples. The other drawback of using this modulator is the limited high temperature tolerance of the diaphragm, which hinders the use of this modulator for the analysis of high boiling components [31].

5. Jet Cooled Non-Moving Dual Stage Cryogenic Modulator

The jet cooled, dual-stage, cryogenic modulator is another type of modulator used in comprehensive two-dimensional gas chromatography to enable the efficient trapping and re-injection of sample components eluting from the first dimension column to the second dimension column. There are basically two types of jet cooled, non-moving modulators. The one described by Beens *et al.* [14] uses two cold CO_2 jets to alternately trap analytes. Oven temperature is used to remobilise the focused spots while the cold jet is off. The other type, a prototype from Zoex corporation (Zoex, Lincoln, NE, USA), which has been employed in this project, has two cold and two warm nitrogen jets used to trap and re-inject the effluents from the first dimension column to the second dimension column.

In the dual stage CO_2 jet modulator, two parts of the modulated capillary column are directly and alternately cooled with the cryogen in order to trap and focus each subsequent fraction, which afterwards is remobilised by the heat from the surrounding oven air. The CO_2 jets consist of two electrical-driven two-way valves that alternately open and close the liquid-CO_2 line through two pieces of capillary and are coupled to the nozzles through which the cold gas is sprayed on the modulated column part. Inserting the jet tubes in a brass socket increases their heat capacity and curbs the prospect of ice formation on the outside of the jets at lower temperatures. Spraying the cold gas directly on the head of the second dimension column cools the column quickly down by about 100°C below the oven temperature. When the valves are closed and the cooling is interrupted, surrounding warm air from the GC oven heats up the cooled part of the modulated capillary to oven temperatures in a very short span (*ca.* 13 ms). A timing device controls the modulation process [14].

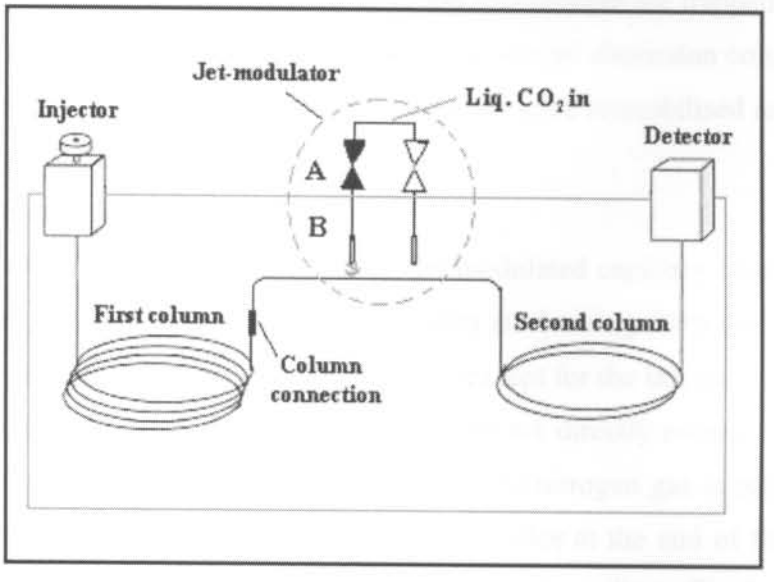

Figure 4.8a A GC×GC system with the dual-stage non-moving CO_2 jet modulator: (**A**) CO_2 valves and (**B**) CO_2 nozzles [14].

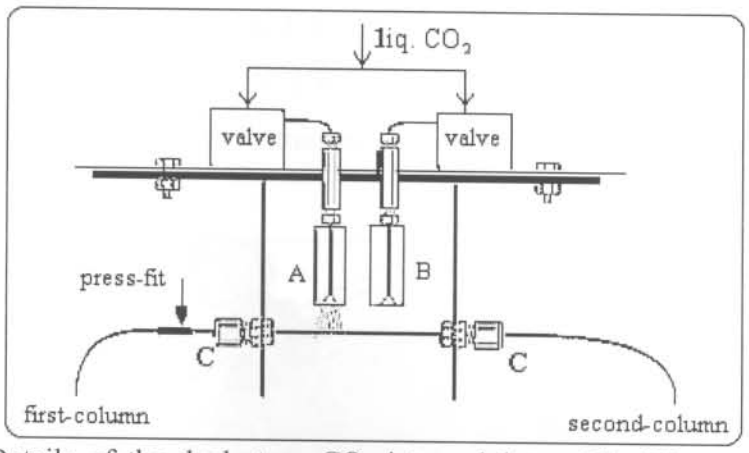

Figure 4.8b Details of the dual stage CO_2 jet modulator: (**A**) CO_2 nozzle on, (**B**) CO_2 nozzle off and (**C**) Tight column unions [14].

In the dual-stage nitrogen cooled and warmed modulator, cool and warm nitrogen gas pulses are used to rapidly alter the temperatures of part of the head of the second dimension column for two-stage modulation. Analytes eluting from the first column are trapped and focused by a continuous stream of cold nitrogen at the head of the second dimension column. Alternately operating hot nitrogen gas jets heat the trapped analytes to be re-mobilised as sharp pulses to the rest of the second dimension column [14].

The cold jet tubes are mounted directly above the modulated capillary from the roof of the GC oven and are insulated inside a vacuum-stainless steel tubing from the top of the liquid nitrogen container unit (dewar) until inside the oven except for the last part, which is exposed to the oven temperatures. Inside the dewar these tubes are directly connected to two copper coils immersed in liquid nitrogen. A continuous flow of nitrogen gas is passed through the cooled coils and is expelled continuously from the nozzles at the end of the stainless steel tubes inside the GC, forming cold spots on the modulated capillary. The hot jet tubes, also mounted from the roof of the GC oven at right angles to the cold jet tubes, are fed with nitrogen gas by two Teflon tubes from the top of the GC. The hot jet tubes have wider diameter than the cold jet tubes to enable them to efficiently heat the cold spot after each cold trapping. To facilitate the heating of the gas, the wider diameter tubes are passed through a drilled-in heating block mounted before the exit of the gas inside the GC oven. All the operations of the modulator, including heating of the heating block are facilitated and monitored by a KT-2001 (Zoex, Lincoln, NE, USA) control board. The level of the liquid

nitrogen in the dewar is controlled by TERAGON liquid nitrogen level controller (TERAGON Research, Sanfrancisco, USA). The GC×GC system is interfaced with the computer *via* an SCB-68 (National Instruments, Austin, Texas, USA) interface modem.

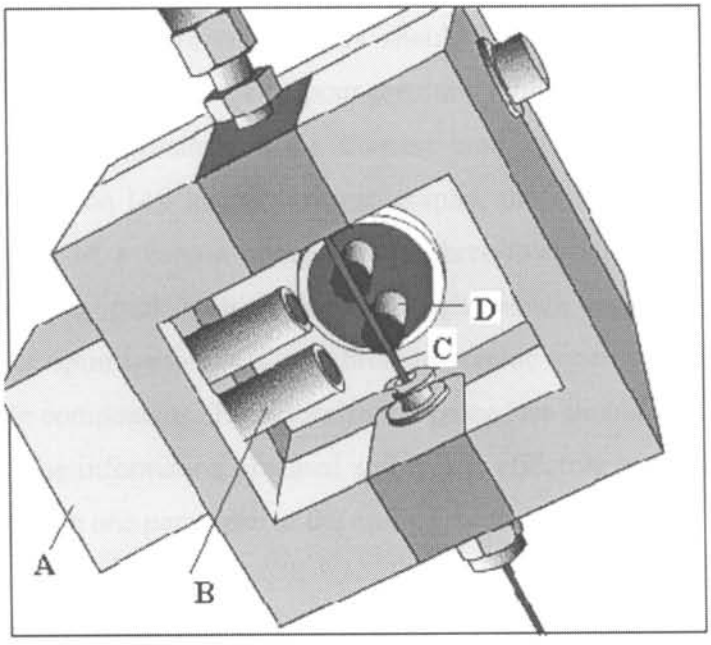

Figure 4.9 The cryogenic jet cooled and warmed dual-stage non-moving modulator. (**A**) Heating block, (**B**) Hot gas jets, (**C**) Modulated capillary and (**D**) Cold gas jets.

The part of the second dimension column in which the modulation takes place is stretched and secured between unions mounted in stainless steel bracket. The tightly stretched modulated capillary is secure from being vibrated by the gas continuously sprayed onto it [14]. Contact of the column with the metallic unions is prevented by passing the column through graphite-vespel ferrules placed on both ends of the tightening Swagelocks.

The dual-stage, non-moving jet modulator is very robust and modulation is performed satisfactorily. Very low temperatures are reached, enabling the modulation of compounds with very low retention factors. As it lacks any moving parts, column breaking during modulation is unlikely [13]. A point of concern is that the extra heating needed to remobilise trapped fractions puts the second dimension column under extra stress and shortens its life span. Moreover liquid nitrogen, although a very effective coolant, is expensive and needs bulky insulation when transported through tubing [14].

Part II: OPTIMISATION OF EXPERIMENTAL CONDITIONS

4.3 Optimisation of Gas Chromatographic Parameters

The purpose of optimising any analytical system is to get the maximum possible information from it. Optimisation in gas chromatography should be done with the ultimate goal of effectively separating the widest range (vapour pressure, molecular mass, polarity, functional group, etc.) of substances possible, in the shortest time, at the lowest cost and with the highest possible resolution [4]. In gas chromatography, the information one gets from the detector output signal of a certain component is three-fold, *viz*. retention time used for qualitative identification, peak width and peak height which together give a measure of quantity. The proper optimisation of a gas chromatographic system involves improving the resolution of sample components. The optimisation procedure should only be carried out if it generally improves the information obtained and if it is effective in terms of cost and time. One should not optimise one parameter at the cost of another [8].

Optimisation in chromatography revolves around the resolution between two peaks called the critical pair and encompasses the optimisation of different variables, which are interdependent. The number of variables which need to be optimised and their interdependency becomes much more complicated and even more difficult in two-dimensional than in linear GC, since two serially coupled columns of different dimensions are employed. However, as we were working under some fixed variables such as column length, internal diameter, carrier gas type, stationary phase composition and film thickness, the number of variables that needed to be optimised around these conditions was brought down to the minimum. Therefore, the linear gas velocity in both columns and the temperature programming rate(s) were the two main parameters that were optimised.

A chromatographic peak is characterised by the retention time (t_R) and the peak width. The complete resolution of two components depends on their having base-line separated Gaussian shaped peaks with an average peak width smaller than their retention time difference. The retention time difference is affected by the type of stationary phase used, the column length, the vapour pressures of the components, column temperature and activity coefficient of the

two species. Mathematically, the resolution of two peaks can be defined by equation ((4.1a) and (4.1b)) [4, 32]:

$$R = \frac{2\Delta t_R}{w_{b1} + w_{b2}} \qquad (4.1a)$$

$$R = \frac{2\Delta t_R}{4(\sigma_1 + \sigma_2)} \qquad (4.1b)$$

Where Δt_R is retention time difference between two peaks

w_{b1} and w_{b2} are the peak widths at base

w_h is width at half height and

σ is the standard deviation

For a Gaussian peak $w_b = 4\sigma$ and $\sigma = (\frac{w_h}{2.355})$

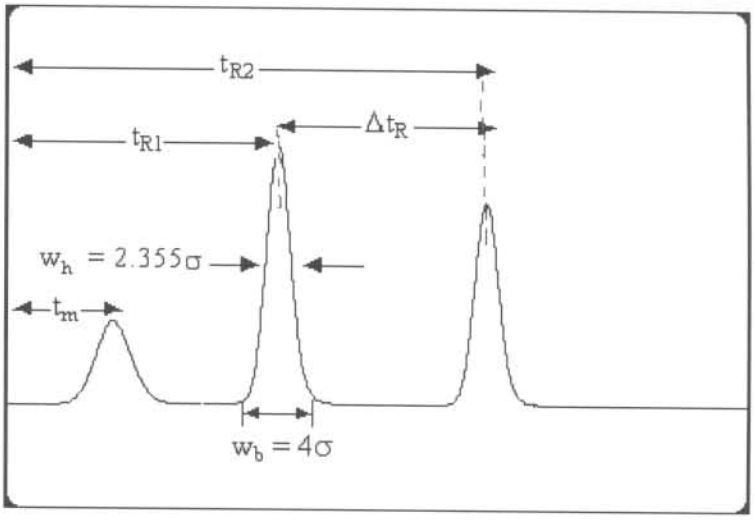

Figure 4.10 Resolution of two peaks: t_m is the dead time, t_{r1} and t_{r2} represent the retention times of peaks one and two.

In comprehensive two-dimensional GC the overall resolution of two neighbouring components is given by the contribution of the resolutions in both dimensions according to the relationship mathematically expressed in equation (4.2) [33]:

$$R \cong \sqrt{(R_1^{\,2} + R_2^{\,2})} \qquad (4.2)$$

If either of the resolutions is greater than one, the final resolution will always be greater than one. Accordingly, the probability of resolving two neighbouring components is more certain in two-dimensional systems than in linear techniques, because two components are much less likely to show similar displacements in the two-dimensional separation space [33].

4.3.1 Optimising the Inlet Pressure for Optimum Linear Gas Velocity[1]

In comprehensive two-dimensional gas chromatography, the first dimension column is chosen to be long and the second dimension column to be short to achieve the desired relative speeds of analysis. The first and second dimension columns normally operate at two different linear gas velocities, as the latter, by virtue of its smaller internal diameter, has a faster linear gas velocity. So, the optimisation of the linear gas velocity (inlet pressure) is to achieve the optimum speed at which the two columns can operate in harmony, that is, the best overall resolution is achieved.

The first column was 28.6 m in length with 250 μm I.D. and 0.250 μm d_f. The second column was 1 m × 100 μm × 0.100 μm d_f. To find the pressure at which the average linear gas velocity is optimum, a series of runs were performed at different pressures. Pairs of peaks were selected and their resolution was calculated using equation (4.1b) for each run. The resolution is preferably calculated from the standard deviation (σ) value, which is easily calculated from the peak width at half-height. It is inconvenient to accurately determine the width at base due to the frequent absence of a smooth baseline. Then the results were plotted against pressure for both columns (figures (4.12) and (4.14)) and the best linear velocity value was selected. In choosing the optimum pressure, the widths of the peaks were also considered. Refer to tables ((4.1) and (4.2)).

[1] The optimisation procedure was conducted using diesel samples. Diesel was chosen for its wide variety of components, which cover almost all the available GC×GC separation space. [*W. Welthagen, A.Z. Zellelow and E. Rohwer, Poster Presentation at the International Symposium on Analytical Science, Analytica 2002, Stellenbosch, South Africa]*

Table 4.1 Peak measurements for the first dimension column

Pressure kPa	t_{m1} sec	D1 L cm	u cm/sec	t_{r1} msec	t_{r2} msec	Δt_r msec	w_{h1} msec	w_{h2} msec	$4\sigma_1$ msec	$4\sigma_2$ msec	R_1
60.00	224	2857	12.75	74686	81582	6896	704	727	1195.75	1234.82	5.67
80.00	173	2857	16.51	59889	65575	5686	483	496	820.38	842.46	6.84
90.00	156	2857	18.31	52333	57402	5069	401	419	681.10	711.68	7.28
100.00	143	2857	19.98	48408	53114	4706	365	383	619.96	650.53	7.41
110.00	130	2857	21.98	45955	50441	4486	320	358	543.52	608.07	7.79
120.00	125	2857	22.86	42281	46433	4152	291	308	494.27	523.14	8.16
130.00	121	2857	23.61	39972	43920	3948	283	296	480.68	502.76	8.03
150.00	100	2857	28.57	35254	38763	3509	228	249	387.26	422.93	8.66
190.00	82	2857	34.84	28783	31675	2892	185	203	314.23	344.80	8.78
220.00	80	2857	35.71	25365	27922	2557	160	174	271.76	295.54	9.01
280.00	68	2857	42.01	20502	22582	2080	140	150	237.79	254.78	8.45
320.00	60	2857	47.62	18065	19889	1824	125	138	212.31	234.39	8.17

*For the meaning of symbols in the table, see text.

$$u = \frac{L}{t_m} \tag{4.3}$$

Where u is the average linear gas velocity in cm/sec

L is the column length in cm and

t_m is the dead time in seconds.

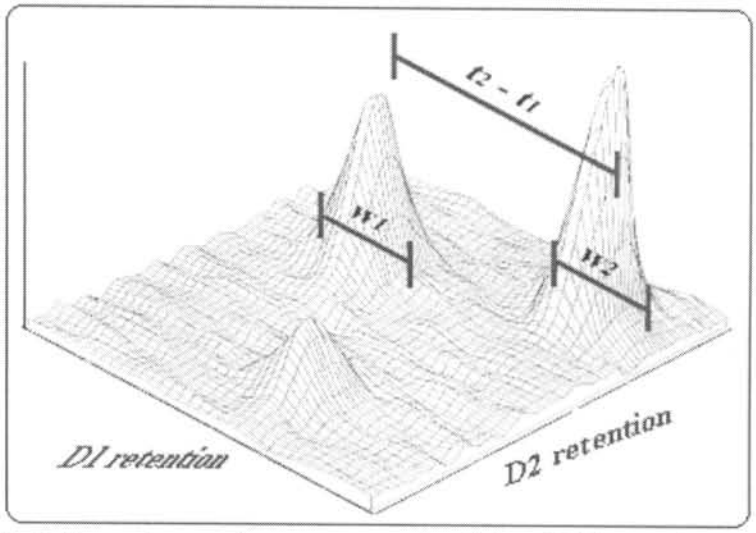

Figure 4.11 First dimension peak measurements: (t_1) and (t_2) are the retention times and (W_1) and (W_2) are the peak widths at half height for peaks one and two in the first dimension, respectively

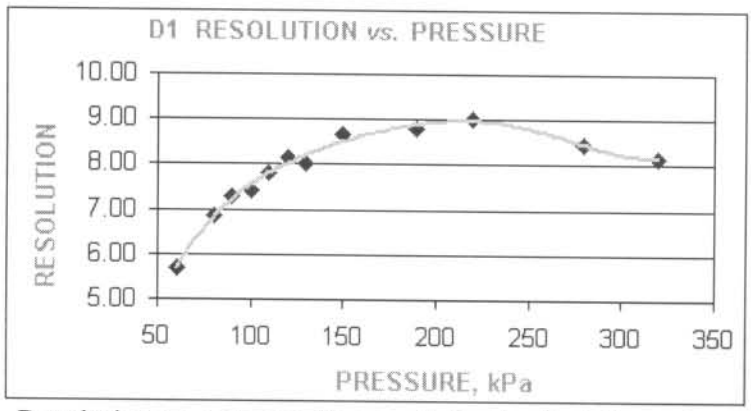

Figure 4.12 Resolution *vs.* pressure line graph for the first dimension column

Table 4.2 Peak measurements for the second dimension column

Pressure kPa	t_{m2} sec	D2 L cm	u cm/sec	t_{r1} msec	t_{r2} msec	Δt_r msec	w_{h1} msec	w_{h2} msec	$4\sigma_1$ msec	$4\sigma_2$ msec	R_2
60	751	105	139.81	2626	2926	300.00	72	90	122.29	152.87	2.18
80	561	105	187.17	2151	2401	250.00	57	65	96.82	110.40	2.41
90	500	105	210.00	2025	2270	245.00	55	61	93.42	103.61	2.49
100	447	105	234.90	1872	2107	235.00	55	60	93.42	101.91	2.41
110	410	105	256.10	1744	1964	220.00	55	60	93.42	101.91	2.25
120	374	105	280.75	1654	1864	210.00	55	60	93.42	101.91	2.15
130	344	105	305.23	1594	1903	309.00	55	60	93.42	101.91	3.16
150	300	105	350.00	1600	1800	200.00	70	75	118.90	127.39	1.62
190	237	105	443.04	1574	1754	180.00	90	90	152.87	152.87	1.18

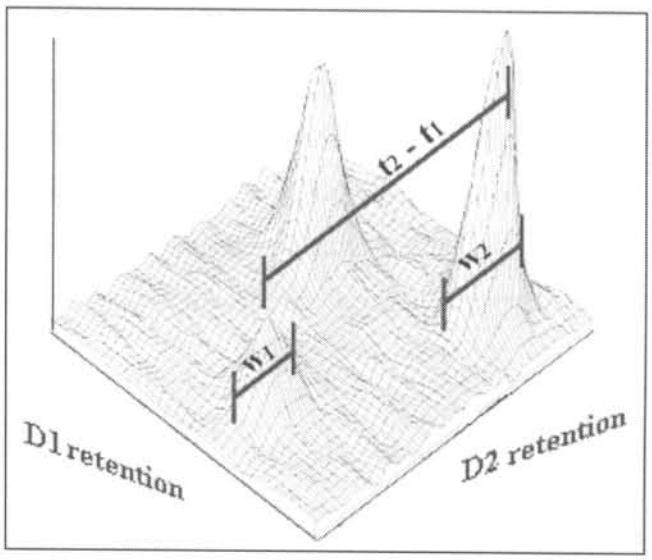

Figure 4.13 Second dimension peak measurements: (t_1) and (t_2) are the retention times and (W_1) and (W_2) are the peak widths at half height for peaks one and two in the second dimension, respectively.

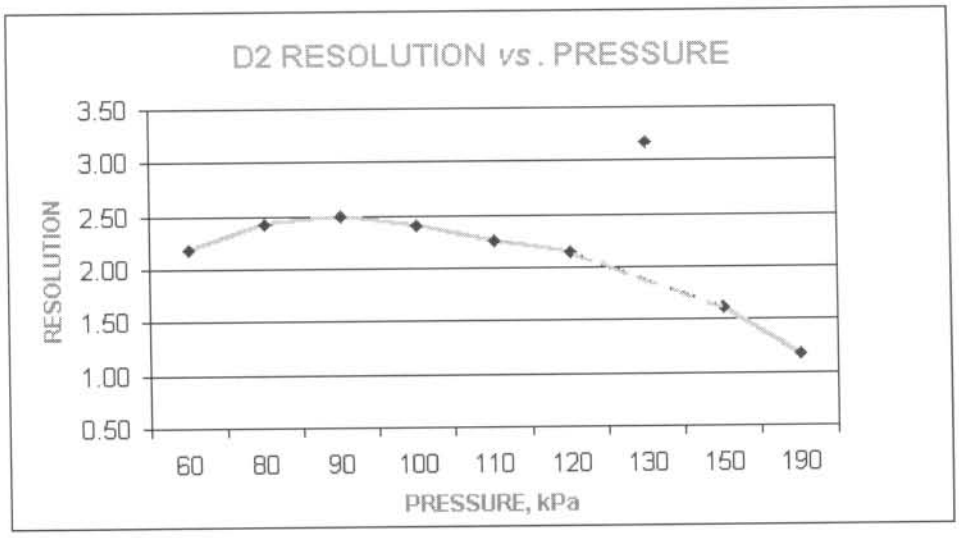

Figure 4.14 Resolution *vs.* pressure line graph for the second dimension column

As can be seen from table (4.1), at low pressures the first dimension column has very broad peaks with relatively low resolution. With increased pressure both the peak widths and resolution between the two peaks improve. On the other hand, if we see the situation in the second dimension column from table (4.2), the peak widths and resolution are at their best between pressures 100 - 130 kPa. The value at 130 kPa is treated as an experimental error.

After considering the above results, a compromise was taken between the conditions in the two columns and the working pressure was set at 110 kPa for all subsequent runs. At this pressure, the overall peak resolution is satisfactory and the peak widths in the first dimension are slightly wider than those achieved at optimum D1 resolution, however, allowing more time for the second dimension run. From a fundamental point of view, the first dimension column is run slightly slower and the second dimension column slightly faster than required for optimum resolution.

4.3.2 Optimising the Column Temperatures

The GC×GC system utilised in this research is equipped with an auxiliary oven (Aux-2) for housing the second dimension column. Therefore, the two columns could be individually temperature programmed. In optimising the temperature, two aspects were considered:

1. **The ramping rate:** The ramping rate affects the retention times of components in general. At faster ramping rates, the retention times of the components are decreased, eluting with increasingly lower retention factors, k. Ramping rates 5°C per dead times (t_m) or lower are required for optimum resolution. The lower rates are not normally, used in linear GC, as it only slows down the analysis. However, in GC×GC, slower first dimension analysis is required in order to have wider peaks and thus more time for the second dimension run. After trying several runs at 1^0C/min, 2^0C/min and 3^0C/min, a 1^0C/min ramping rate was found to be most appropriate for analysing the essential oil samples at hand.

2. **The temperature difference between the two columns**: Operating the two columns at different temperatures has a distinctive advantage in manipulating the retention time of components in the second dimension column. Increasing the temperature difference between the two columns (running the second column at higher temperatures) reduces the second dimension retention times of more polar components. However, in this case, the second dimension separation between less polar analytes is decreased (too low k values). Reducing the temperature difference towards the end of the run increases the second dimension retention time but improves the separation of less-polar high boiling components [34]. Accordingly, the second auxiliary oven was operated at 30^0C higher than the main GC oven for most of the run time. The difference is decreased slowly, and eventually the main oven temperature catches-up with the auxiliary oven temperature towards the end of the run. This temperature programme also ensures that the second, less stable, polar column, does not exceed its maximum allowable temperature at the end of the run. The final temperature programming rate used for the first column was from 30°C (2 min. hold) – 160°C at 1°C/min, then ramped to 250°C at 5°C/min (5 min hold). The second column was temperature programmed from 60°C (2 min hold) – 190°C at 1°C/min, and ramped to 250 at 5°C/min.

4.4 Optimising the Modulator for Better Performance

The prime task of a modulator is to produce narrow solute injection peaks. Pulsed peak widths of <100 ms have been reported [35]. The required final second dimension peak width

is a function of the second dimension column length and other operating conditions including temperature and carrier gas velocity. To obtain maximum resolution from the second dimension column, the modulator injection bandwidths should be much narrower than the widths produced by the column itself. This is often difficult to achieve in the case of the fast, almost unretained peaks in isothermal GC. The shapes of the second dimension injection profiles are also important. Although peak asymmetry could be the result of column performance and activity of solutes in the column, it might also indicate the inability of the modulator in delivering sharp symmetrical bands to the second dimension column.

The dual-stage modulator controls two trapping-remobilisation steps. In the first step a fraction of the analytes is trapped by the cold jet and remobilised when the first hot pulse blows on the trapped spot. Then the analytes are re-trapped by the second cold jet and after a while the second hot pulse remobilises the spot to be re-injected into the rest of the second dimension column. The accuracy of the duration of the warm pulses and the pulse delay between the two jets affects the re-injection and the final appearance of the chromatograms. Short pulses may help to introduce sharp injections, but too short a pulse may also not effectively and completely release and transfer trapped solutes. If the pulse is too long the injection band broadens and the second dimension peaks become asymmetrically distorted and tail. On the other hand a proper setting of the pulse delay, the time between the first pair of pulses and the next, is also equally important. Pulsing with too short delay reduces the efficiency of the cold jets to effectively trap and a long delay may induce freezing of the modulated capillary which will make it difficult for the hot pulses to release the trapped components. The up-stream and down-stream warm jet pulses were pulsed with 2 seconds break between them to effectively release the cold-trapped solutes from the first and then the second stages of the modulated capillary.

The modulation period was set at 6 seconds. This modulation period was long enough to allow most components to be eluted from the second dimension column. There was no serious wrap-around effect of polar compounds, especially when using the medium polar second dimension column (Rtx-1701). The wrap-around effect increases when a polar second column (Rtx-Wax) is used because of the tendency of the polar stationary phase to hold polar

components longer. The components spread out, and the more polar components show retention times longer than the set 6 seconds modulation period.

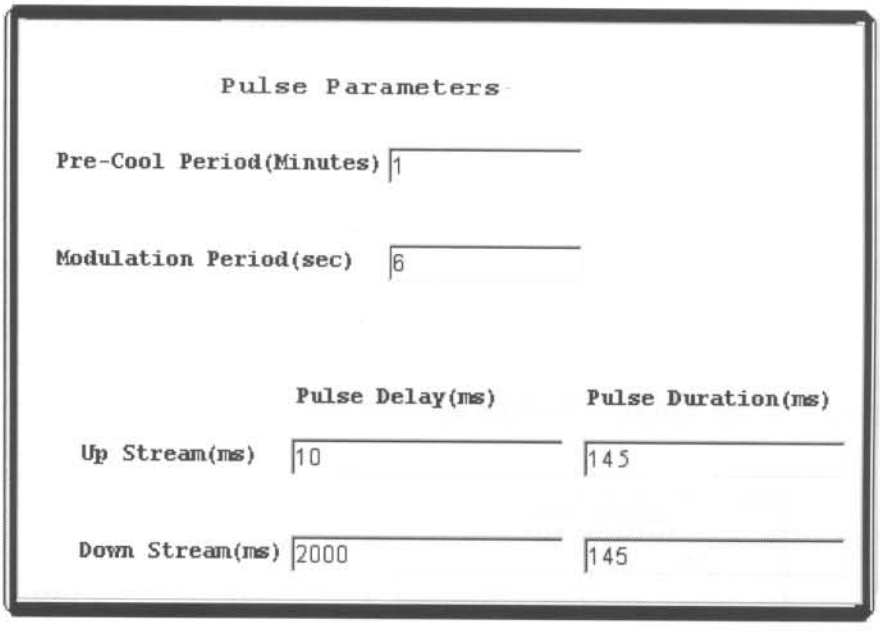

Figure 4.15 The hot pulse parameters.

The temperature of the heating block responsible for heating the hot jet tubes inside the oven is set at a constant temperature of about 250°C. Therefore, the hot jets are hot enough when they exit the nozzle so that they can effectively release the trapped solutes.

A further optimisation of the modulator involves the flow rates of the cold jets. The cold jet flow rate is optimised for two reasons: to maximise its solute trapping efficiency and to reduce liquid and gaseous nitrogen consumption and thus the associated cost. The temperature of the cold gas stream increases with the distance from the nozzle depending on the gas flow rate and the temperature of its surrounding. The higher the gas flow rate or the lower the temperature of the surrounding air, the lower the temperature of the cold gas jets [13]. The use of appropriate cold jet flow rates guarantees that solute fractions are properly trapped before being released by the hot pulses. The cold nitrogen flow was kept between 11 – 12 l/min, depending on the oven temperature. At this flow the cost of nitrogen (liquid + gas) was about R20.00 per analysis.

Excessive cooling was observed not only to prevent effective re-injection but it also induces changes in retention times, especially in the first dimension. In such cases a cold spot apparently occurs in the column just before the first modulation stage, giving rise to additional retention as shown in table (4.3). This type of problem is very hard to detect as it does not show up in the shape of either the first or second dimension peaks. It only becomes apparent when accurate and reproducible first dimension data is required for peak identiffication. The surface temperature of ferrules and Swagelocks in the modulator bracket was monitored during runs, by attaching thermocouples, to make sure that their temperature is above the oven temperature.

Table 4.3 Variation in first dimension retention times of *n*-alkanes due to improper modulator optimisation.

Compound	D1 retention times, minutes		
	Optimised modulator	Cold modulator	Δt_r, minutes
Nonane (C-9)	20.2	20.9	0.7
Decane (C-10)	32.5	33.7	1.2
Undecane (C-11)	46.6	47.7	1.1
Dodecane (C-12)	60.7	61.9	1.2
Tridecane (C-13)	74.7	76.1	1.4
Tetradecane (C-14)	87.8	89.1	1.3
Hexadecane (C-16)	112.1	113.8	1.7

Another point that needs mentioning in using this modulator is the humidity and moisture level inside the laboratory or inside the GC oven. At higher humidity levels, water vapour might condense and create frost/ice on the modulated capillary, especially at lower oven temperatures. This reduces the effective release of the trapped solutes by the hot jets. In such cases it is essential to dry the oven by pumping in dry nitrogen. Keeping silica pebbles in the oven floor also helps to ward off moisture. More discussions on the performance of the modulator will follow in the section on discussion of results (refer to (5.4), pages 5-12 to 5-15).

4.5 Stationary Phase Selection for Essential Oil Analysis

The selection of appropriate stationary phases in gas chromatography is a crucial issue as the efficiency and precision of a chromatographic column depends on the general characteristics and coating efficiency[2] of the stationary phases. Marriott *et al.* [36] have outlined the following as important points to be looked into when developing and choosing appropriate stationary phases for essential oils analyses:

- The phases should be thermally and chemically stable.
- They should give greater selectivity in the separation of components by different phase chemistry. For example, as in the two different phase columns in GC×GC.
- They should allow better efficiency by making a more regular surface coating, or producing a thinner film coating.

In developing such phases:

- Specific components should be incorporated to allow new interactions such as those available with chiral selectors.
- Different technologies could also be used to optimise the available phases to the specific regions of the analysis that require better resolution.

Although a thermally stable column indicates its reliability over extended use, most essential oils only require column temperatures of about 200°C for complete elution of components. Still this problem is more evident in the case of polar stationary phases, which show more susceptibility to high temperatures and hence have a shorter life span when compared to non-polar phases that show more efficiency and thermal stability over an extended period of time. The proper analysis of any sample depends heavily on how efficient and selective the stationary phase is [36].

The availability of a wide range of stationary phases gives an analyst the freedom of choosing the appropriate phase(s), which can properly handle the sample matrix at hand.

[2] **Coating Efficiency** (CE%) is a measure of how smoothly and homogeneously the stationary film has been deposited on the column's internal wall.

Chiral phases are essential when enantiomer separation is required, such as in authentication of the purity and quality of an essential oil or in the complete characterisation of essential oil profiles [36].

Due to their ability to fulfil the above-mentioned characteristics, silicones are the most widely used stationary phases in gas chromatography. They exist in three phases: silicone oils, which are linear polysiloxanes; silicone gums, which are linear high molecular weight polysiloxanes and silicone rubbers, which are the cross-linked gums [37].

Gum phases are preferred to liquid phases due to their ability to efficiently and easily coat on capillary walls and give higher theoretical plate numbers [37]. Silicone gum apolar stationary phases, including SE-30, SE-52-, SE-54 and OV-73, are the most frequently used phases in perfumes and essential oils analysis. In addition to the above-mentioned qualities and characteristics, these phases exhibit high inertness, low bleeding and excellent column-to-column reproducibility. The purity of the stationary phases is high and the structure well defined. The drawback of these phases comes from their low polarity [1], which prevents them from separating non-oxygenate essential oil components from oxygenated counterparts of the same volatility.

The use of the medium polar pure polypropylene glycol (PPG) or more polar polyethylene glycol (PEG) phases, especially in two-dimensional gas chromatography coupled with apolar phases, is taken as an alternative. Another alternative is to use high molecular weight (HMW) PEG and its nitroterephthalic esters (FFAP phases), but these phases show increased activity compared to pure polyethylene glycol coatings. Aldehydes, for example, are very sensitive to acid-base effects and show reversible or irreversible adsorption on immobilised films and therefore tail or disappear completely. Therefore, for the analysis of essential oils and perfumes a normal PEG column is preferred [1]. In comprehensive two-dimensional gas chromatography, the coupling of gum phase capillary columns (*e.g.* HP-1) with medium (*e.g.* RTX-1701) or high polar (*e.g.* Rtx-Wax) phases is the solution adopted in the analysis of essential oils. The performances and results of the medium polar (Rtx-1701) and polar (Rtx-Wax) second dimension columns are given in more detail in the next chapter.

References

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5 EXPERIMENTAL RESULTS AND DISCUSSIONS

5.1 Experimental Conditions used in the Analysis of the Essential Oils

In the optimised GC×GC system, 0.1 µl of essential oil sample was manually injected at a split ratio of 1:125. The essential oils were used as supplied without any dilution. The injector, operating in the split mode, was kept at 250°C. The first column (28.6 m × 250 µm I.D. × 0.250 µm d_f, HP-1, Hewlett Packard Corp., USA) was temperature programmed from 30°C (2 min. hold) – 160°C at 1°C/min, then ramped to 250°C at 5°C/min (5 min hold) for all the samples except *Tagetes minuta*. For this essential oil the temperature was programmed from 30°C (2 min hold) - 210°C at 1°C/min, then ramped to 250°C at 5°C/min (5 min hold). The second column (1 m x 100 µm I.D. x 0.1µm d_f, Rtx-1701 or Rtx-Wax, Restek International, USA) was temperature programmed from 60°C (2 min hold) – 190°C at 1°C/min, and ramped to 250 at 5°C/min. For *Tagetes minuta* it was raised up to 230°C at 1°C/min and ramped to 250 at 5°C/min. The FID detector was held at 300°C for all the runs. The carrier gas was H_2 and N_2 was used as a make-up gas. A constant inlet pressure of 110 kPa was used throughout.

Data acquisition was performed *via* a computer interfaced with the 6890A gas chromatograph (Agilent Technolgies, Wilmington, DE, USA) by SCB-68 interface board (National Instruments, USA). LabView Version 2.0x comprehensive two-dimensional gas chromatography operating program (National Instruments, USA) was used to acquire the GC×GC data. This software also translates the raw data after each analysis into text format for export to other software. Chromatograms were plotted by the use of transform (Research Systems, Noeys Version V2.0) and MATLAB Version 6.0.0.88 Release 12 (Mathworks Inc., USA) software packages.

5.2 The Power of Comprehensive Two-Dimensional Gas Chromatography

As already explained in the previous chapters, comprehensive two dimensional gas chromatography (GC×GC) has distinct advantages over linear gas chromatography, *viz.*, high peak capacity, increased sensitivity, higher resolution power, and the provision of two independent retention times for reliable component identification [1].

The high peak capacity in GC×GC is a result of the added separation dimension, which simplifies the analysis of complex samples. In GC×GC, members of the same homologous series are progressively separated due to their volatility differences in the first non-polar dimension and show similar second dimension retention times. The second dimension separation, independent of the first dimension separation, enables the compounds to form a class-type arrangement Accordingly, groups of peaks are formed occupying well-defined first and second dimension retention times, depending on the complexity and make-up of the sample analysed. Non-polar compounds, like hydrocarbons, form the first group occupying the left hand side of the two dimensional space. These are followed by medium polar components, such as monocyclic aromatic compounds, aldehydes and ketones, which exhibit higher second dimension retention times than hydrocarbons. More polar compounds including polycyclic aromatic compounds, alcohols and acids show the longest second dimension retention times and form their own distinct group in the right hand side of the GC×GC chromatogram [1, 2]. This is exemplified in figure (5.1), where components of a standard mixture were separated into their respective classes by GC×GC.

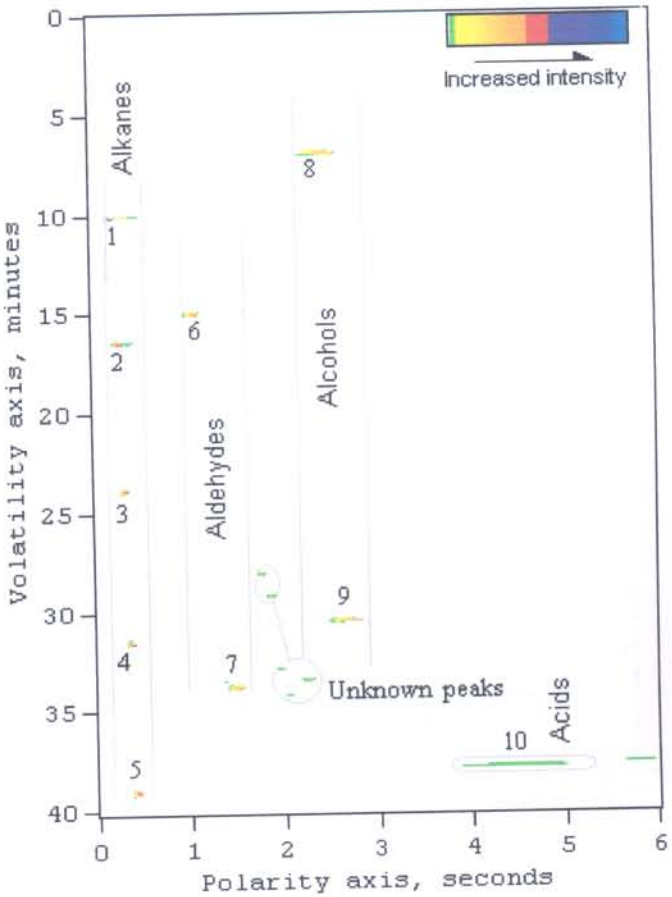

Figure 5.1 Component classes of a standard mixture separated by GC×GC[1], forming distinct groups: (**1 – 5**) are C-8 to C-12 n-alkanes, (**6**) Heptanal, (**7**) Citronellal, (**8**) 3-methyl-1-butanol, (9) Linalool, and (**10**) is Octanoic acid.

From the two alternatively used second dimension columns, the more polar Rtx-Wax column produces peaks that are well spread in the two-dimensional separation space. In the case of the medium polar Rtx-1701 column the peaks are more crowded in the second dimension, as can be clearly seen from figure (5.2). It is also evident in this figure that components with similar volatility are separated in the second dimension due to their polarity differences. The advantage of GC×GC in this case is self-evident. Had a linear system been used, for example, the circled peaks in the figure would have not been separated clearly. As each group has almost the same first dimension retention times, the compounds would have co-eluted as single peaks.

[1] GC×GC conditions: GC oven 30°C (2 min. hold) – 110°C at 2°C/min., auxiliary oven: 60°C (2 min. hold) – 140°C at 2°C/min. The second dimension column was Rtx-Wax.

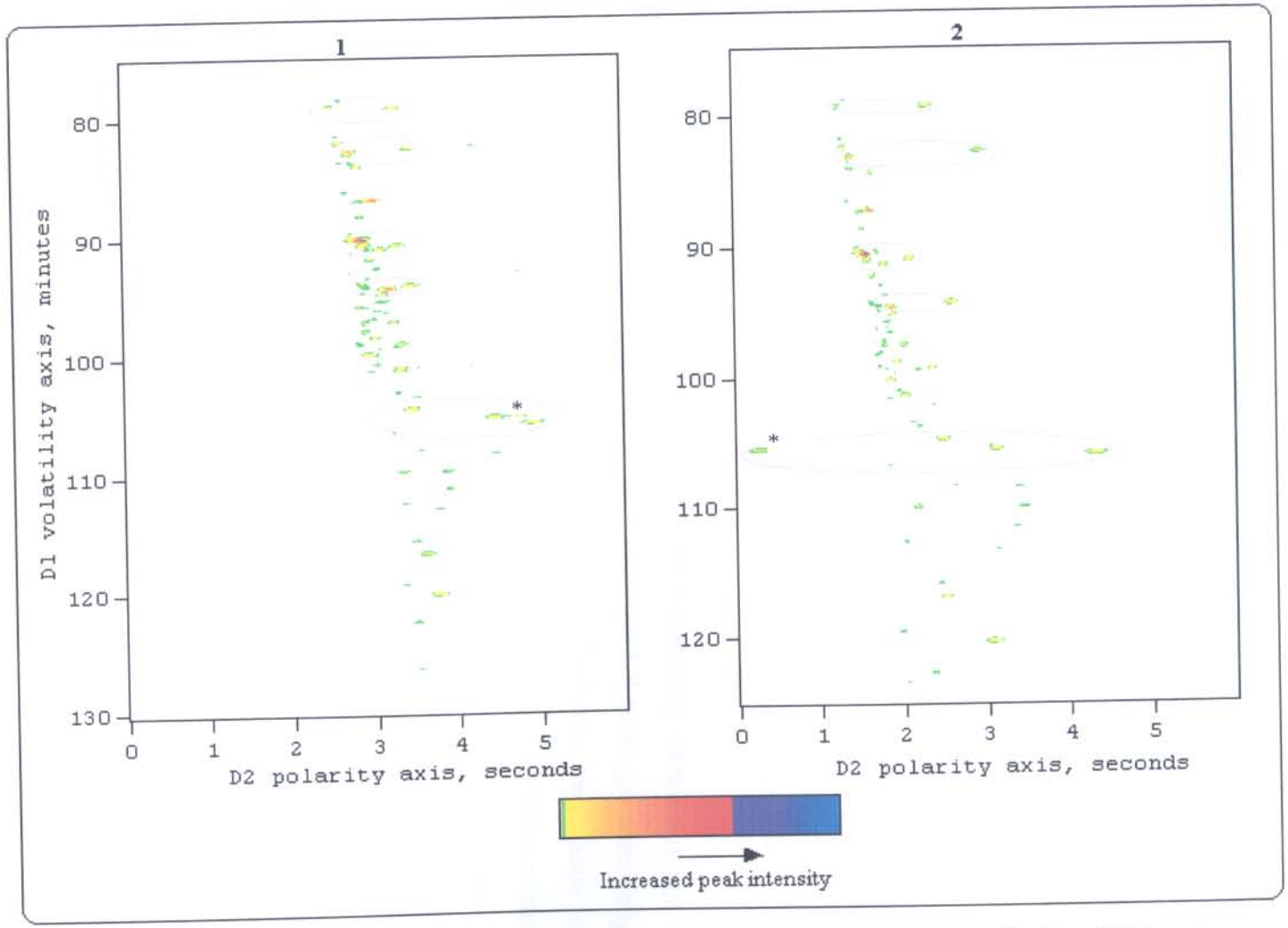

Figure 5.2 Extracted parts of GC×GC two-dimensional plots of Bourbon Geranium. (**1**) Rtx-1701 and (**2**) Rtx-Wax second dimension columns, and (*) Wrap-around has occurred for this compound in the case of Rtx-Wax second dimension column.

5.3 Qualitative Identification of Essential Oil Components by Gas Chromatography – Time-of-Flight Mass Spectrometry (GC-TOFMS)

Some of the most important parameters obtained from a chromatogram are the retention times, often used for the identification of components. Retention times are functions of many chromatographic parameters such as temperature programming rate, column dimension, stationary phase type, film thickness and linear gas velocity (inlet pressure). A change in any of these parameters brings about a change in retention times. The attempt and success of using retention times for the purpose of qualitative identification of compounds, therefore, depends on maintaining all these factors as constant as possible.

Retention time locking is a new method of reproducing retention times of identical components from instrument to instrument (between HP 6890 gas chromatographs) and from one laboratory to another using the same nominal columns, temperature programming rates and dead time, regardless of the column exit pressure and detection method used [3]. Reproducing retention times between GC-FID and GC-MS runs has been notoriously difficult because, in the latter case, the column exit pressure is normally at 0 kPa, giving rise to changes in absolute and relative retention times. Retention times are locked by adjusting the column head pressure using the RTL calibration procedure of the RTL HP ChemStation Software (ChemStation Rev.A.08.03 [847], Agilent Technologies). Adjusting the head pressure also adjusts the dead time, so that dead times also become the same when pressures are locked.

Instrument to instrument analysis methods are translated using the 'Mxlator' method translation software (GC Method Translation Software Version 2.0a, Hewlett Packard). Two methods are mutually translatable if the columns used have the same stationary phase and the same phase ratio. For exact reproduction of retention times, a constant speed gain, S, of one ($S = t_{R1}/t_{R2}$) is selected [3]. Other values of S are used to expand or compress the time axis of a chromatogram with full maintenance of peak positions.

The method translation software calculates the inlet pressure of the instrument being locked to a given chromatogram. The calculated pressure gives an approximate, but not an exact

match of the original retention time. Exact retention times are only obtained by 'retention time locking' the new method with the original method. In this procedure, a range of inlet pressures (around the calculated value) is plotted against experimentally observed retention times and the correct inlet pressure is found by interpolation to match the exact retention time of the original method [4].

The locking of the first dimension GC×GC retention times with those from a GC-MS has not been attempted before and deals with even larger difficulties in column exit pressure. In this case the exit pressure of the GC×GC first dimension is at around 100 kPa above atmospheric (required to drive the 100 μm I.D. second dimension column).

The potential rewards of successfully locking GC×GC and GC-TOFMS retention times are large, as it would effectively give both a polarity retention time as well as an accurate-mass mass spectrum of single components eluting from the (first dimension) gas chromatogram.

In this project, an attempt was made to use retention times of components analysed on a gas chromatography – Time-of-flight mass spectrometry (GC-TOFMS) to identify the same components analysed by GC×GC, using the retention time locking (RTL) and method translation procedures.

A hydrocarbon standard mixture was run using the optimised GC×GC system. Then, to get the initial GC-TOFMS target pressure the GC×GC parameters with the dead time and the GC-TOF parameters were put into the GC method translation tool. From the software the initial calculated pressure was about 16 kPa. Using this initial pressure a series of five runs were made at ±10% and ±20% of the target pressure (12, 14, 16, 18 and 20 kPa[2]) to help retention time lock the method. Then, all the retention times of the hydrocarbons with the associated pressures were entered into the RTL Software. This procedure determines, by interpolation, the value of the head pressure that has to be used in the new method, *i.e.* the GC-TOFMS for reproduction of the first dimension GC×GC retention times [4].

[2] The pressures used are the nearest whole number pressures to the calculated ±10% and ±20% of the target pressure. The GC-TOFMS accurate mass instrument used was not controlled by the ChemStation software. Under the software used only whole number pressure values are allowed.

Out of the five GC-TOFMS runs made the retention times of most of the hydrocarbons from the 14 kPa run were more close to the GC×GC retention times (run at 110 kPa), as can be seen from table (5.1). Moreover, at this pressure the retention time of nonane, C-9, from GC-TOFMS exactly locks with its GC×GC retention time. Based on this, a test run of the actual essential oils was done, but the retention times were a lot lower than the GC×GC retention times. Accordingly, more runs were made at higher GC-TOFMS inlet pressures. At 18 kPa the retention times of the essential oil components were close enough to enable the correct correlation of peaks between the two instruments. This pressure was used for all the essential oil runs and subsequent component identifications, although the retention time correlation was not entirely satisfactory.

Table 5.1 Retention times from the original GC×GC method and GC-TOFMS runs

Hydro-carbon	GC×GC t_R (min.), at 110 kPa	GC-TOFMS retention times (min.) at different pressures in kPa				
		12	14	16	18	20
C-8	11.1	11.09	10.96	10.83	10.45	10.17
C-9	**20.14**	20.40	**20.14**	20.04	19.35	18.75
C-10	32.54	32.94	32.60	32.46	31.61	30.85
C-11	46.60	47.19	46.77	46.73	45.65	44.70
C-12	60.70	60.66	60.26	59.99	59.38	58.84
C-13	74.64	75.29	74.78	74.75	73.64	72.64
C-14	87.78	88.51	87.95	87.91	86.77	85.79
C-15	112.10	112.74	112.18	112.08	111.04	110.14

Note the good retention time correlation between GC×GC retention times and those of GC-TOFMS at 14 kPa.

5.3.1 Identified Essential Oil Components

The essential oil components were identified using their GC×GC[3] and GC-TOFMS retention time correlations and accurate-mass mass spectra (designated as 'a' in the tables) and from relative retention of the compounds reported in the literature. In the case of lemongrass (*Cympobogon citratus) and Artimisia afra* additional identification of the components was obtained from GC×GC-TOFMS[4] runs of the two samples (designated as

[3] The GC×GC runs were made using the HP-1 – Rtx-1701 column set.

[4] The GC×GC-TOFMS runs were done by courtesy of Leco (USA) using Rtx-5 (30 m × 250 μm × 0.25 μm) first dimension column and DB-WAX (1 m × 100 μm × 0.1 μm) second dimension column. Oven temperature: 55°C (0.2 min. hold) – 155°C at 1°C/min. Second oven: 75°C (0.2 min. hold) – 170°C at 1°C/min.

'b' in the tables). Peaks observed only in the GC×GC chromatograms but not in the GC-TOFMS runs were left out (especially for *Tagetes minuta*, Bourbon Geranium, and *Cymbopogon flexuosus*). Some peaks identified from the GC×GC-TOFMS runs, that could not be correlated with any GC×GC counterparts were also left out from the list (tables (5.2) to (5.6)).

Table 5.2 Identified Essential Oil Components of Bourbon Geranium (*Pelargonium capitatum ×p. radens)*

Peak no.	GC×GC		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	t_{r1}, min	t_r, min	Δt_r, min	%		
1.	1.7	22.60	22.50	0.10	-0.44	α-pinene	a, [ref. 5, 6]
2.	2.0	30.00	30.00	0.00	0.00	β-myrcene	a, [ref. 6]
3.	2.1	30.90	30.89	0.01	-0.03	phellandrene	a
4.	2.5	33.00	33.08	-0.08	0.24	p-cymene	a
5.	2.3	33.80	33.80	0.00	0.00	β-ocimene	a, [ref. 6]
6.	2.2	34.20	34.25	-0.05	0.15	Limonene	a
7.	3.5	44.00	45.26	-1.26	2.86	Unknown	---
8.	2.9	45.50	45.77	-0.27	0.59	Unknown	---
9.	2.9	47.70	47.74	-0.04	0.08	Unknown	---
10.	4.1	50.70	51.60	-0.90	1.78	p-Menthan-3-one	a, [ref. 5, 6, 7]
11.	4.2	62.00	61.08	0.92	-1.48	Citronellol	a, [ref. 5, 6,7]
12.	4.1	62.40	62.48	-0.08	5.90	Citronellol acetate	a
13.	4.2	65.50	67.92	-2.42	5.42	Geraniol	a, [ref. 5, 6, 7]
14.	3.5	68.40	69.20	-0.80	3.20	Citronellyl formate	a, [ref. 5, 6, 7,]
15.	3.5	71.60	72.32	-0.72	2.23	Unknown	---
16.	3.3	78.90	79.68	-0.78	0.99	Citronellyl propionate	a, [ref. 5, 6]
17.	3.5	82.40	82.53	-0.13	0.16	α-copaene	a
18.	2.8	82.70	83.38	-0.68	0.82	β-bourbonene	a, [ref. 5, 6]
19.	3.0	86.80	87.55	-0.75	0.86	*Trans* caryophyllene	a, [ref. 5, 6, 7]
20.	2.9	90.10	91.14	-1.04	1.15	Guaiadene	a, [ref. 5, 6]
21.	3.2	94.20	95.16	-0.96	1.02	β-cadinene	a
22.	3.1	94.70	95.48	-0.78	0.82	Gurjunene	a
23.	3.2	97.10	97.62	-0.52	0.54	Unknown (similar to 91.14)	---
24.	3.0	98.30	98.86	-0.56	0.57	Germacrene D	a, [ref. 5, 6]
25.	3.3	98.90	99.42	-0.52	0.53	Calamenene	a
26.	3.0	99.70	100.37	-0.67	0.67	σ-cadinene	a, [ref. 7]
27.	3.4	101.00	101.51	-0.51	0.50	Citronellyl butyrate	a, [ref. 5, 6]
28.	4.5	105.00	105.04	-0.04	0.04	Unknown	---
29.	5.0	105.50	106.18	-0.68	0.64	Unknown	---
30.	3.7	116.50	116.89	-0.39	0.33	Geranyl butyrate	a, [ref. 5, 6, 7]
31.	3.9	119.90	120.45	-0.55	0.46	Unknown	---

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra.
[ref.] References in square brackets indicate literature where these compounds have been reported.

Table 5.3 Identified Essential Oil Components of Kakiebos (*Tagets minuta*)

Peak no.	GC×GC retentions		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	tr_1, min	t_r, min	Δt_r, min	%		
1.	*	*	4.30	*	*	4-methyl-pentene	*
2.	*	*	4.80	*	*	Cyclohexane	*
3.	1.6	14.3	14.01	0.29	-2.03	Ethyl-2-methyl butyrate	a, [ref. 8, 9, 10]
4.	1.8	27.2	27.01	0.29	-1.06	Sabinene/alpha-pinene	a, [ref. 8, 9, 10]
5.	2.0	34.5	34.47	0.03	-0.09	Limonene	a, [ref. 9, 10]
6.	2.1	36.5	36.41	-0.11	0.30	*Cis*-ocimene	a, [ref. 8, 9, 10]
7.	3.1	37.5	37.52	-0.02	0.05	Dihydrotagetone	a, [ref. 8, 9, 10]
8.	2.6	41.4	41.09	0.11	-0.27	Geranial	a
9.	3.1	48.5	48.19	0.21	-0.43	Unknown	---
10.	3.3	49.9	49.63	0.07	-0.14	*Cis*-tagetone	a, [ref. 8, 9, 10]
11.	3.6	50.5	50.53	-0.03	0.06	*Trans*-tagetone	a, [ref. 8, 9, 10]
12.	3.6	51	50.75	-0.05	0.10	Unknown	---
13.	4.4	57.9	58.10	-0.50	0.87	(2-methylprop-1-enyl)-cyclohexa-1,5-diene	a
14.	4.4	58.8	59.03	-0.43	0.73	Unknown(isomer of 58.10	a
15.	3.9	61.3	61.35	-0.35	0.57	Ocimenone (isomer unknown)	a, [ref. 8]
16.	4.1	62.2	62.17	-0.27	0.44	Carvacrol	a
17.	5.2	81.8	81.73	-0.23	0.28	Unknown	---
18.	2.8	87.1	87.05	-0.25	0.29	*Trans* caryophylline	a, [ref. 8, 10]
19.	3.0	91.2	91.20	-0.40	0.44	Unknown	---
20.	4.3	105.2	105.11	-0.51	0.49	Unknown	---
21.	4.7	155.5	154.71	-0.21	0.14	Piperitone	a
22.	4.6	161.7	160.06	-0.16	0.10	Unknown	---
23.	5.1	161.7	160.52	-0.22	0.14	Unknown	---
24.	5.2	166.3	164.56	-0.06	0.04	Unknown (base peak 83)	---
25.	5.2	171.0	167.86	0.34	-0.20	Unknown (base peak 83)	---

* Peaks not observed in the GC×GC chromatogram.

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra.

[ref.] References in square brackets indicate literature where these compounds have been reported.

Table 5.4 Identified Essential Oil Components of Lemongrass (*Cymbopogon flexuosus)*

Peak no.	GC×GC retention		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	t_{r1}, min	t_r, min	Δt_r, min	%		
1.	1.2	24.1	23.68	0.42	-1.74	Camphene	a, [ref. 11, 12]
2.	2.8	28.3	27.76	0.54	-1.91	6-methyl-5-heptene-2-one	a, [ref. 12, 13]
3.	2.7	30.2	29.96	0.24	-0.79	Myrcene	a, [ref. 6, 11,12, 13 ,]
4.	1.6	34.5	34.21	0.29	-0.84	Limonene	a, [ref. 6,11, 12, 13]
5.	2.8	40.1	39.63	0.47	-1.17	4-nonanone	a, [ref. 13]
6.	3.0	44.4	44.18	0.22	-0.50	Linalool	a, [ref. 6, 11, 12, 13]
7.	3.5	49.1	48.67	0.43	-0.88	Unknown	---
8.	4.4	55.0	54.60	0.40	-0.73	Unknown (similar to 48.67)	---
9.	4.5	62.5	63.10	-0.60	0.96	Neral	a, [ref. 6, 11, 12, 13]
10.	4.5	66.7	67.70	-1.00	1.50	Geranial	a, [ref. 6, 11, 12, 13]
11.	0.5	74.3	74.70	-0.40	0.54	Methyl geranate	a
12.	0.9	79.1	82.58	0.22	-0.27	Geranyl acetate	a, [ref. 6, 11, 12, 13]
13.	2.5	98.7	98.59	0.11	-0.11	Cadinene (unknown isomer)	a, [ref. 12, 13,]
14.	4.0	105.6	105.13	0.47	-0.45	Unknown	--

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra.

[ref.] References in square brackets indicate literature where these compounds have been reported.

Table 5.5 Identified Essential Oil Components of Lemongrass (*Cymbopogon citratus*)

Peak no.	GC×GC		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	t_{r1}, min	t_r, min	Δt_r, sec	%		
1.	1.9	28.10	27.86	0.24	-0.85	6-Methyl-5-hepten-2-one	a, b, [ref. 6, 14]
2.	0.7	29.20	---	---	---	2,3-Dehydro-1,8-cineols	b
3.	0.5	30.10	30.47	-0.37	1.23	β-Myrcene	a, b, [ref. 6, 14, 15]
4.	0.9	34.60	---	---	---	Cymene	b, [ref. 15]
5.	1.3	35.50	---	--	---	Eucalyptol (1,8-cineole)	b, [ref. 6, 14]
6.	0.6	36.20	36.18	0.02	-0.06	Limonene	a, b, [ref. 6, 14]
7.	0.7	37.60	37.47	0.13	-0.35	(Z)-β-ocimene	a,b, [ref. 6, 14, 15]
8.	2.2	42.50	42.59	-0.09	0.21	α-Thujone	b, [ref. 6]
9.	2.1	43.10	---	---	---	Nonanal	b, [ref. 6]
10.	2.0	43.80	---	---	---	Citronellal	b, [ref. 6, 15]
11.	2.1	44.40	---	---	---	Verbenol	b, [ref. 14]
12.	1.9	44.40	44.17	0.03	-0.07	Linalool	a, b, [ref. 6, 14, 15]
13.	2.0	46.10	46.05	0.05	-0.11	Decanal	a, b
14.	2.4	48.90	48.76	0.14	-0.29	α-terpineol	a, b, [ref. 6, 14, 15]
15.	2.1	52.20	52.27	-0.07	0.13	Methylcyclohexyl-ethanal	a
16.	2.1	54.70	54.77	-0.07	0.13	Oxiranecarboxaldehyde, 3-methyl-3-(4-methyl-3-pentenyl)	b
17.	2.4	62.10	---	---	---	Nerol	b, [ref. 6, 14, 15]
18.	3.3	62.10	63.18	-1.08	1.74	Neral	a, b, [ref. 6, 14, 15]
19.	2.5	65.70	---	---	---	Geraniol	b, [ref. 6, 14, 15]
20.	3.4	66.30	67.66	-1.36	2.05	Geranial	a, b, [ref. 6, 14, 15]
21.	2.1	70.70	---	---	---	2-Undecanone	b, [ref. 6, 14]
22.	1.9	71.80	72.08	-0.28	0.39	Geranyl formate	a, b
23.	5.2	73.80	74.78	-0.98	1.33	2,7-Dimethyl-2,6-octanediol	a, b
24.	5.5	78.50	79.17	-0.67	0.85	Epoxy-linalooloxide	a, b
25.	1.5	81.20	---	---	---	Neryl acetate	b
26.	1.9	82.50	82.89	-0.39	0.47	Geranyl acetate	a, b, [ref. 6]
27.	1.2	89.90	---	---	---	Caryphyllene	b, [ref. 15]
28.	2.3	96.80	97.04	-0.24	0.25	2-Tridecanone	a, b, [ref. 14]
29.	3.0	105.20	---	---	---	Caryophyllene oxide	b
30.	2.9	109.40	---	---	---	Selina-6-en-ol	b

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra
(b) Peaks identified by GC×GC-TOFMS.
[ref.] References in square brackets indicate literature where these compounds have been reported.

Table 5.6 Identified Essential Oil Components of African Wormwood (*Artemisia afra)*

Peak no.	GC×GC		GC-TOFMS	Difference		Identified Components	Method of identification
	t_{r2}, sec	t_{r1}, min	t_r, min	Δt_r, min	%		
1.	1.5	14.3	---	---	---	Santolina triene	b
2.	1.1	14.9	---	---	---	5-methyl-5-hexen-2-one	b
3.	1.1	15.6	---	---	---	Lilac alcohol B	b
4.	1.3	21.1	---	---	---	Tricyclene	b, [ref. 16]
5.	1.4	22.3	---	---	---	α-pinene	b, [ref. 16, 17]
6.	1.6	23.5	23.65	-0.15	0.64	Camphene	a, b, [ref. 16, 17]
7.	1.8	26.7	---	---	---	Sabinene, thujene	b, [ref. 16]
8.	3.0	27.9	---	---	---	1-Butanol, 3-methyl-, propanoate	b
9.	2.9	30.9	---	---	---	1-Octen-3-ol	b
10.	2.3	32.8	33.03	-0.23	0.70	p-cymene	a, [ref. 16, 17]
11.	2.3	33.8	33.96	-0.16	0.47	1,8-cineole	a, b, [ref. 16, 17]
12.	2.8	38.2	38.31	-0.11	0.29	Isopropyl triazole	a
13.	2.9	42.1	---	---	---	α-Terpinoline	b, [ref. 16]
14.	3.9	43.1	44.27	-1.17	2.71	α-thujone	a, b, [ref. 16, 17]
15.	3.8	44.5	45.38	-0.88	1.98	β-thujone	a, b, [ref. 16, 17]
16.	4.1	47.2	47.48	-0.28	0.59	Camphor	a, b, [ref. 16, 17]
17.	4.0	51.5	---	---	---	Borneol	b, [ref. 16]
18.	3.1	53.5	---	---	---	α-Thujenal	b
19.	2.7	54.8	55.04	-0.24	0.44	α-terpineol	b, [ref. 16]
20.	3.5	64.5	---	---	---	Myrthenal	b, [ref. 16]
21.	4.4	70.7	---	---	---	*Cis*-Caryophyllene	b

(a) Peaks identified by their GC×GC and GC-TOFMS retention time correlations and mass spectra.
(b) Peaks identified by GC×GC-TOFMS.
[ref.] References in square brackets indicate literature where these compounds have been reported.

Retention time locking makes peak identification more accurate and allows comparing of results from different instruments. It permits transferring of methods from one column to another. It makes communication of results a lot easier and it is a good way of checking that temperature, flow, and column are working properly [4]. The procedure of retention time locking and method translation is fairly user friendly once you have the appropriate instruments and the necessary software. The level of accuracy obtained for retention time locking of different one-dimensional GC systems, with the same or different detectors, is very high as reported in the literature [3, 18].

The difficulty of trying to lock GC×GC columns with the GC column in the GC-TOFMS system is evident as can be seen from the results in the tables above. One of the requirements in RTL is to use the same nominal columns, but GC×GC uses not one but two different polarity and dimension serially coupled columns. Separation is not only a function of volatility but also a function of polarity. Basically, it is only possible to match the first

column of the GC×GC with the GC-TOFMS column. Discrepancies in retention times can arise due to the short polar second dimension column. Another issue is the temperature of the second dimension column, which was maintained at higher temperatures than the first dimension column. Retention time as a function of temperature therefore might be slightly affected by this temperature difference.

The determination of accurate retention times of components is also affected by the peak shape. In determining the retention times of standards it is possible to regulate the peak shapes by injecting the right amount of sample to obtain only non-overloaded, Gaussian shaped peaks. In using actual samples difficulties arise due to variations in the quantity of the individual components in the sample. Some of the components produce fairly Gaussian shaped peaks, others produce totally overloaded peaks with typical 'fronting'. The shapes of such peaks could be improved by injecting dilute samples, but this is done at the cost of losing information on the minor peaks.

In practice in retention time locking of these two systems was not simple since quite a few factors affect the procedure. The search for better understanding of these factors to get better retention time correlation requires more work and it is too early to give any concluding remarks regarding the matter. More work is still underway to solve the problem.

5.4 GC×GC Run to Run Reproducibility and Overall Efficiency

The efficiency of a comprehensive two-dimensional gas chromatographic system can be measured by the complexity of samples that can still be effectively separated into the individual chemical components. Reliable and reproducible results can only be achieved if the proper functioning of all parts of the separation system is ensured. The modulator has to effectively trap, focus and re-inject sub-samples quantitatively into the second column. The detector has to have a broad dynamic range and has to be very sensitive so that it can effectively detect all solutes, which are present in the sample in a wide range of concentrations. The columns used must be stable over a wide temperature range, have a low bleed and be able to effect good separation of the analytes.

To check on the separation effectiveness of the GC×GC system, the precision and consistency of some chromatographic peak parameters can be monitored. These parameters include: reproducibility of retention times, peak widths and peak shapes.

5.4.1 Retention Times and Peak Width Reproducibility

Retention time is a function of column dimension, stationary phase chemistry, film thickness, temperature programming rate and carrier gas velocity. Under constant conditions of temperature programming rates and linear gas velocity, a certain column or a set of coupled columns must produce reproducible retention times. The consistency of the retention times is most important especially in instances where retention times are used for pattern recognition, or if reliable identification of individual components using standards is being performed [19].

To test the efficiency of the GC×GC system utilised in this project, a series of replicate runs were performed using the HP1-RtxWax column set under optimised conditions. Then, in order to compare the retention times in both dimensions and the second dimension peak widths, some peaks were randomly chosen. The selected peaks represent the different volatility and polarity ranges exhibited by the essential oil components.

Table 5.7 GC×GC run-to-run reproducibility* of selected peaks (*Cymbopogon flexuosus*)

Peak no.	Compound name	D1 retention times, minutes			D2 retention times, seconds			D2 peak widths (W_h), milliseconds		
		Mean	STD	%RSD	Mean	STD	%RSD	Mean	STD	%RSD
1.	Camphene	24.10	0.09	0.32	0.82	0.07	9.13	48.00	2.45	5.10
2.	6-methyl-5-heptene-2-one	28.20	0.14	0.41	2.76	0.08	2.90	120.00	5.48	4.56
3.	Limonene	34.60	0.09	0.22	1.12	0.07	6.68	52.00	4.00	7.69
4.	4-nonanone	40.06	0.10	0.23	1.72	0.07	4.35	72.00	4.00	5.56
5.	Linalool	44.44	0.16	0.31	4.72	0.12	2.47	205.00	10.00	4.88
6.	Unknown	52.44	0.16	0.30	3.02	0.32	10.76	144.00	4.90	3.40
7.	Unknown	54.98	0.12	0.14	3.38	0.12	3.45	142.00	7.48	5.27
8.	Geranyl acetate	82.66	0.39	0.40	3.04	0.30	9.89	120.00	6.12	5.10
9.	Cadinene	98.86	0.10	0.10	1.86	0.08	4.30	69.00	2.00	2.90
10.	Unknown	105.64	0.12	0.11	3.10	0.11	3.53	120.00	5.48	4.56
Mean				**0.25**			**5.75**			**4.90**

*The values in the table are the mean of five GC×GC runs. More results for all the essential oils are given in the Appendix.

As can be inferred from table (5.7), the first dimension retention times showed good reproducibility with a mean relative standard deviation of only 0.25%. The first dimension retention times stayed fairly constant and the actual deviation in the retention times was in the range of 6 – 12 seconds (0.1 – 0.2 minutes). For example, for the first peak the retention times for the five runs were: (24.1), (24.2), (24.2), (24.0) and (24.0) minutes[5].

The second dimension retention times and peak widths showed greater variation than that observed in the first dimension retention times. This is mainly because the second dimension retention times, peak shapes and widths were, to a certain degree, affected by the modulator performance. One of the problems of working with the dual stage jet modulator is the precise setting of the cooling rate, pulsing frequency and heating of the hot pulses. Shifting in retention times and irregular peak shapes occurred whenever there were slight differences in modulator performance due to inefficient trapping, too much cooling or inefficient heating of the trapped fractions. Occasional cold spots in the modulator bracket, before or after the two modulation stages or between the two stages, create irregularities in retention times (especially first dimension retention times) and peak shapes.

The precision of second dimension retention times is affected by the reproducibility of modulation start time between the different runs. As it was difficult to monitor the modulation start time with the software used in this project, the retention times had to be realigned for some of the runs after the completion of each run [19]. The peak widths at half height observed in all the runs were quite impressive, and range between 45 milliseconds for peak number one and 200 milliseconds for peak five (table (5.8)) which was the broadest of all the selected peaks.

Shellie *et al.* [19] reported that the first dimension retention times and peak widths at half height show excellent reproducibility for runs done in the same day. As is shown in table (5.8), the retention times and peak widths at half height reproduce almost precisely. Variations in retention times and peak widths are more visible when comparisons are made between runs done on different days, as is the case in table (5.7).

[5] The retention times and peak width at half height reproducibility study was done for all the oils, and more results are given in the Appendix.

Table 5.8 Comparison of peaks from runs done in the same day (***Cymbopogon flexuosus)***

Peak no.	Compound name	t_{r1} in minutes			D2 W_h in milliseconds		
		Run 1	Run 2	Δt_r	Run 1	Run 2	ΔW_h
1.	Camphene	24.0	24.0	0.0	45	45	0.0
2.	6-methyl-5-heptene-2-one	28.0	28.1	0.1	110	120	10.0
3.	Limonene	34.5	34.5	0.0	50	50	0.0
4.	4-nonanone	39.9	40.0	0.1	70	70	0.0
5.	Linalool	44.2	44.3	0.1	200	200	0.0
6.	Unknown	52.5	52.5	0.0	140	135	5.0
7.	Unknown	55.0	55.0	0.0	140	140	0.0
8.	Geranyl acetate	82.9	82.3	0.6	110	*	*
9.	Cadinene	98.9	98.8	0.1	65	65	0.0
10.	Unknown	105.7	105.7	0.0	110	110	0.0

*The geranyl acetate peak in the second run gave a very broad and flat peak due to sudden change in the performance of the modulator (improper trapping and re-injection).

5.4.2 Second Dimension Retention Times *vs.* the Polarity of Second Dimension Columns

The time sample components stay in the second dimension column of a GC×GC system is influenced by the second column dimensions (length, inner diameter and film thickness) the temperature and the linear flow rate of the carrier gas. Under fixed column dimensions, only the column temperature and linear flow rate can be manipulated. Running the second dimension column at higher temperatures than the first dimension column helps to reduce the retention time of the more polar compounds, but this reduces the separation efficiency of the less polar components. Reduced temperature differences between the two columns increases retention time in the second dimension column, but improves the second dimension separation between medium polar components [20, 21].

The second dimension retention times, as a function of component class, showed differences between the two second dimension columns used. Non-polar and medium-polar compounds like non-oxygenated terpenes showed minimum second dimension retention times on both columns. Compounds with high hydrogen bonding ability but with moderate dipole moments (*e.g.* alcohols) showed high retention times on the Rtx-Wax second dimension column and moderate retention on the Rtx-1701 second dimension column. Compounds with large dipole moments but with low hydrogen bond ability (ketones and aldehydes) displayed high retention times on the Rtx-1701 second dimension column and moderate retention times on the Rtx-Wax second dimension column [22].

Table 5.9 Comparison of second dimension retention times of some components of the essential oil of *Cymbopogon flexuosus*

Peak no.	Compound name	t_{r1}, minutes	t_{r2}, seconds	
			Rtx-1701	Rtx-Wax
1.	Camphene	24.1	1.2	0.7
2.	Limonene	34.4	1.6	1.0
3.	4-Nonanone	40.0	2.8	1.6
4.	Linalool	44.3	3.0	4.5

The variation in the second dimension retention times observed in table (5.9) is a function of the chemical class to which the components belong. Camphene and limonene, non-polar terpene hydrocarbons, showed minimum second dimension retention in both columns. Nonanone, a ketone, is retained more in Rtx-1701 while linalool (an alcohol) has higher second dimension retention in Rtx-Wax.

In some cases more polar compounds may show second dimension retention times greater than the modulation period (>6 seconds), complicating the determination of second dimension retention times. In such cases adding the modulation period to the second dimension retentions and subtracting the same amount of time from the first dimension retentions can adjust the retention times. In cases of wrap-around the modulation period can be increased to find the actual second dimension retention times. A bigger temperature difference between the two dimensions can also be used to elute all solutes in one modulation period (6 sec. in this case). Wrap-around, besides giving wrong retention times may affect the quantification and identification of components if the wrap-around is causing overlapping of peaks [19, 22, 23]. The essential oils analysed here did not show any extensive wrap-around, only occasional wrap-around was observed like the peak in figure (5.2), marked with an asterisk.

5.5 Qualitative Comparison of Lemongrass Samples

One of the uses of comprehensive two-dimensional gas chromatography is the pattern recognition of different samples for quality control and forensic investigations [24]. Qualitative comparison of the same products of different origin can be accomplished by comparing the detailed GC×GC chromatograms of the samples. In this way, differences can

be observed by overlaying the different chromatograms on top of each other or by specifically looking at the patterns of peaks obtained from the different samples. Differences in peak intensities may also indicate the quantitative differences between the same compounds from sample to sample.

The chemical composition of essential oils from the same type of plant may differ both qualitatively and quantitatively depending on the place of origin, extraction method used, harvest season and weather. This difference plays a role in the pricing of the oils [25, 26]. Knowing the qualitative and quantitative chemical composition of an essential oil helps to determine whether the oil is pure, or a blend, or has been adulterated with synthetic lower cost compounds. If the gas chromatographic analysis of a known oil gives extra peaks, which have not been reported before, it is possible that the oil might have been tampered with. Synthetic additions of compounds, which are part of the sample, can be detected by the trace amounts of synthesis impurities, especially when the impurities are not naturally found in the essential oil [26].

The citral rich (60 - 85%) essential oil of lemongrass is dominated by monoterpene hydrocarbons and oxygenated monoterpenes (alcohols, aldehydes, esters and ketones) in various proportions [6, 14, 15]. The quality of this oil depends on the amount of (*cis* and *trans*) citral it contains; the minimum market requirement is 75% [, 14, 15]. In this study, a total of seven different lemongrass essential oil samples, obtained from six farms, were analysed and their GC×GC chromatograms were compared. To make the comparison, seven peaks, that visibly showed noticeable variations in their peak intensities, were chosen and their relative peak intensities were compared.

Table 5.10 Normalised peak areas (FID responses, arbitrary units) of selected compounds from the essential oil of lemongrass

Compound name	Sample number						
	1	2	3	4	5	6	7
Myrcene	0.135	0.066	0.093	0.273	0.141	0.243	.049
Limonene	0.088	0.038	0.061	0.328	0.182	0.267	0.037
Ocimene	0.101	0.056	0.191	0.172	0.148	0.269	0.063
Neral (*cis*-citral)	0.084	0.113	0.120	0.136	0.189	0.167	0.191
Geranial (*trans*-citral)	0.077	0.082	0.115	0.131	0.164	0.165	0.267
Undecanone	0.079	0.019	0.121	0.227	0.289	0.216	0.050
Geranylacetate	0.165	0.284	0.332	0.033	0.014	0.043	0.130

The relative peak intensities of the selected compounds were found to vary amongst all seven samples. Although samples 1 and 2 were from the same farm, the components from the two samples showed significant differences in their relative peak intensities (table (5.10) and figure (5.3)). These two samples also showed the lowest relative (*cis/trans*) citral content of all of the seven samples compared (figure (5.4)). Samples 4 and 6 showed high relative contents of myrcene and limonene. Sample 7 showed the lowest relative amounts of myrcene and limonene of all the seven samples, but it had the highest relative (*cis/trans*) citral content followed by samples 5 and 6 (figure (5.4)). For all of the seven samples, the relative amount of undecanone and geranylacetate varied greatly. For example, the relative ratio of undecanone between sample 5, with highest relative undecanone content, and sample 2, with lowest relative undecanone content was 15 : 1 (table (5.10)).

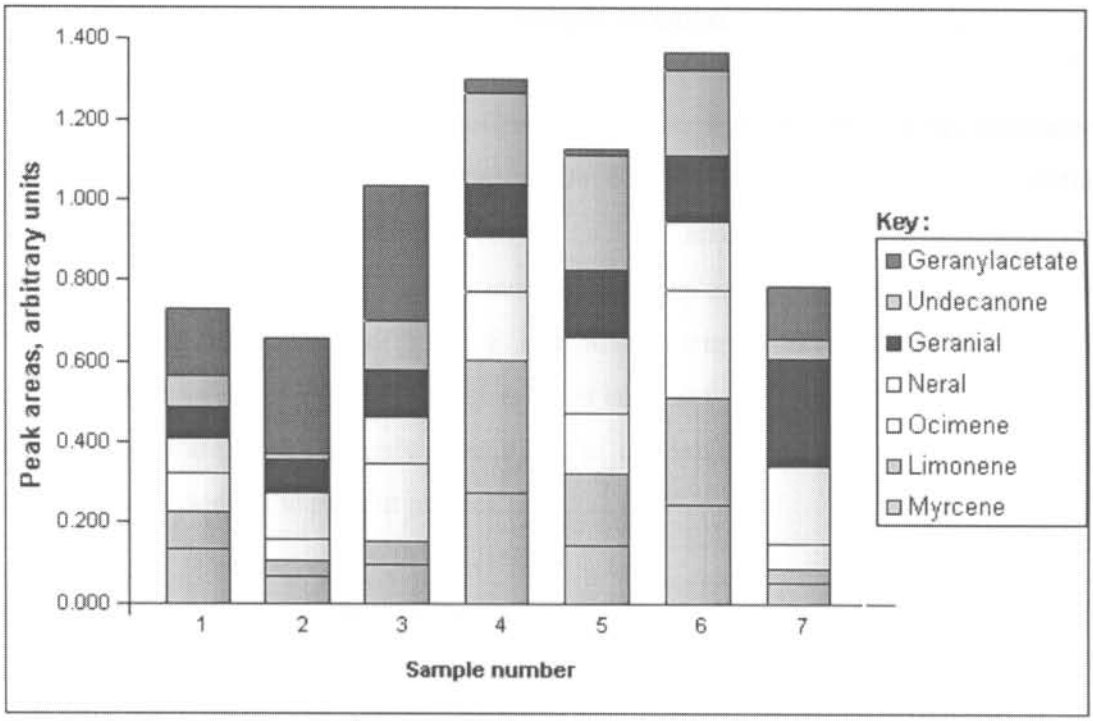

Figure 5.3 Normalised peak areas (FID responses, arbitrary units) of selected compounds of the essential oils of lemongrass. (Peak areas not corrected for run-to-run variation in 0.1 µl nominal injection volume).

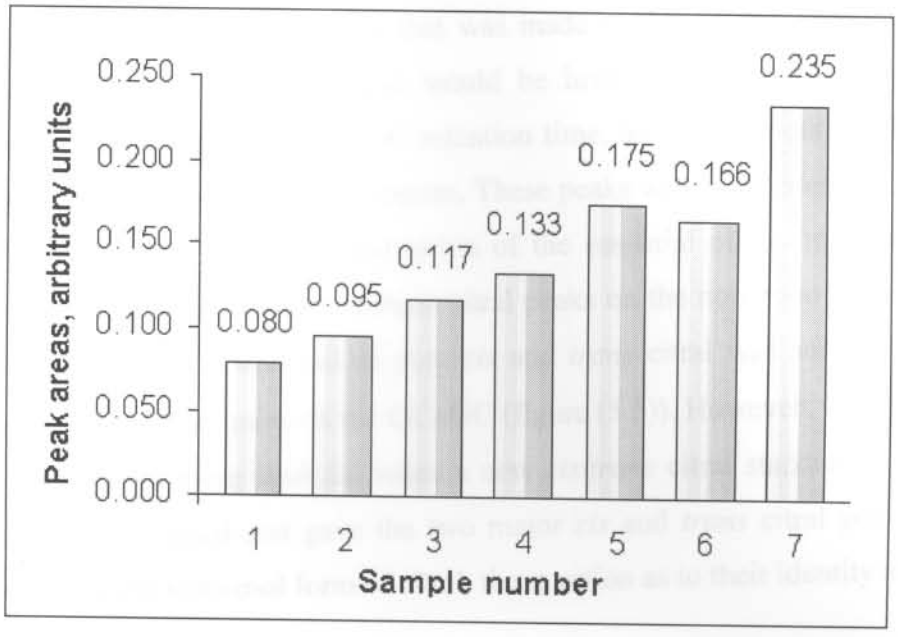

Figure 5.4 *cis* + *trans*-Citral sample-to-sample variation.

The sole purpose of the above comparisons was to determine if there are any qualitative[6] or relative quantitative differences between the components of the different essential oil samples. The information obtained from the GC×GC analyses was able to provide enough information to make the above comparisons. To make a complete assessment and determine the percentage abundance of all the individual compounds requires the quantitative determination of all the components present in each sample. In the absence of quantitative analysis software, that has only recently been available from the company ZOEX, this could not be done in the present project.

5.6 Observation on the *cis/trans* Citral Peak Pair

The high resolving power of comprehensive two-dimensional GC as a function of the two separation parameters, *viz*. volatility and polarity, is able to isolate compounds with very similar characteristics. Compounds of the same volatility but with slight polarity differences are easily resolved. The use of a chiral second dimension column will also help to separate enantiomers [27]. From the beginning of this project, we observed two peaks with first

[6] Taking sample 6 as a reference (it is an export quality oil), no new peaks were observed in any of the other lemongrass (*Cymbopogon citratus*) essential oils that have not been seen in sample 6.

dimension retention times similar to those of the *cis* and *trans* citral peak pair in lemongrass. We were keen to find out whether those peaks were possibly the result of a continuous keto-enol tautomerism process that was made visible by the very fast second dimension analysis. Such a transformation would be invisible in a slow GC run if it happened at a rate much faster than the GC retention time, as the GC peak would merely reflect the average movement of the two isomers. These peaks were not observed during the GC-TOFMS analyses done for the identification of the essential oil components, due to their overlap with the more abundant *cis/trans* citral peaks on the non-polar DB-1 column. To further investigate this, a standard of pure *cis* and *trans* citral was analysed, and the results showed the same peak pairs on the GC×GC (figure (5.5)). However, we still had our reservations and became more doubtful when a new *cis/trans* citral standard (95% citral, Sigma Aldrich) was analysed and gave the two major *cis* and *trans* citral peaks only. If those peaks were not the keto-enol form of citral, the question as to their identity arose.

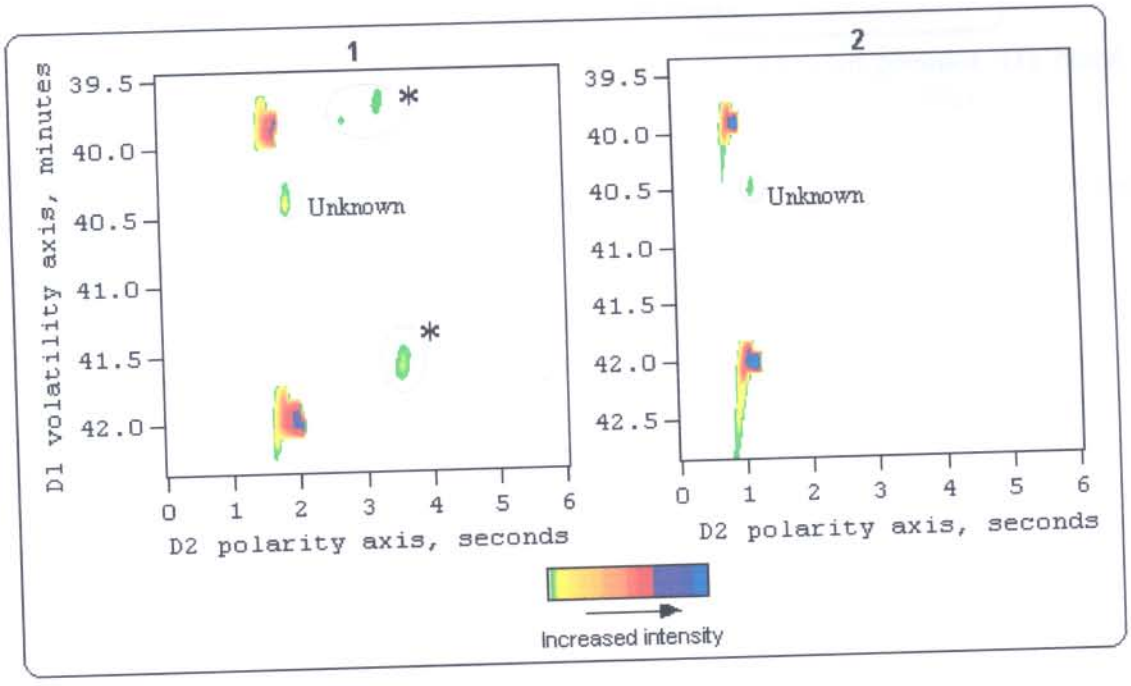

Figure 5.5 *cis/trans*-Citral standard: (**1**) Old standard, (*) Extra peaks in old *cis/trans* citral standard and (**2**) New standard without the extra peaks.

The volatility of the two alcohols, nerol and geraniol, is very close to that of the *cis*-citral (neral) and *trans*-citral (geranial), respectively. Therefore, a standard of these alcohols was

co-injected with the new *cis/trans* citral standard to determine if the alcohol peaks elute in the same position as the peaks obtained from the old *cis/trans* citral standard (figure (5.6)).

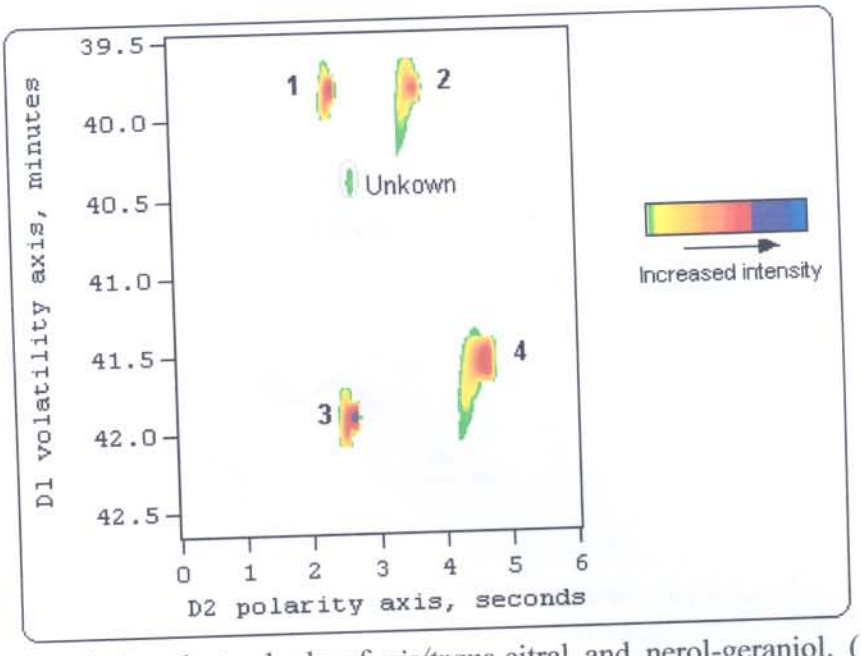

Figure 5.6 Co-injected standards of *cis/trans*-citral and nerol-geraniol. (1) Neral, (2) Nerol, (3) Geranial and (4) Geraniol. Second dimension column is Rtx-Wax.

As expected, the alcohol peaks eluted at the same retention times as the unknown peaks in the old *cis/trans* citral standard. These peaks in the lemongrass essential oil sample were identified as nerol and geraniol, respectively, by GC×GC-TOFMS. Nerol and geraniol have indeed been identified in the essential oil of lemongrass [6, 14, 15]. The fact that these peaks cannot be seen in a conventional GC on a DB-1 column, clearly demonstrates the power of GC×GC for essential oil analysis. Normally, *cis/trans* citral is catalytically hydrogenated to give these alcohols [28] as in figure (5.7).

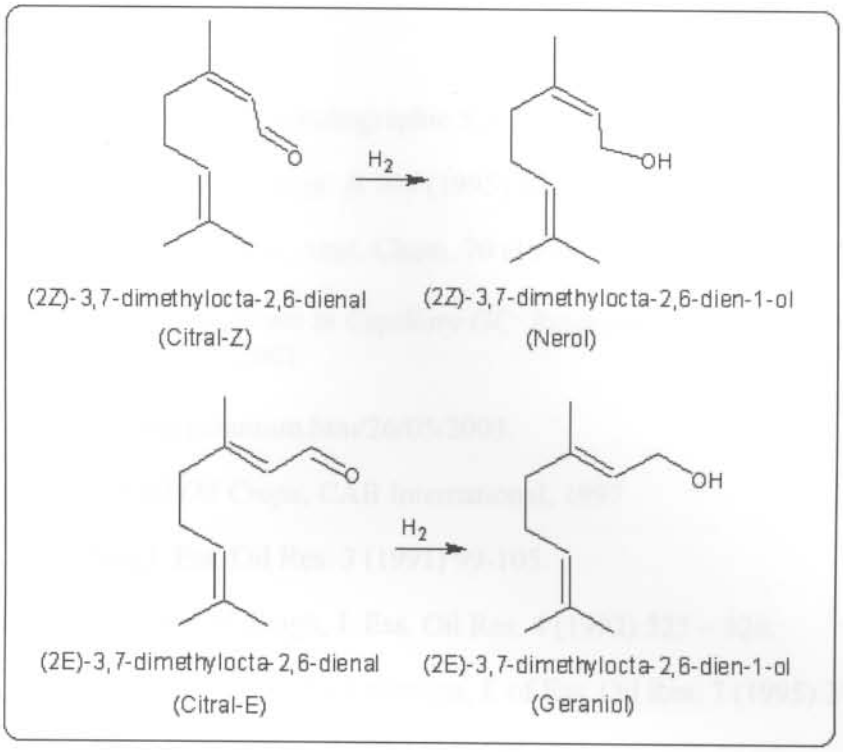

Figure 5.7 Catalytic hydrogenation of *cis* and *trans*-citral to nerol and geraniol, respectively [28].

Without running the pure standard of *cis/trans* citral, it could be considered that the partial reduction to nerol and geraniol was an artefact produced by the hydrogen carrier in the hot injector. Obtaining only the aldehyde peaks from the pure standard disproves this argument and proves that the small amounts of nerol and geraniol are indeed contained in the lemongrass essential oil (and in the impure old standard).

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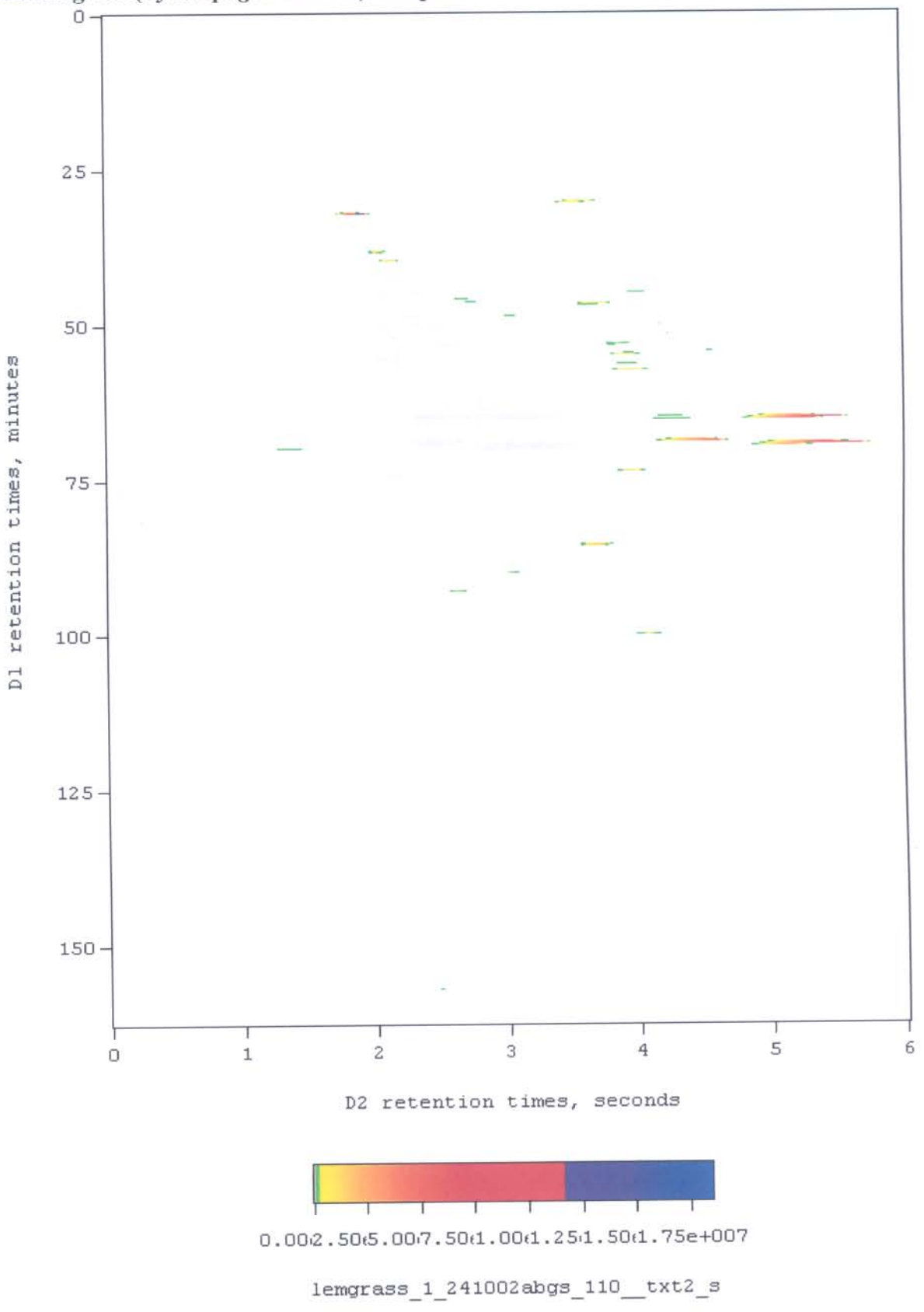
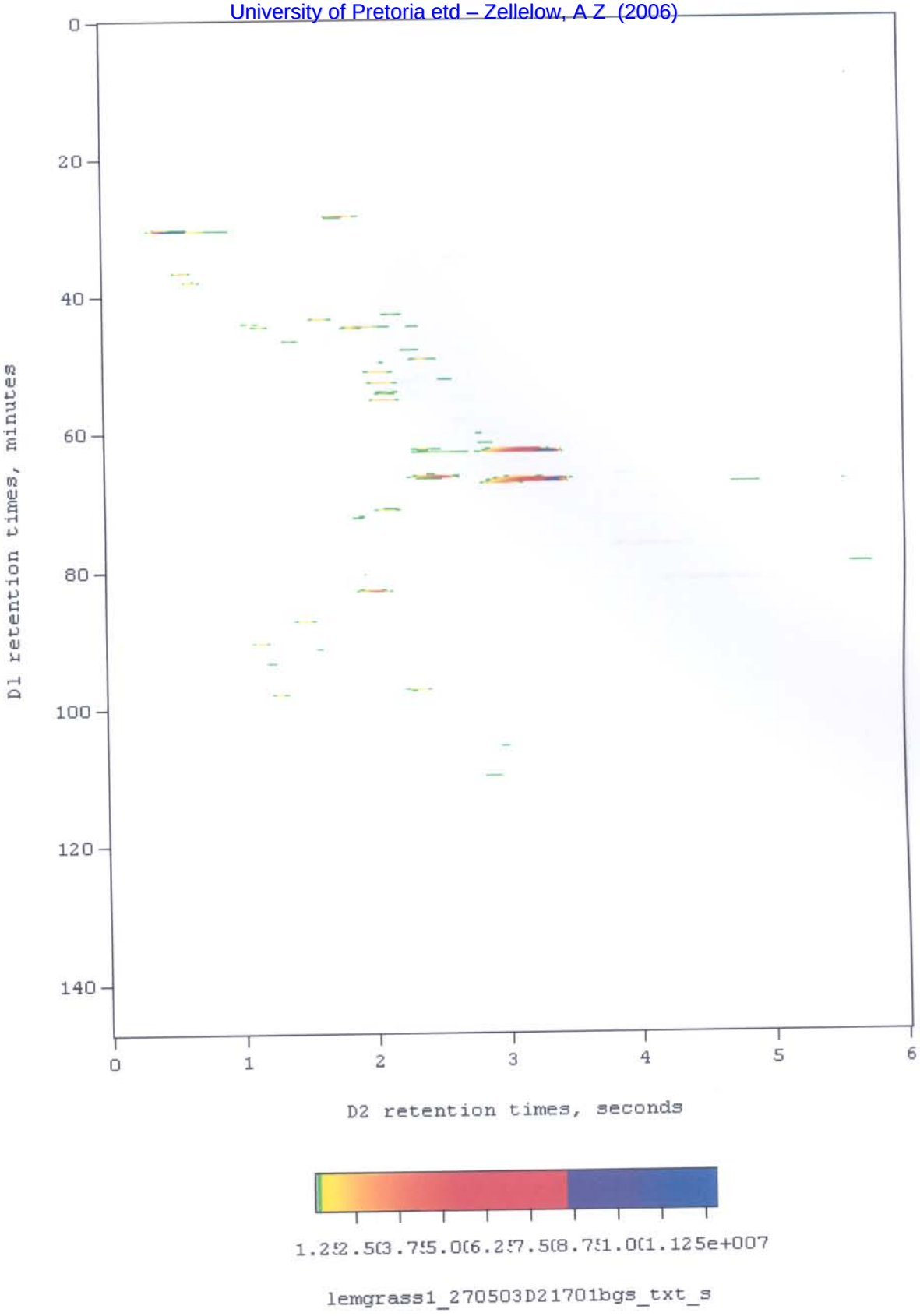
APPENDIX

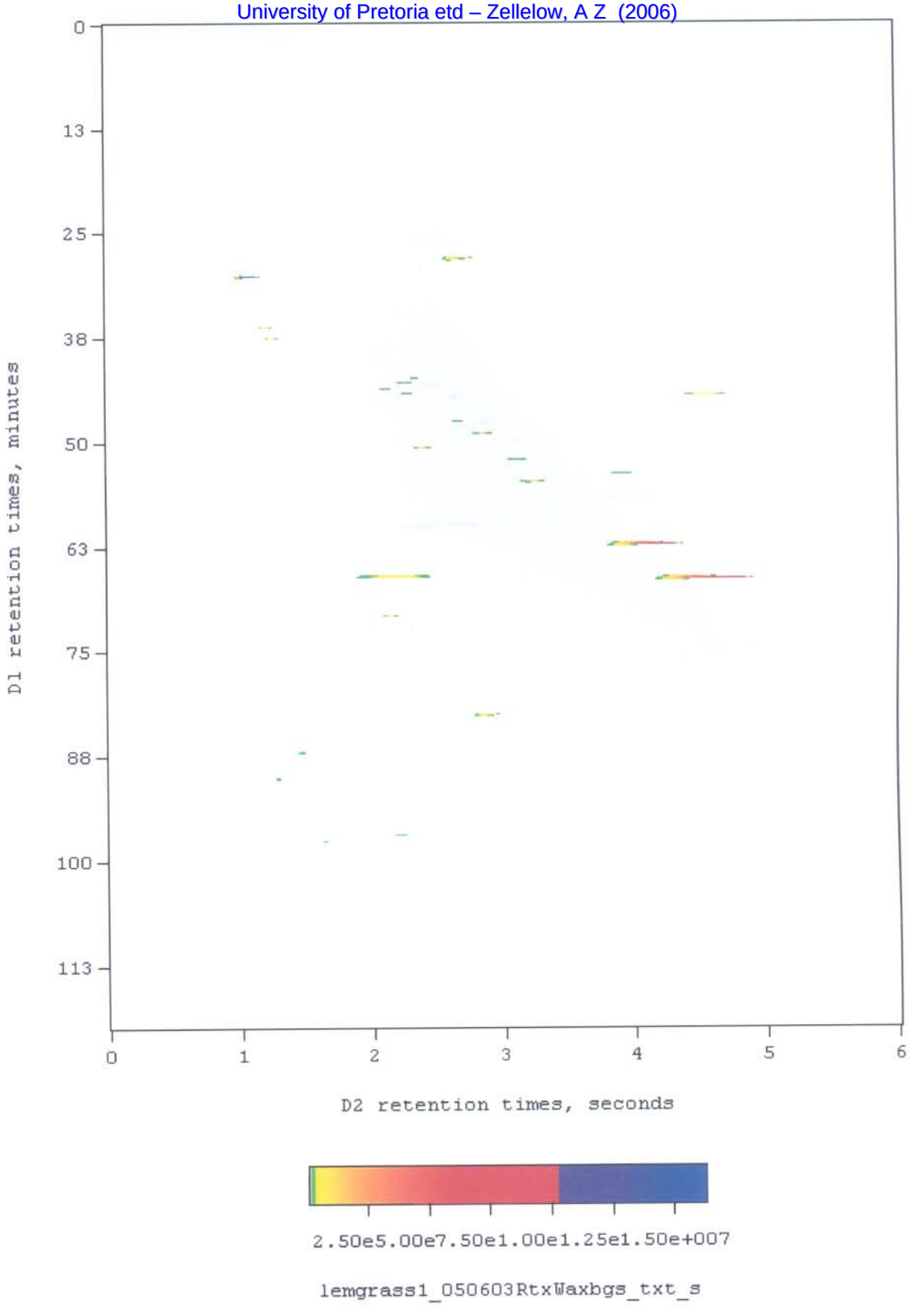
Key to file names: at the bottom of each chromatogram there is a file name, which consists of the name of the sample, the date it was run and the type of the second dimension column. A letter 'a', 'b', or 'c' after the name of the second dimension column indicates the number of the same sample runs in the same day. If there is a 'bgs' at the end of the file name, it indicates that the second dimension retention times have been re-aligned. Some file names also include the pressure (110) at the end of the file name. For example, the file name '**lemgrass_1_241002abgs_110_txt2_s'** indicates: lemongrass sample 1 run on 24/10/2002, 'a' it was the first lemongrass sample 1 run on that day, 'bgs' the second dimension retention times have been re-aligned, '110' it was run at 110 kPa and the file is a text file (all files are text files). The scales (s) on the X-Y axes are time scales, the first dimension (D1) volatility axis retention times are in minutes and the second dimension (D2) polarity axis retention times are in seconds.

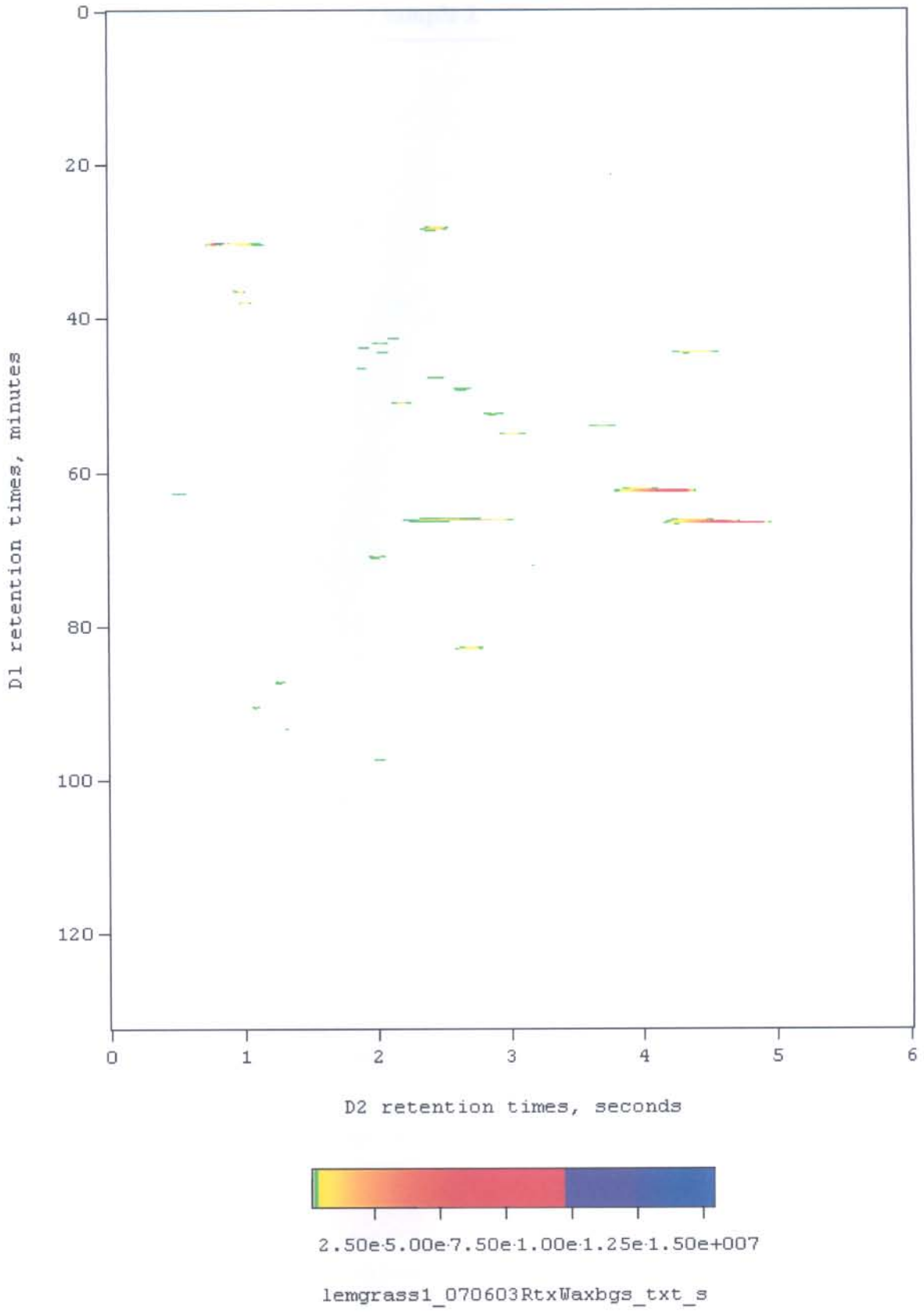
The coloured bar at the bottom of the chromatograms is the key indicating the intensity (abundance) of the components in the sample, from the lowest (green) to the highest (blue).

Lemongrass (*Cymbopogon citratus*) sample-1









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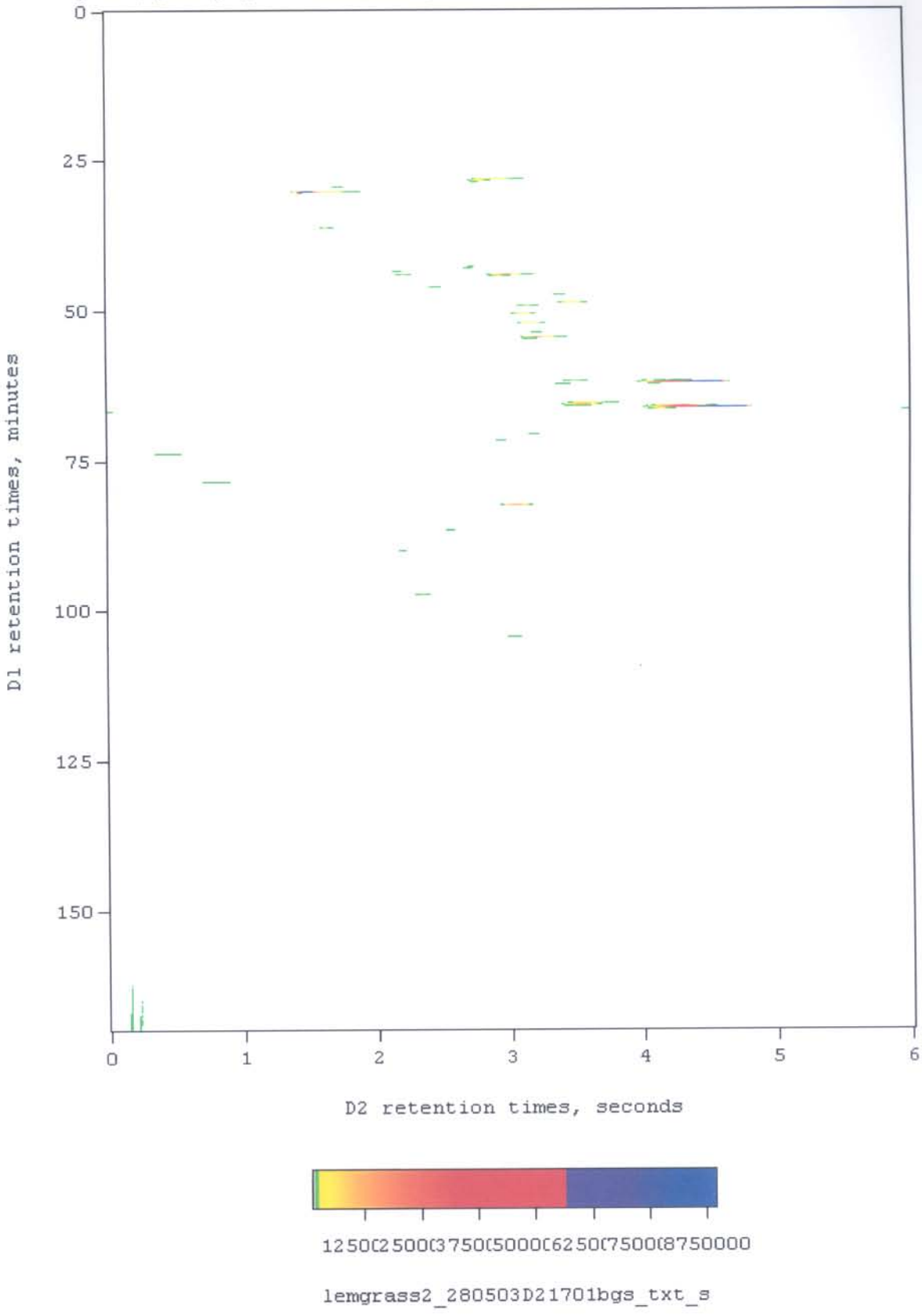
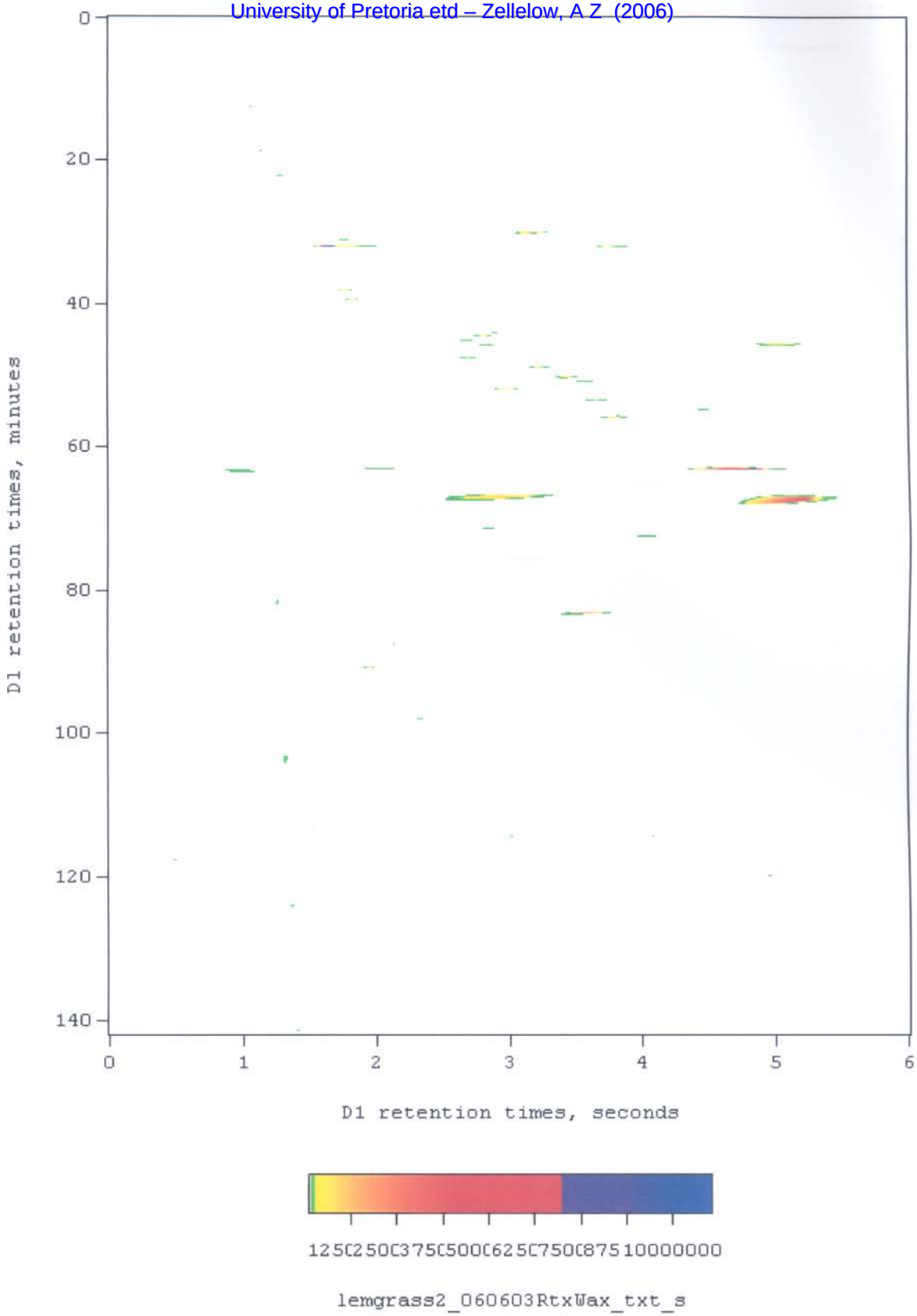
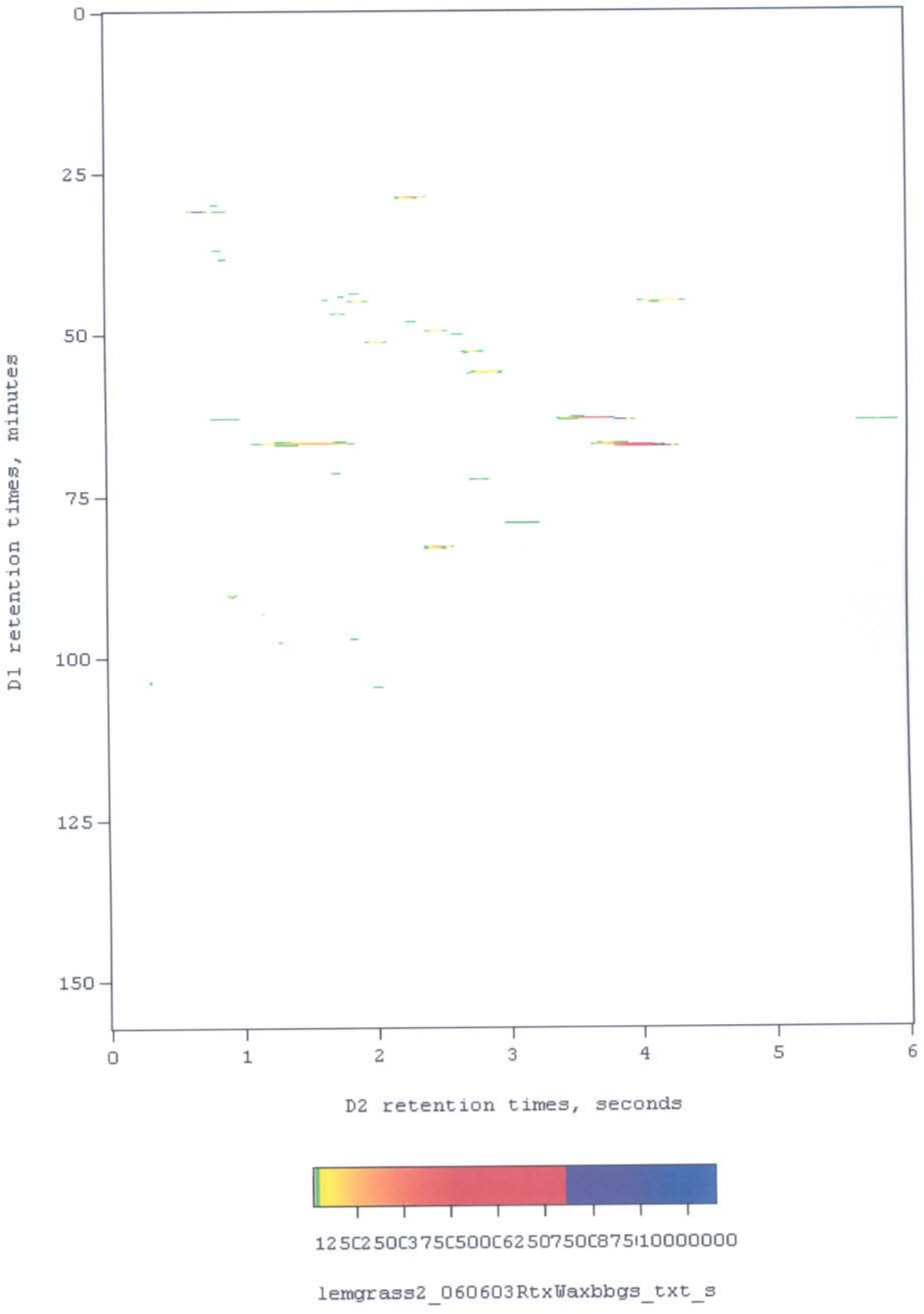
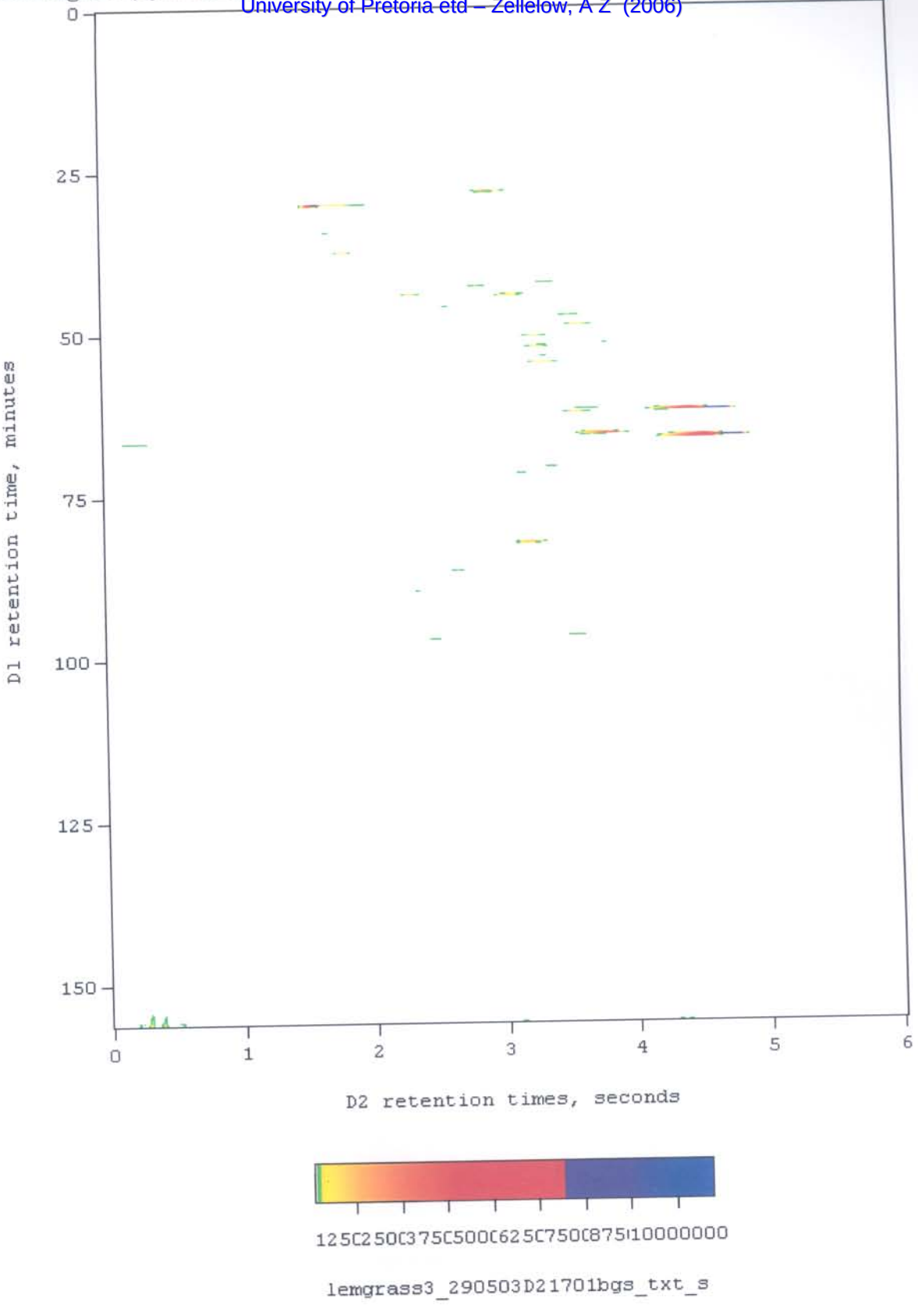
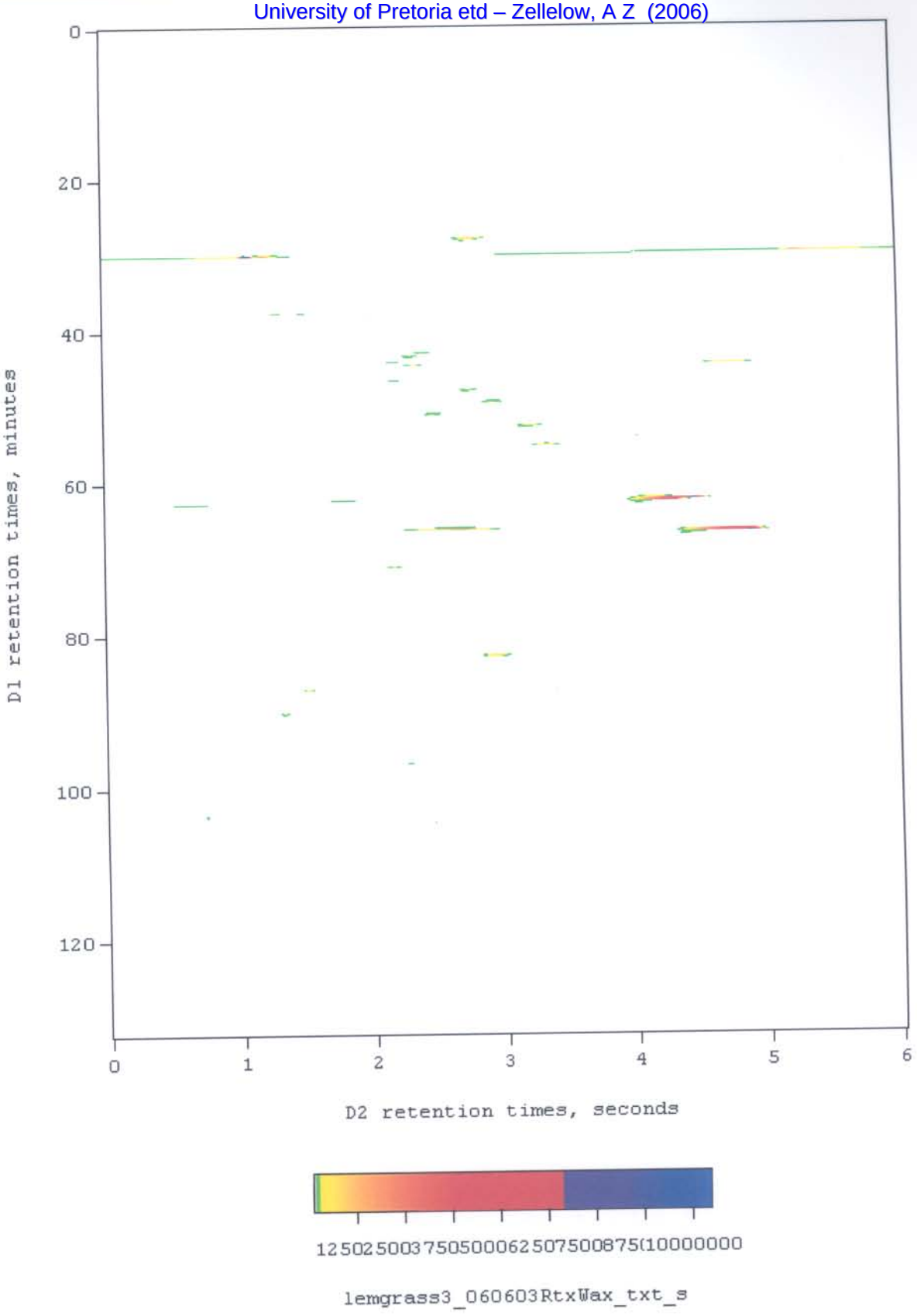


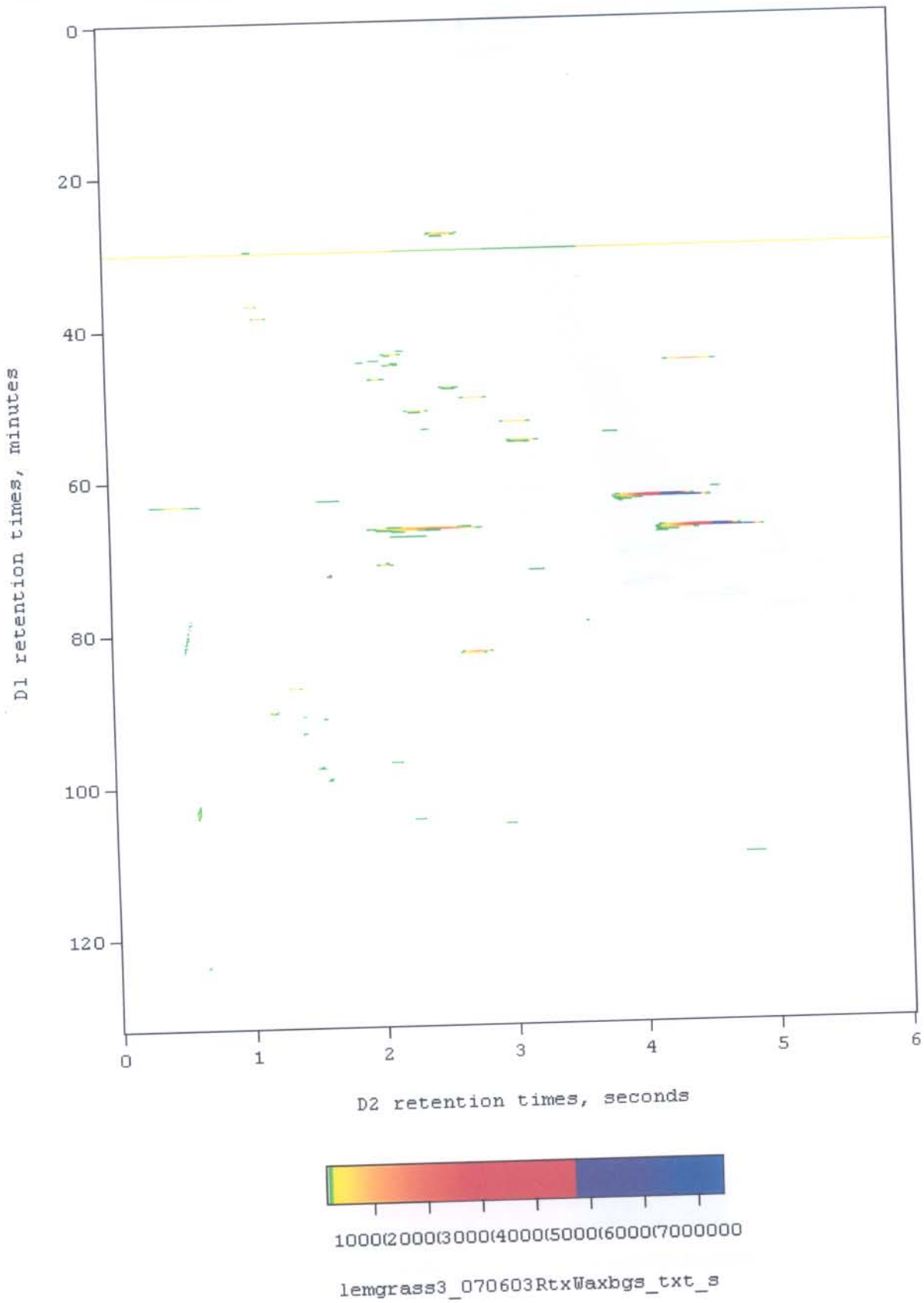




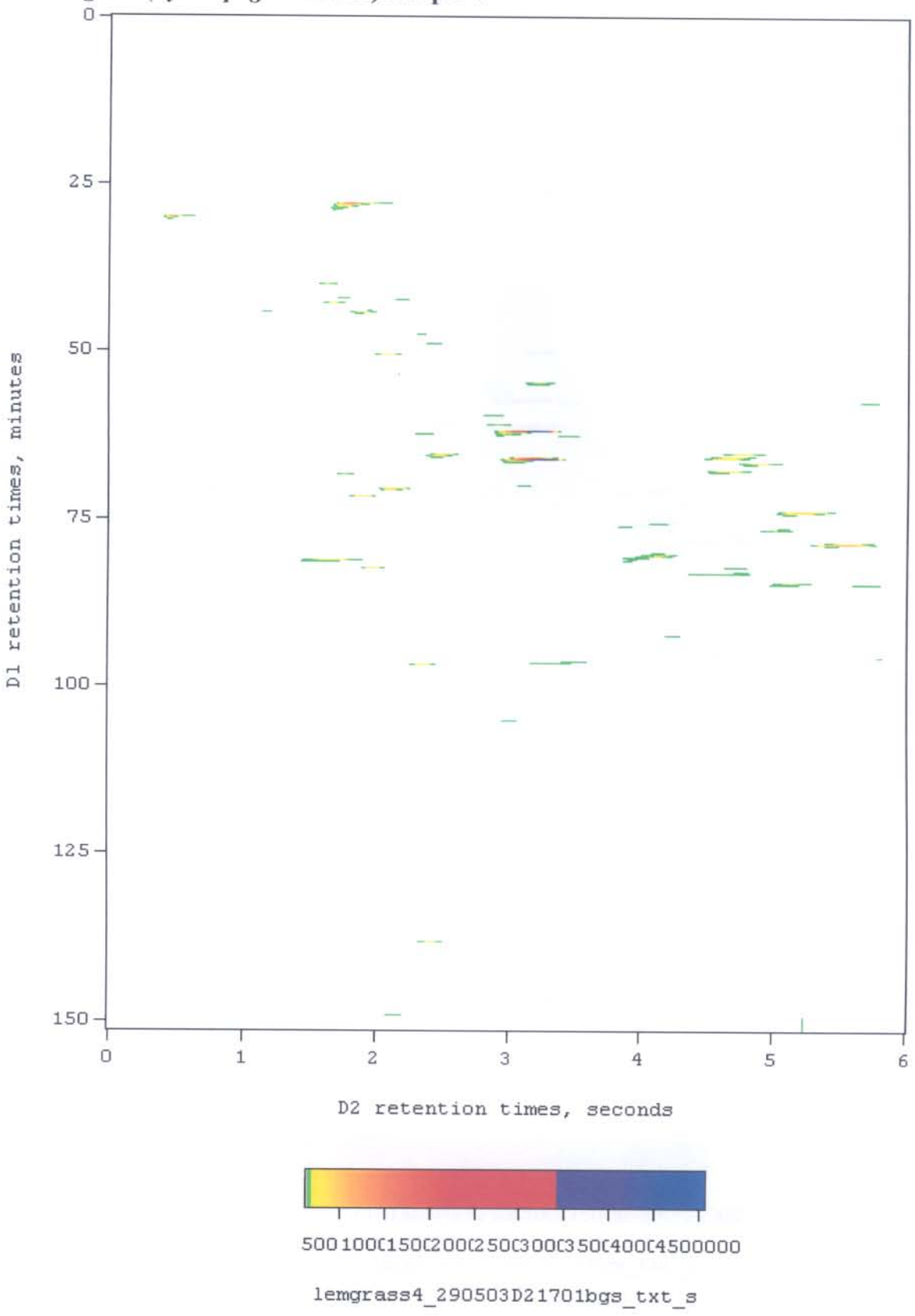
Lemongrass (*Cymbopogon citratus*) sample-3

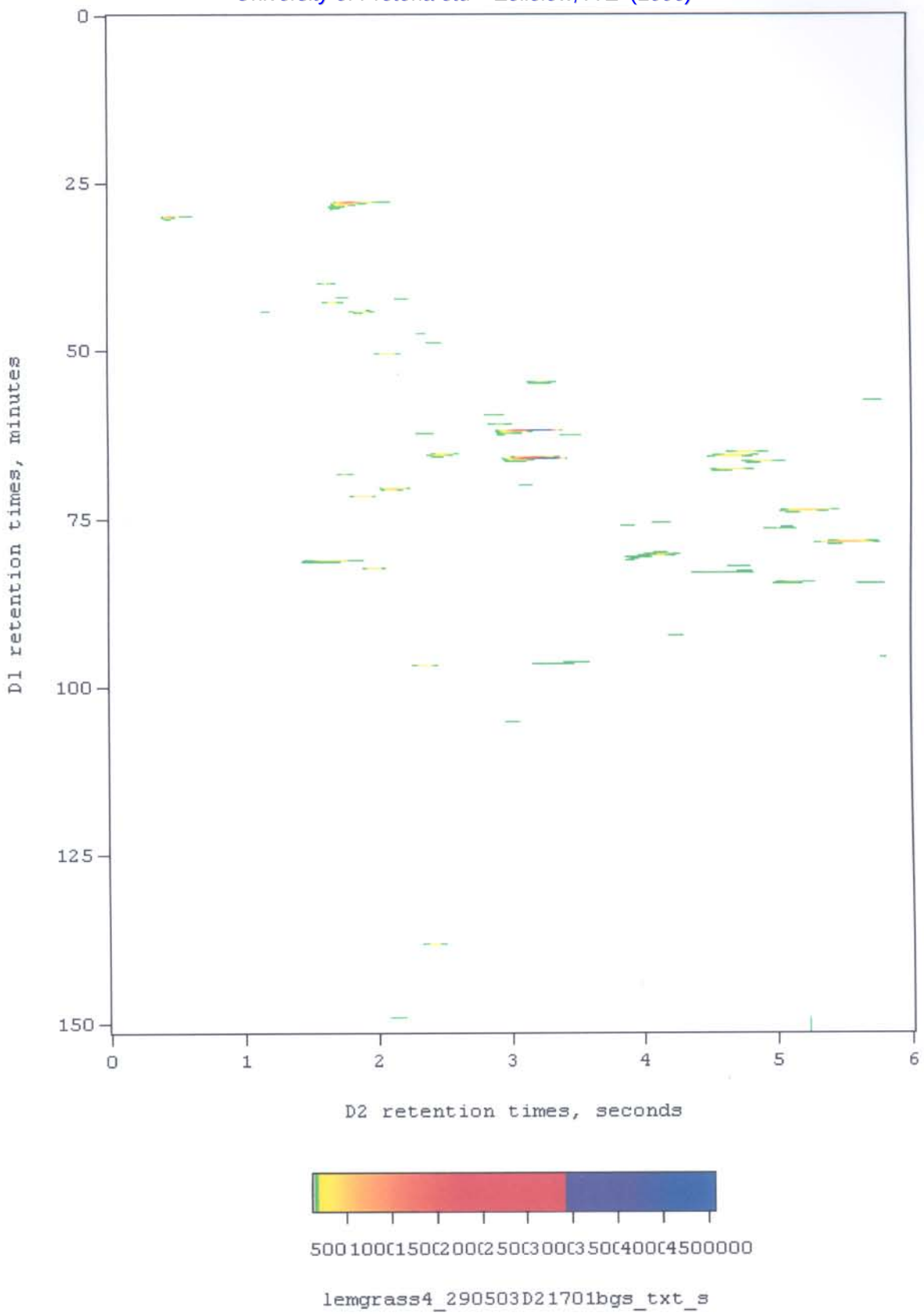


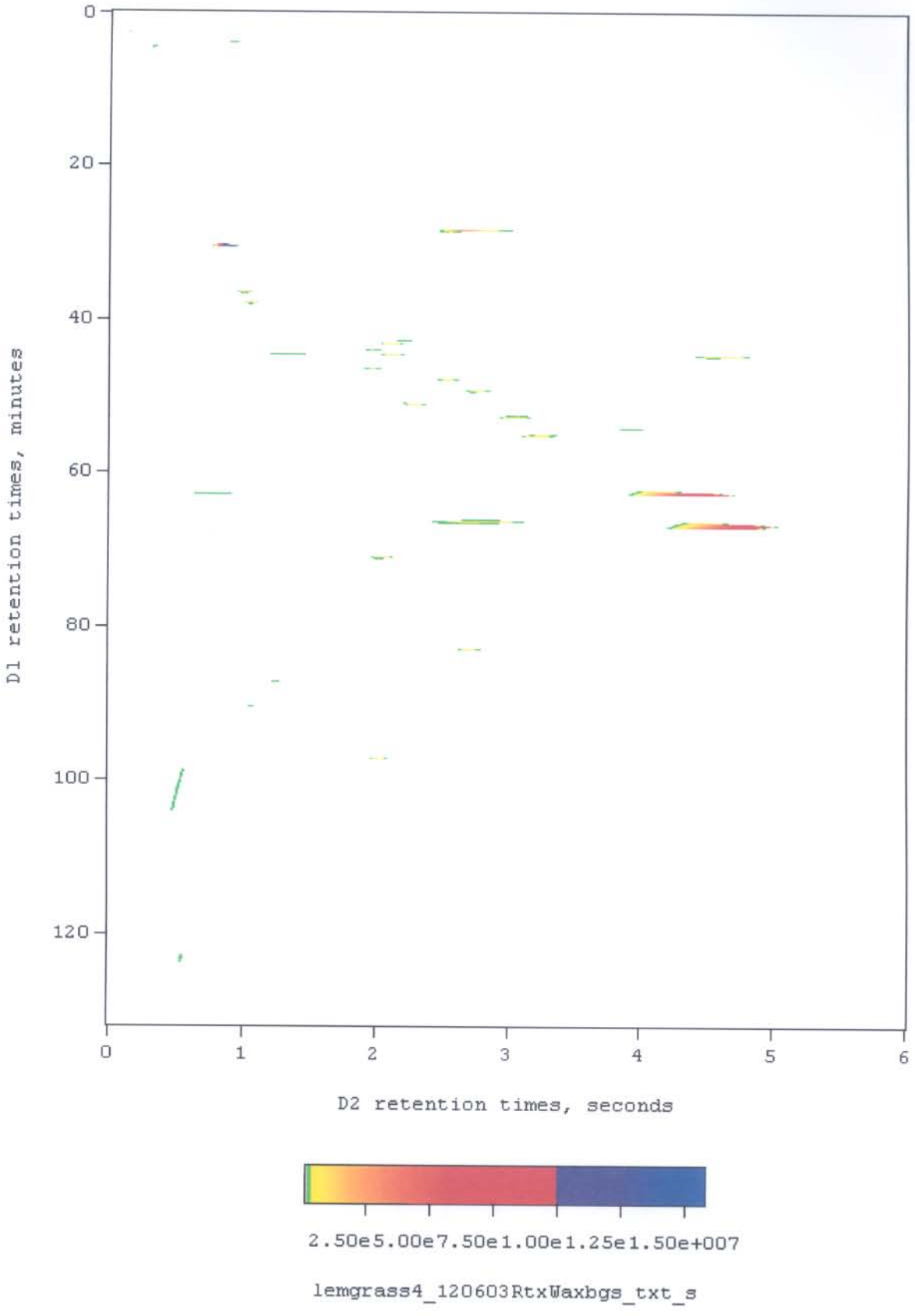


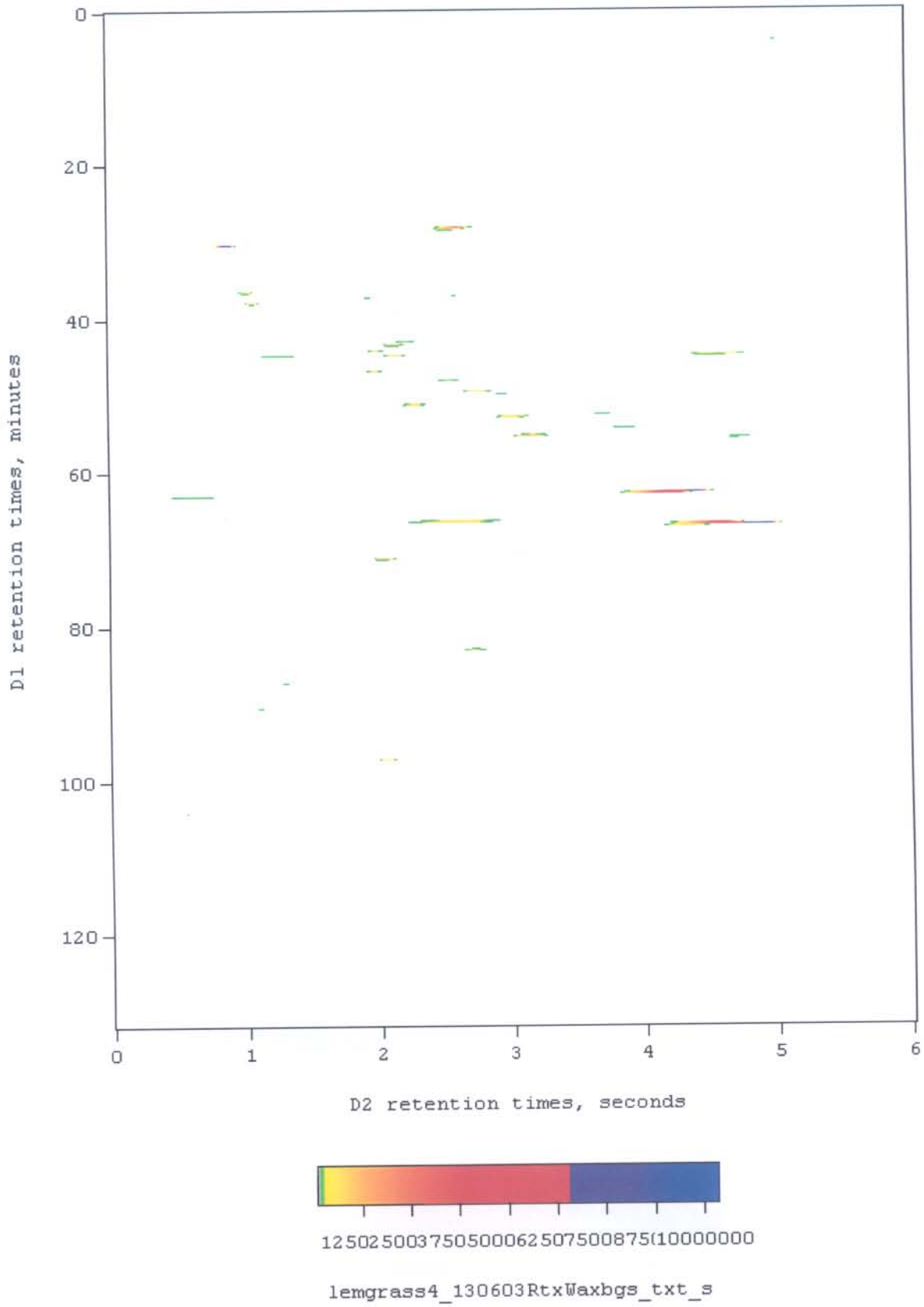


Lemongrass (*Cymbopogon citratus*) sample-4

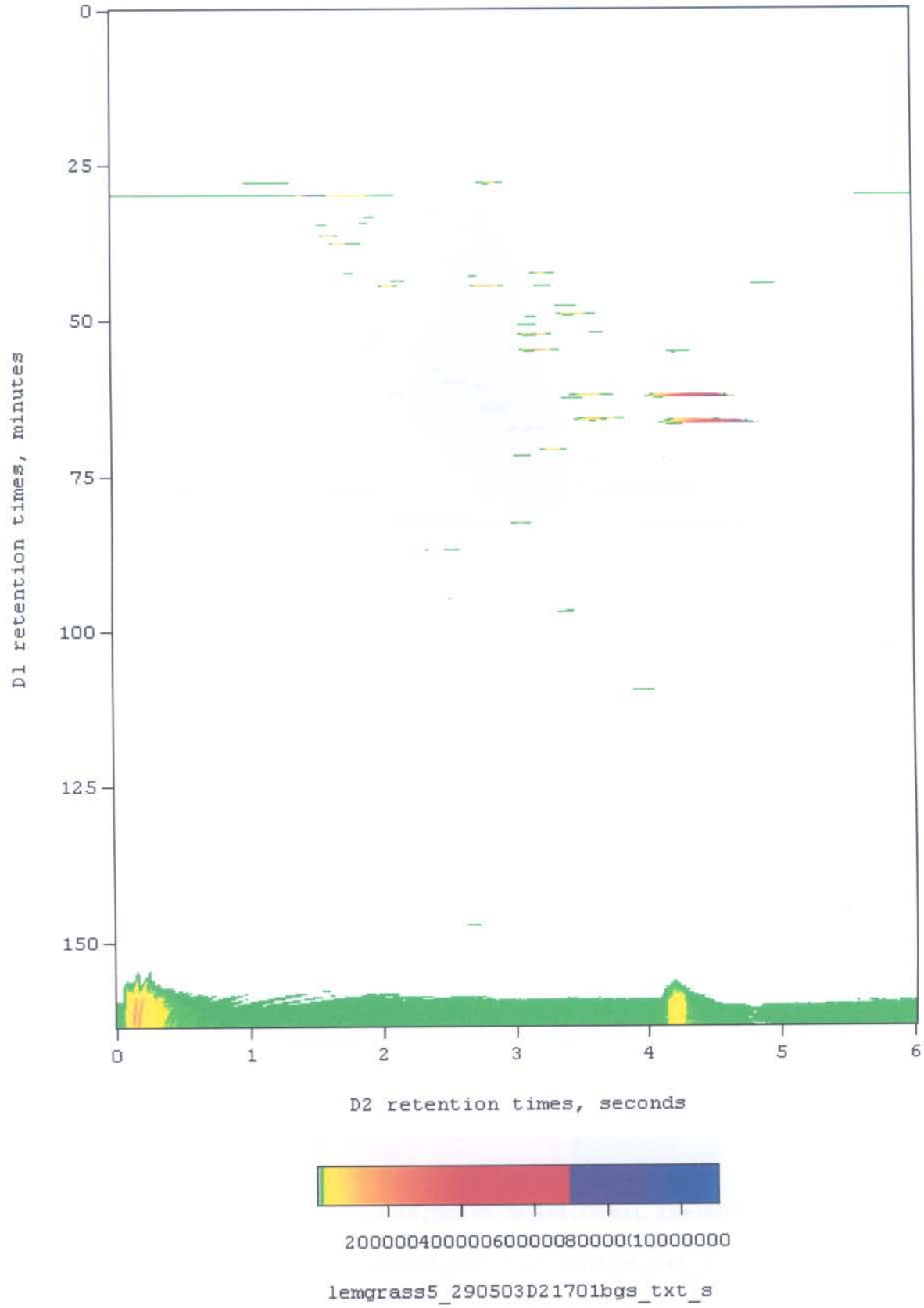


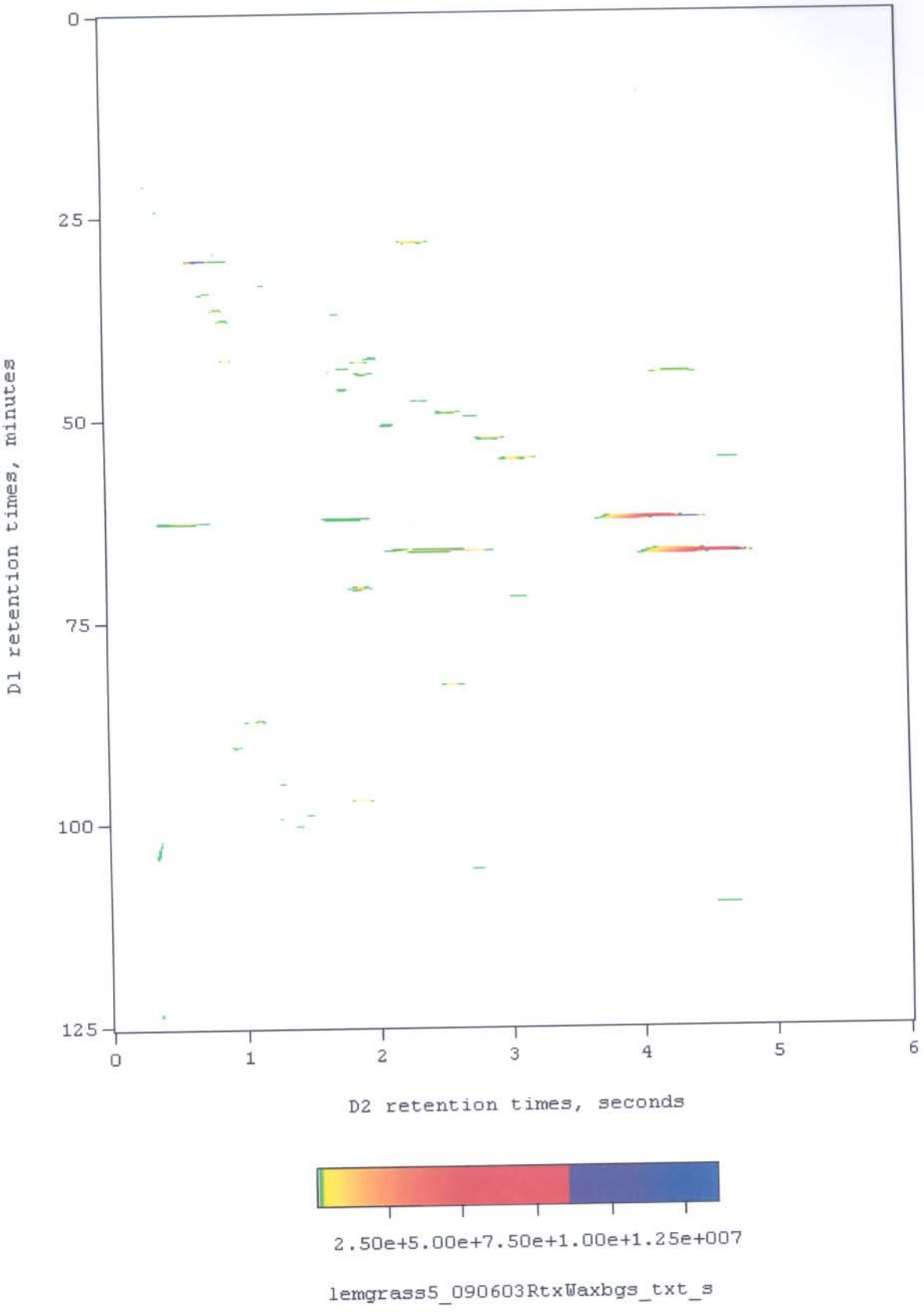


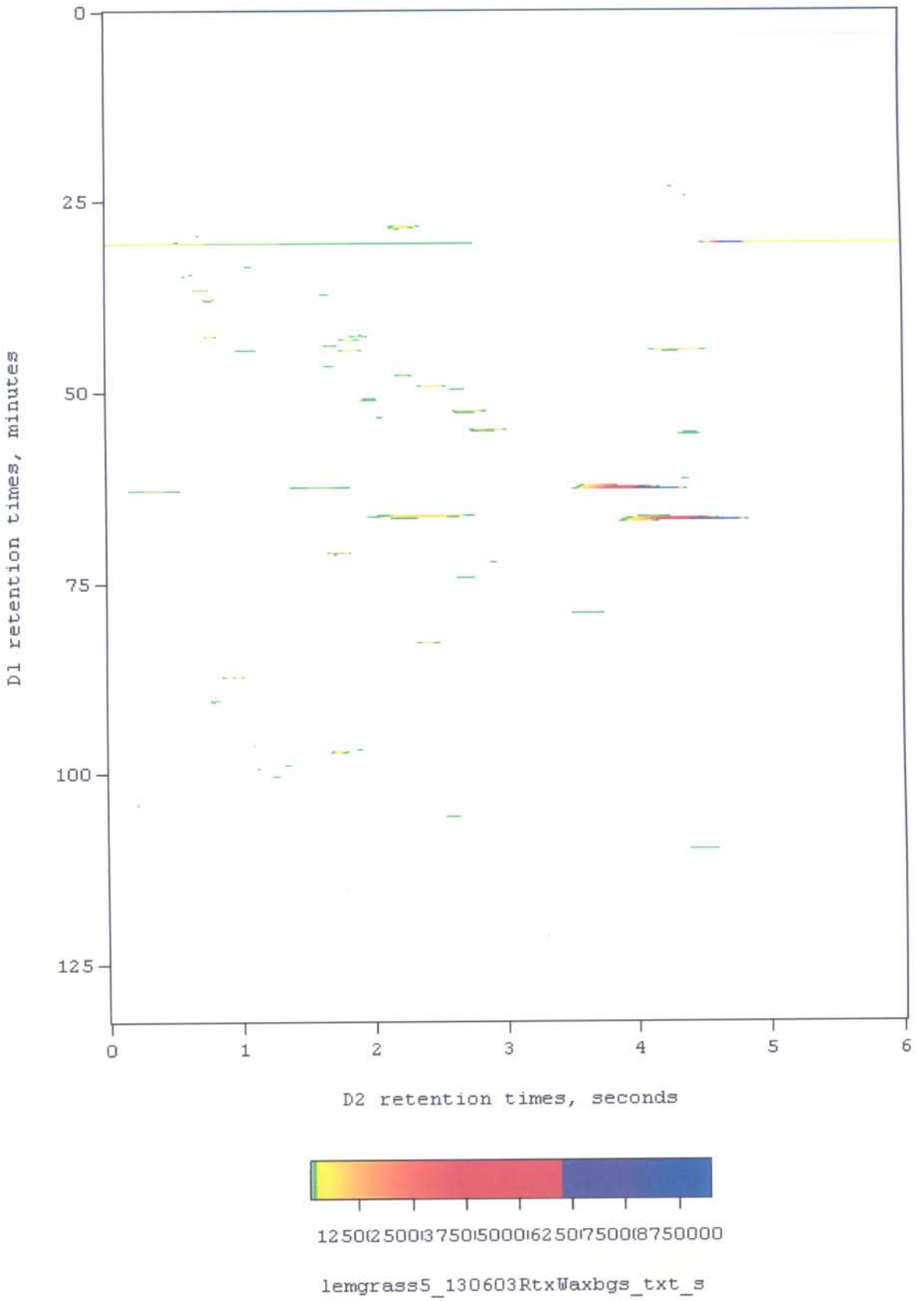




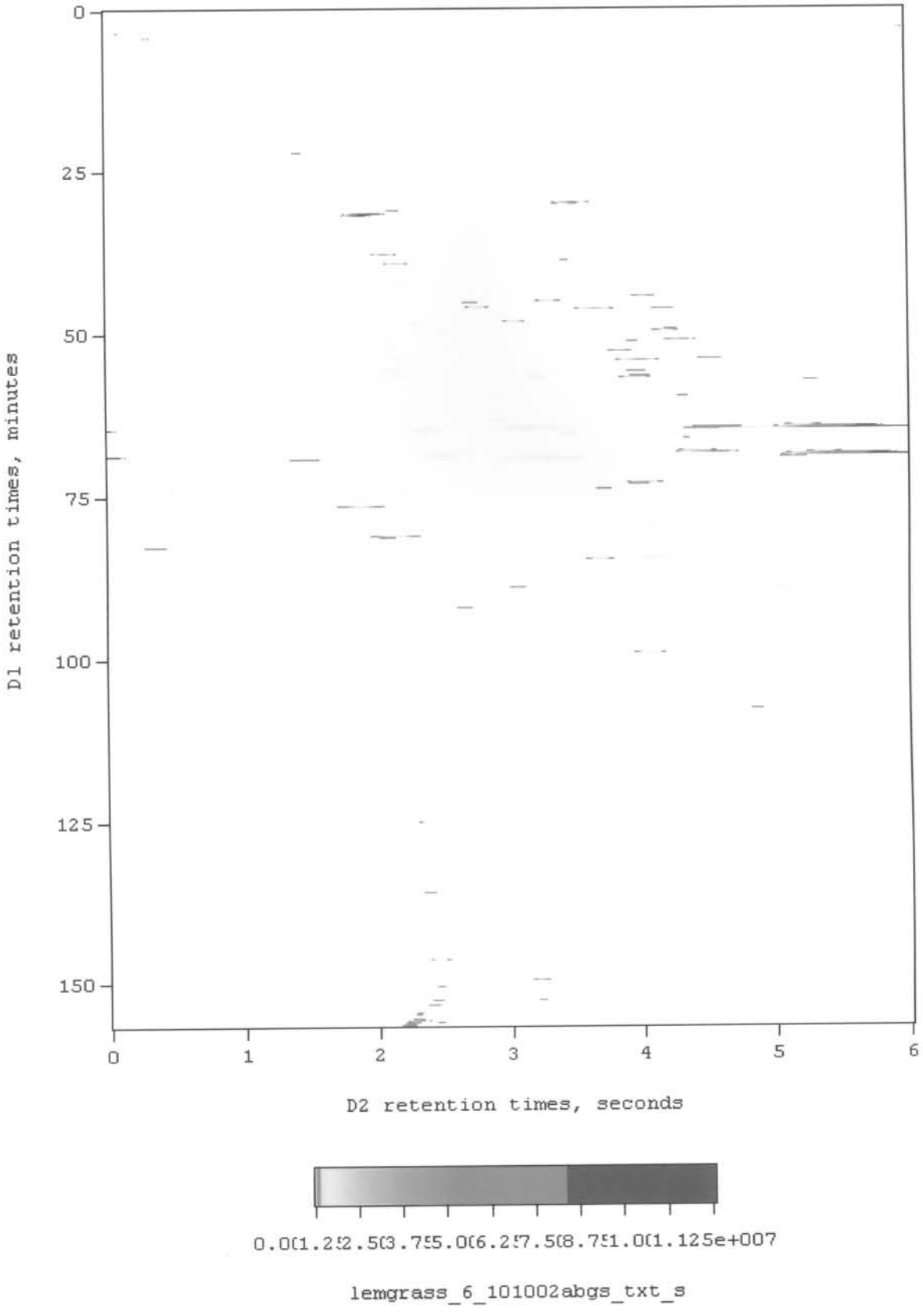
Lemongrass (*Cymbopogon citratus*) sample-5

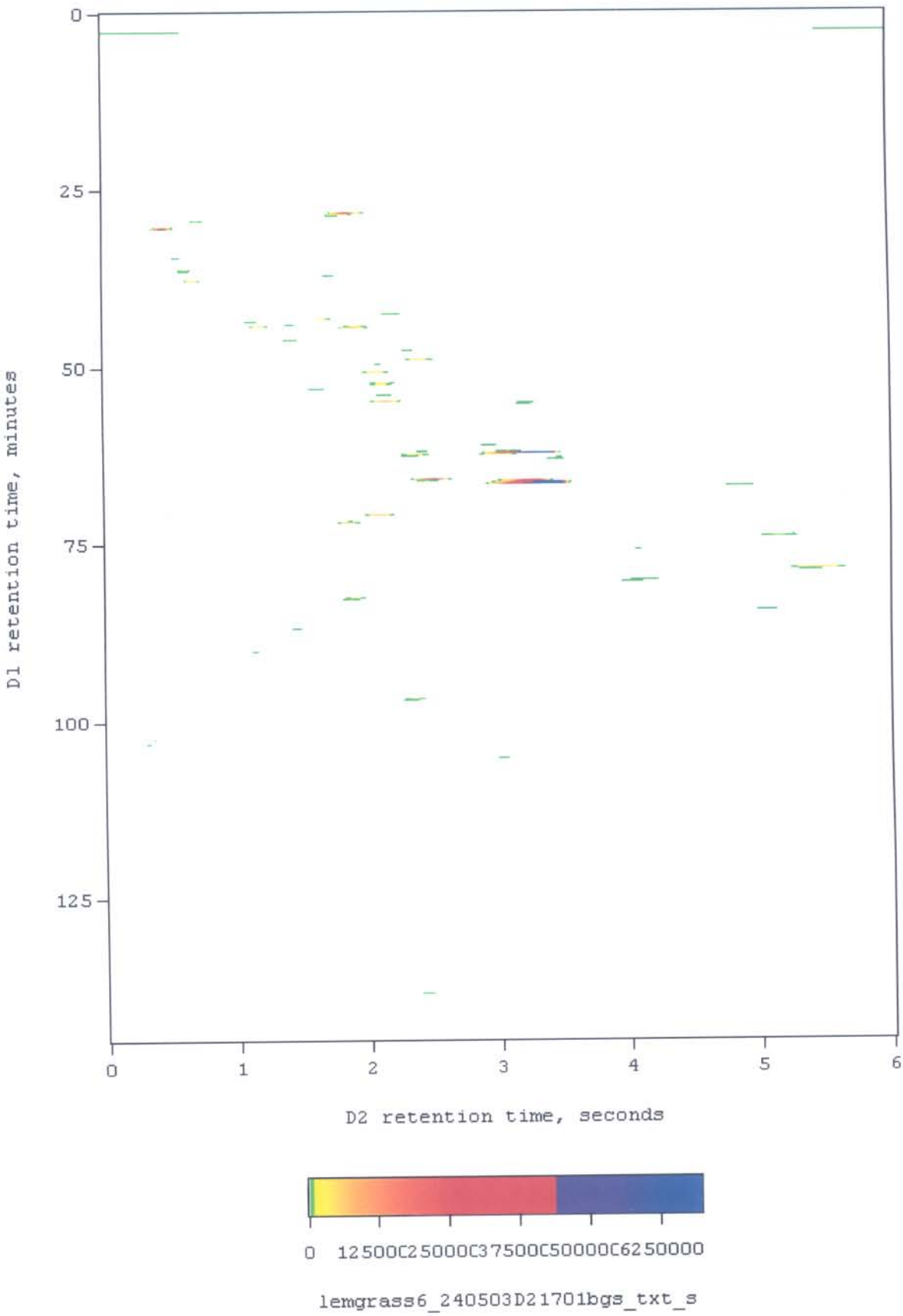


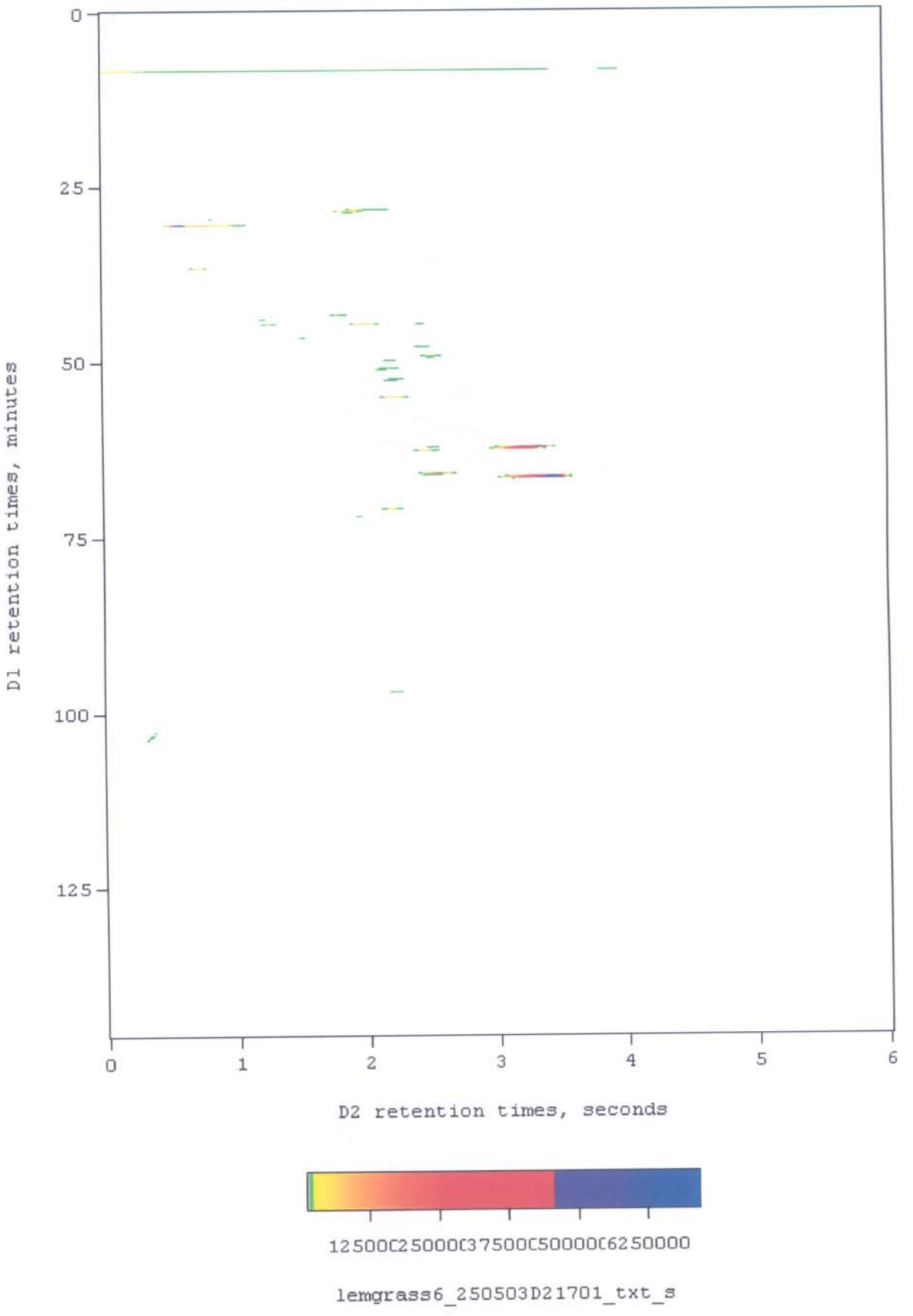


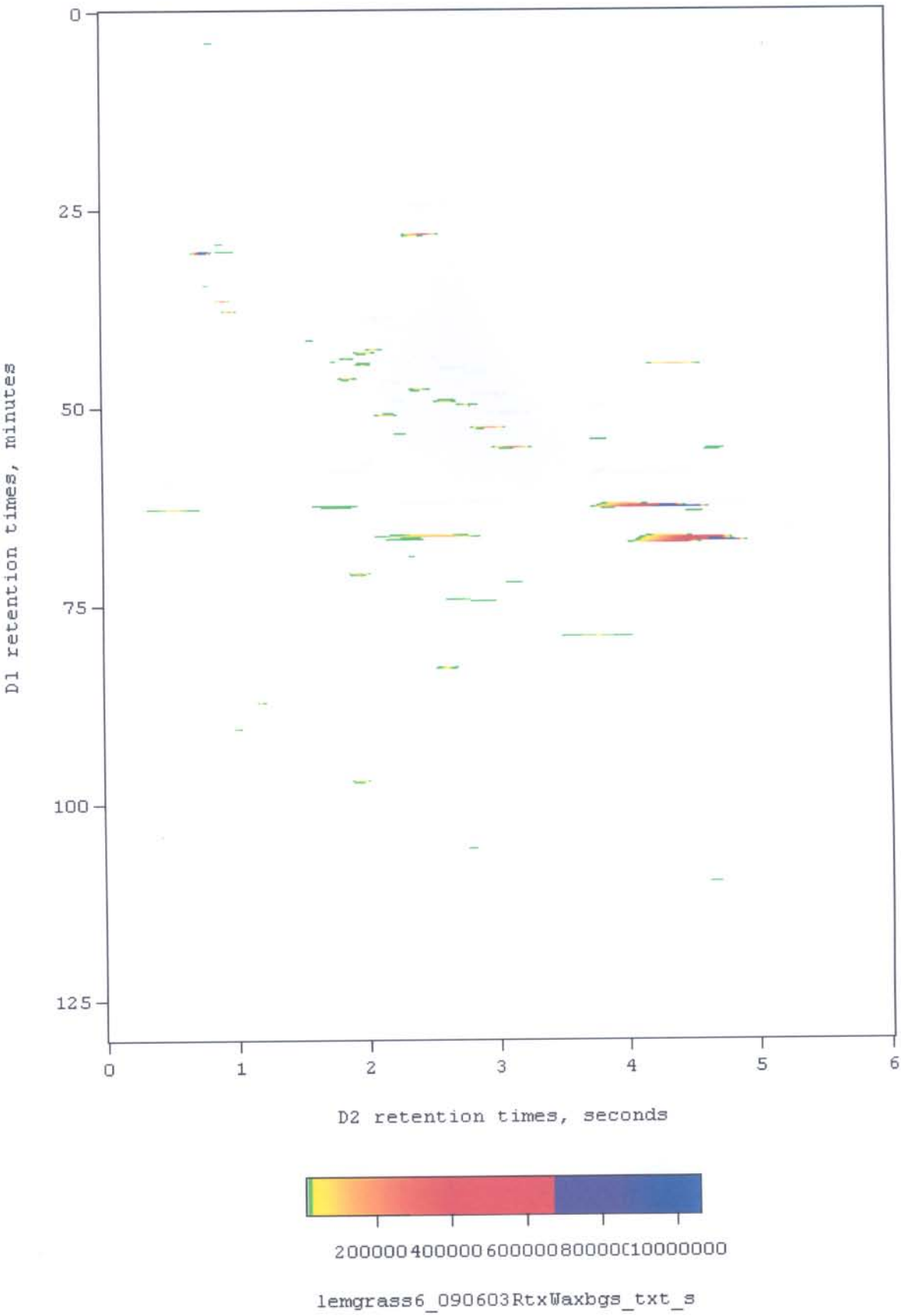


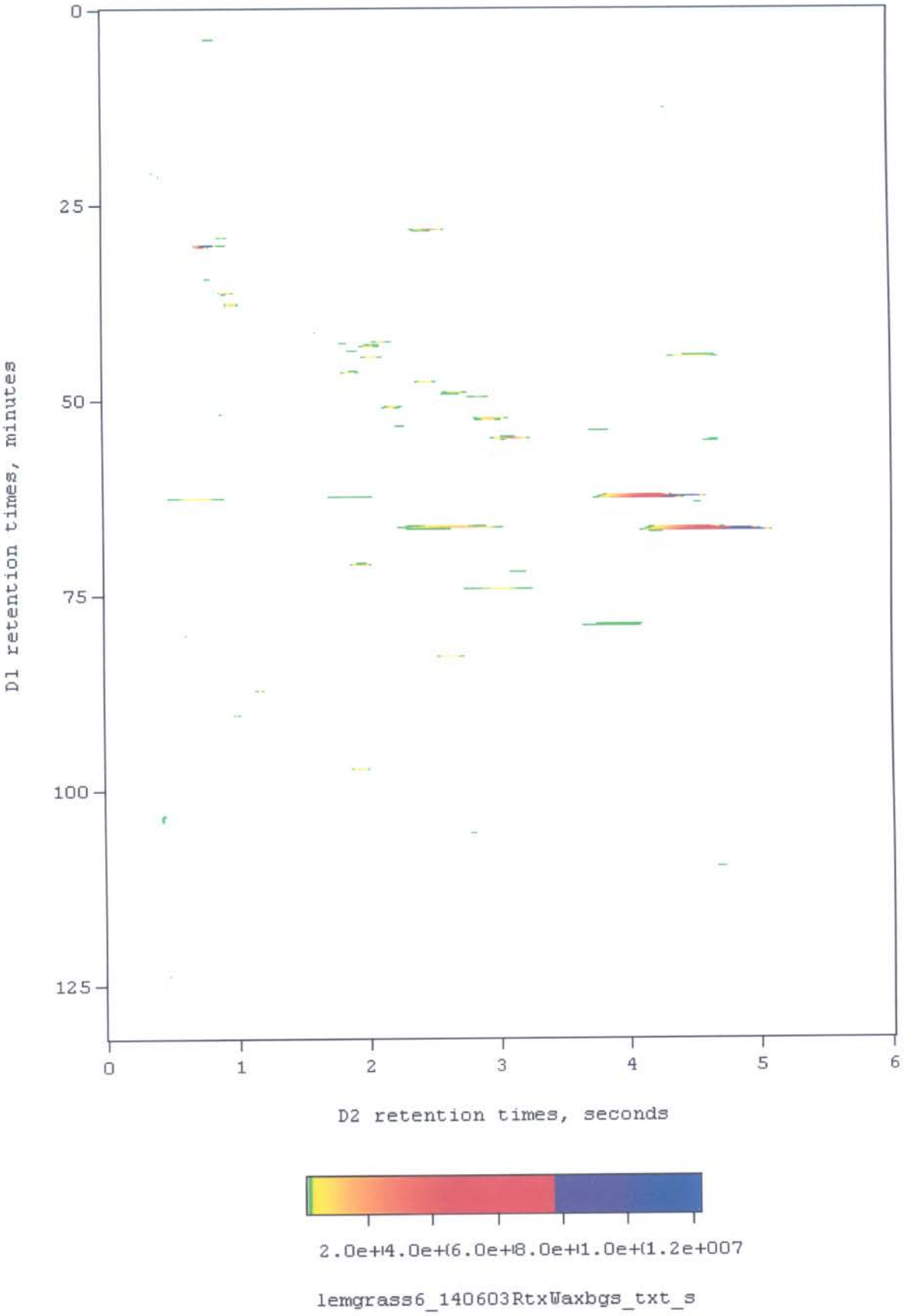
Lemongrass- (*Cymbopogon citratus)* sample-6

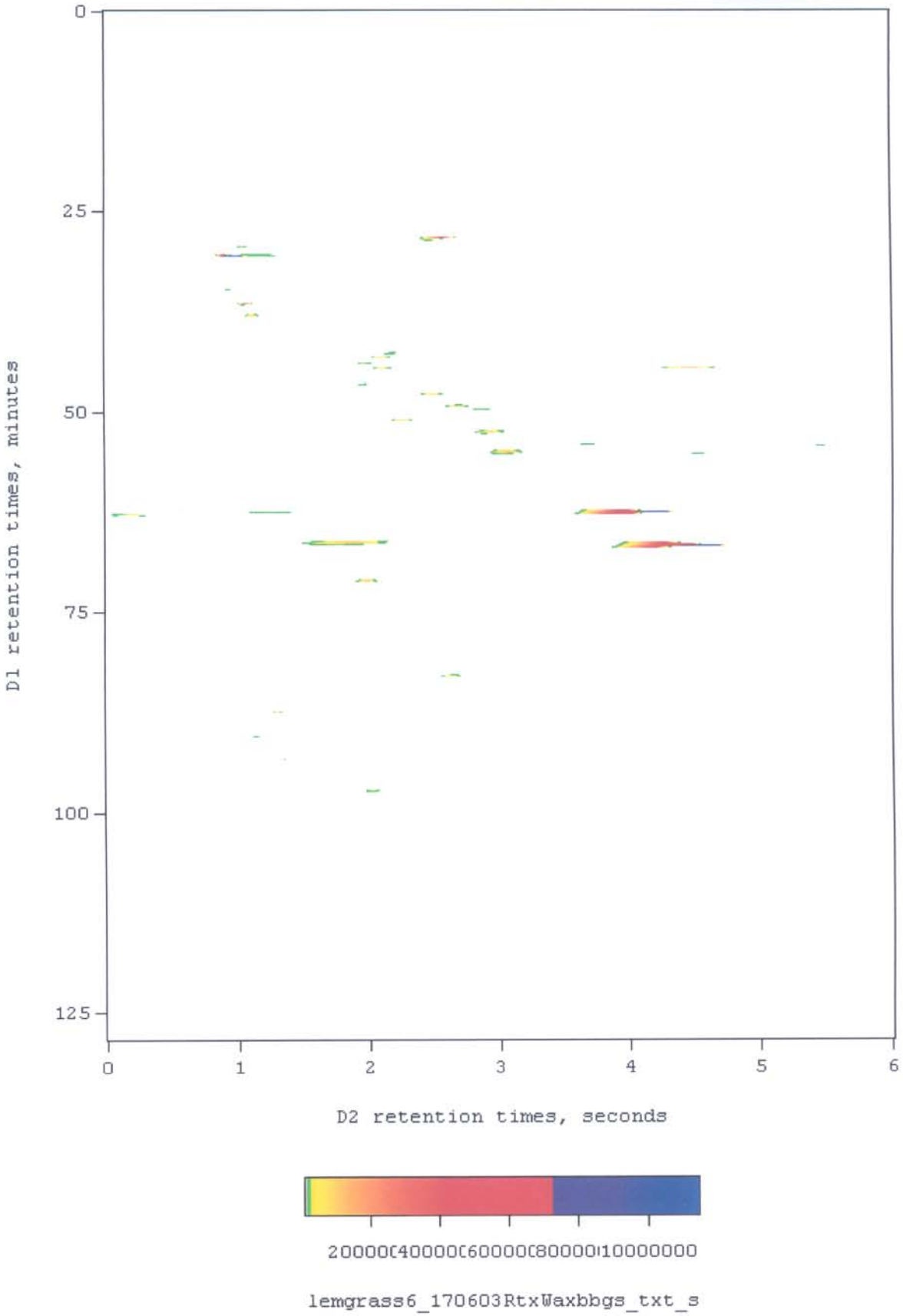


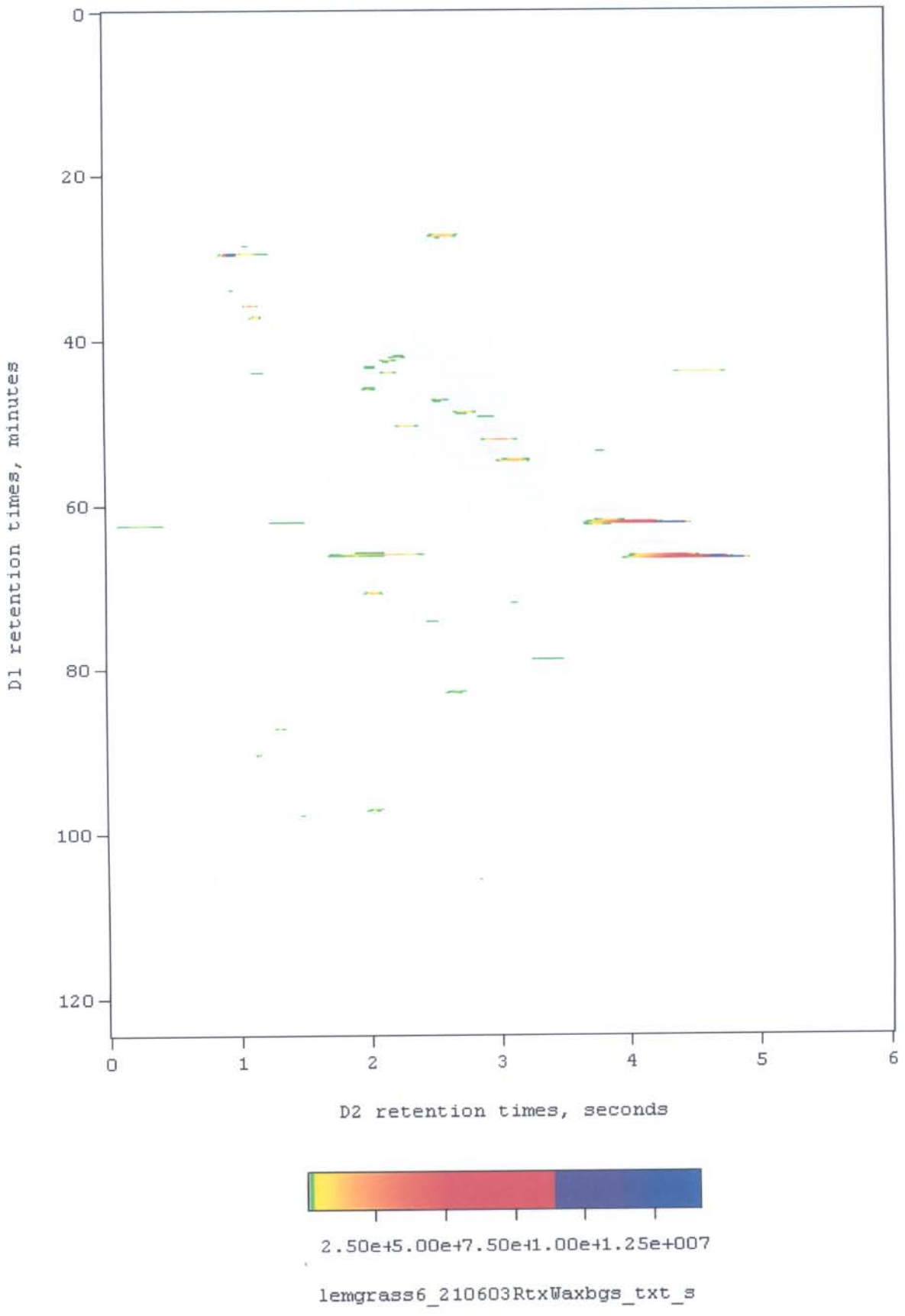


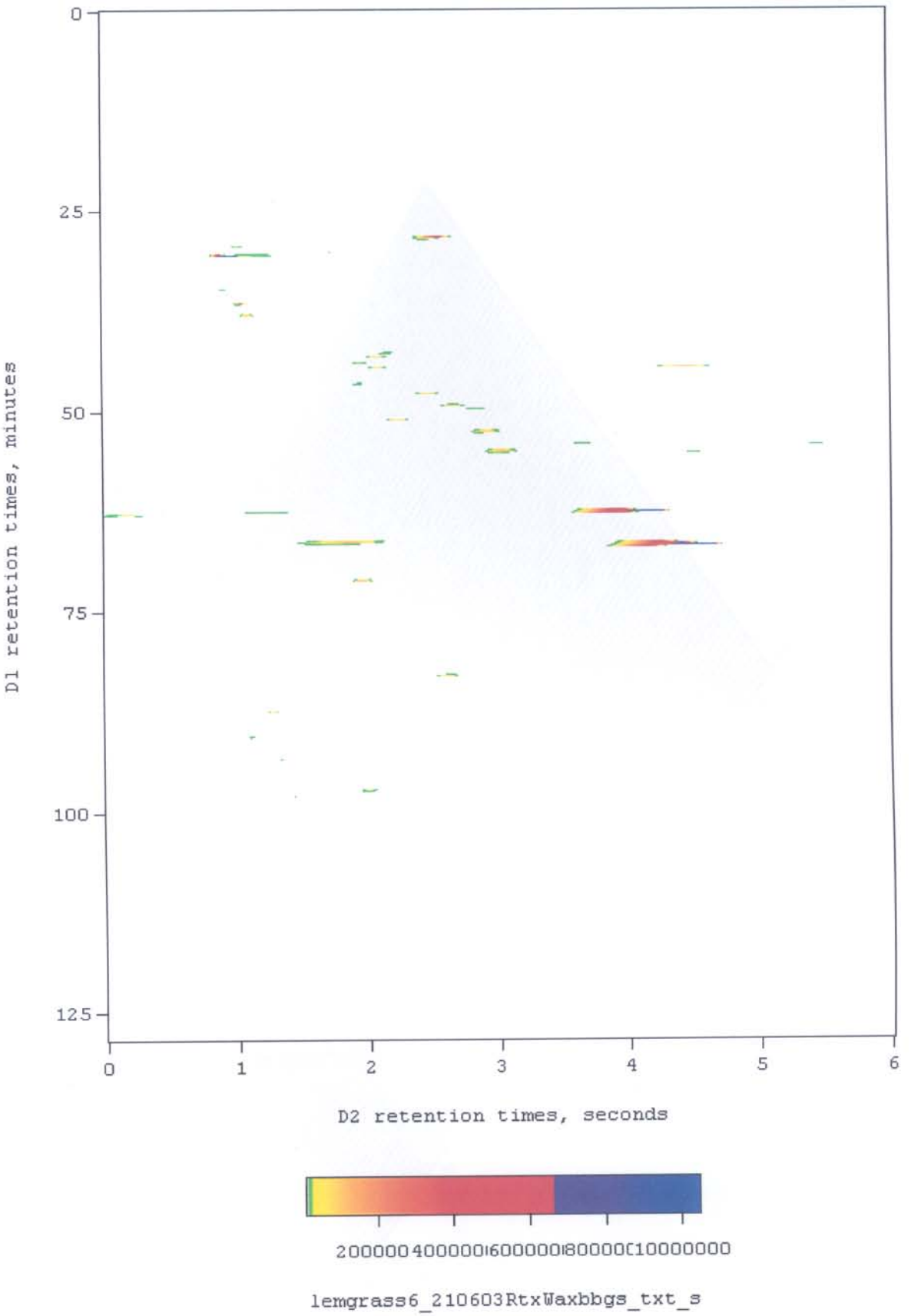


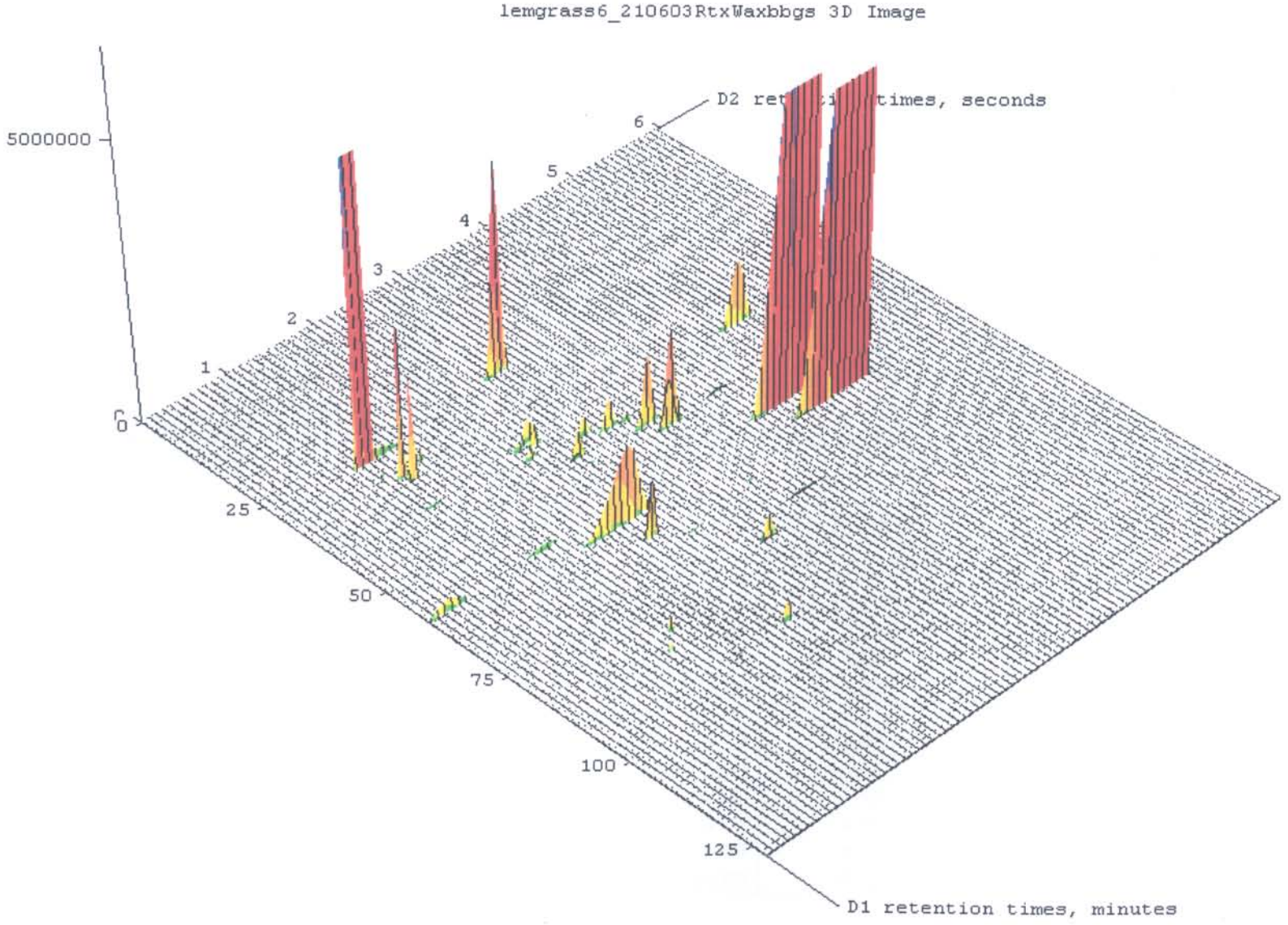











lemgrass6_210603RtxWaxbbgs 3D Image
5000000
0
1
2
3
4
5
6
D2 retention times, seconds
25
50
75
100
125
D1 retention times, minutes

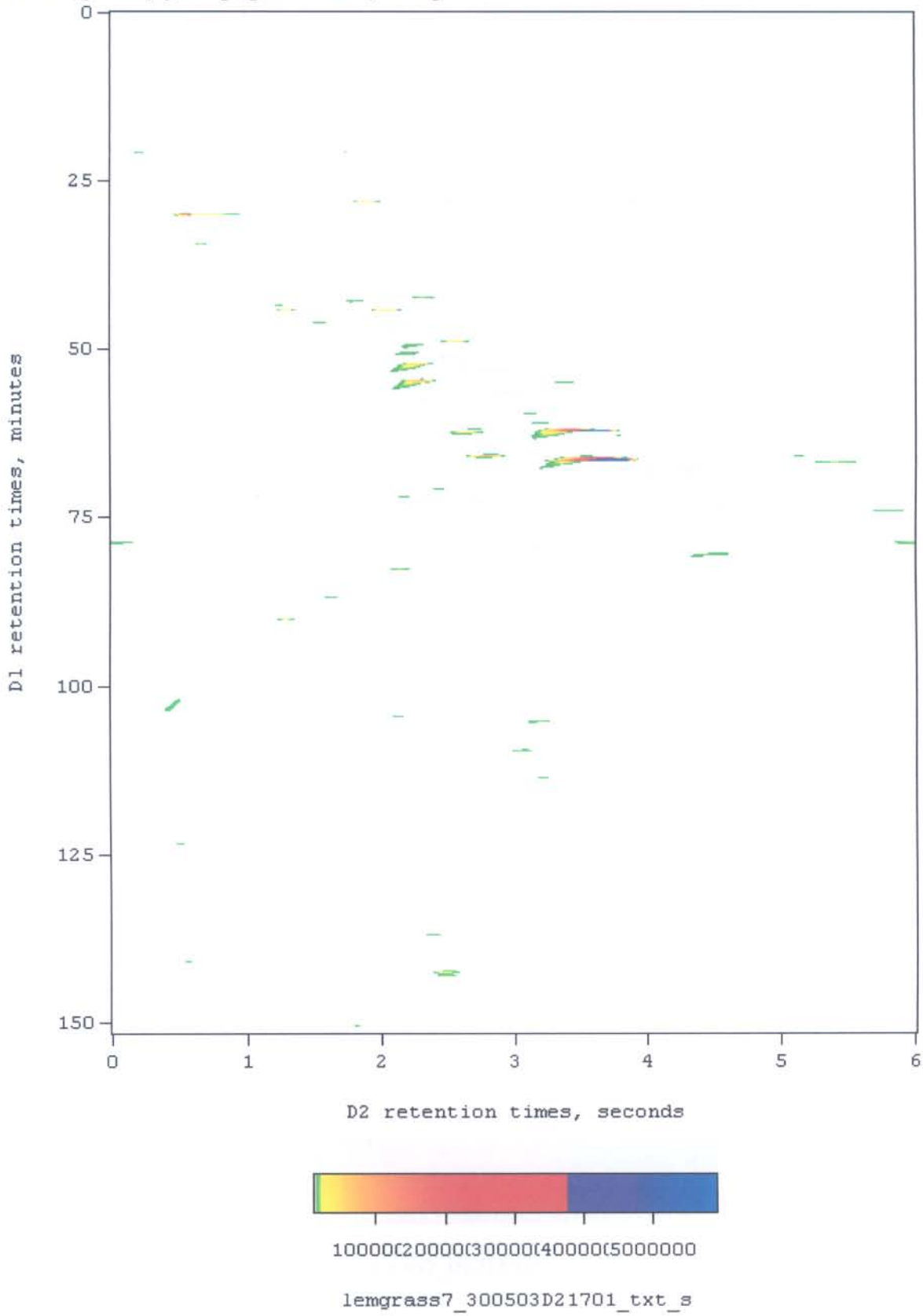
Lemongrass (*Cymbopogon citratus*) run-to-run peak reproducibility

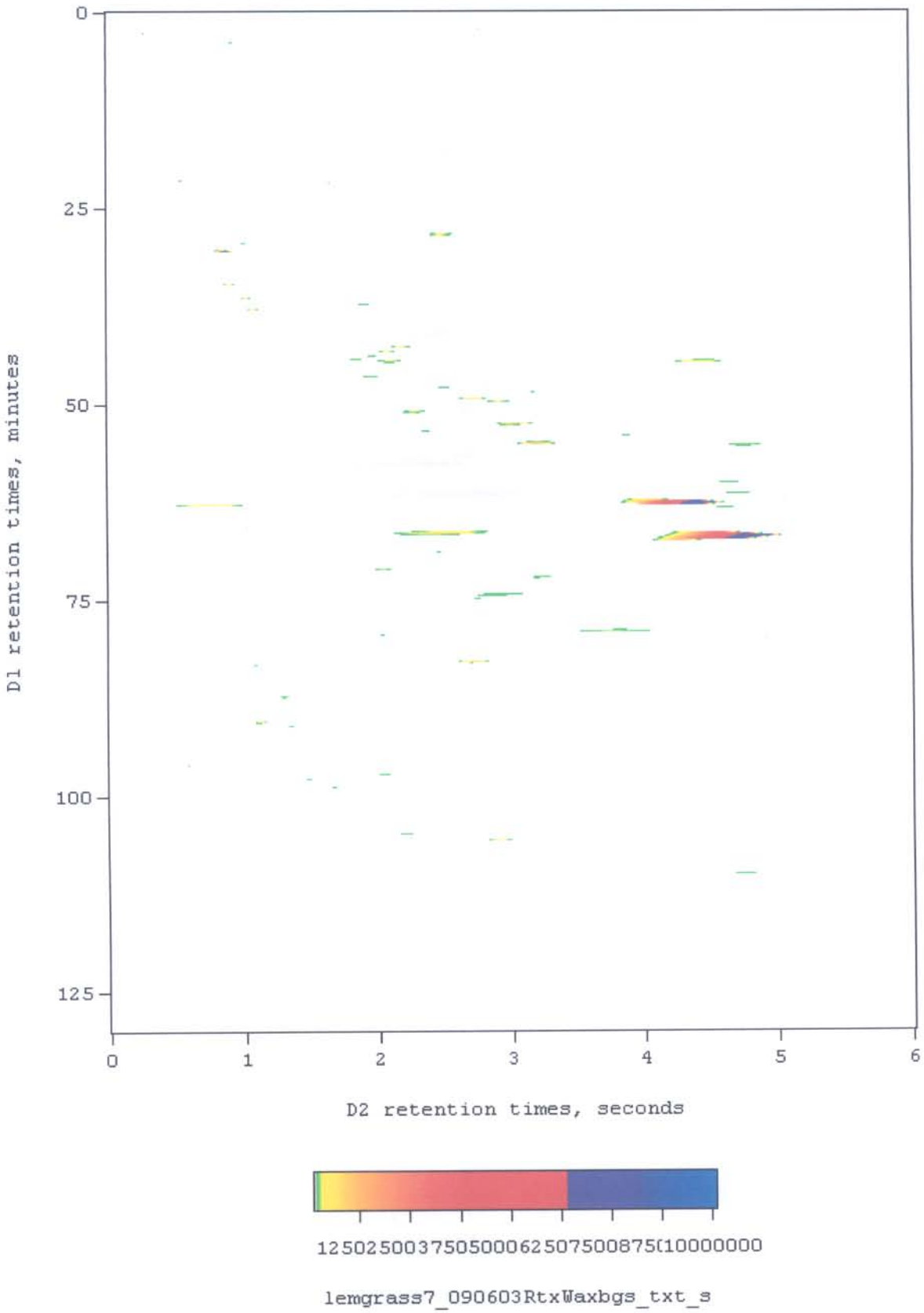
Peak	D1 retention times in minutes					D2 retention times in seconds					D2 Peak width at half height, msec				
no.	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1.	28.1	28.0	28.1	27.2	28.1	2.5	2.6	2.6	2.6	2.5	130	160	125	130	120
2.	30.3	30.2	30.4	29.4	30.4	0.8	0.8	1.0	1.0	1.0	100	110	140	90	100
3.	36.4	36.3	36.5	35.6	36.4	0.9	0.9	1.1	1.1	1.0	55	55	60	50	55
4.	37.8	37.6	37.8	37.0	37.8	1.0	1.0	1.1	1.2	1.1	55	60	60	50	60
5.	44.5	44.3	44.4	43.2	44.4	4.4	4.5	4.5	4.6	4.5	200	220	200	200	200
6.	49.1	49.1	49.2	48.7	49.1	2.6	2.7	2.7	2.8	2.7	120	120	135	120	130
7.	50.8	50.8	50.9	50.3	50.9	2.2	2.2	2.3	2.1	2.2	100	100	105	100	100
8.	52.5	52.4	52.4	52.0	52.4	2.9	3.0	3.0	3.0	2.9	120	140	150	135	120
9.	55.0	54.9	54.9	54.6	54.9	3.1	3.1	3.1	3.2	3.1	140	150	150	140	130
10.	71.0	71.0	71.0	70.7	71.1	2.0	2.0	2.0	2.0	2.0	90	90	80	80	80
11.	82.8	82.9	82.9	82.7	82.9	2.6	2.6	2.6	2.7	2.6	120	120	125	100	105
12.	97.0	97.2	97.2	97.0	97.2	1.9	1.9	2.0	2.0	2.0	100	90	90	70	80

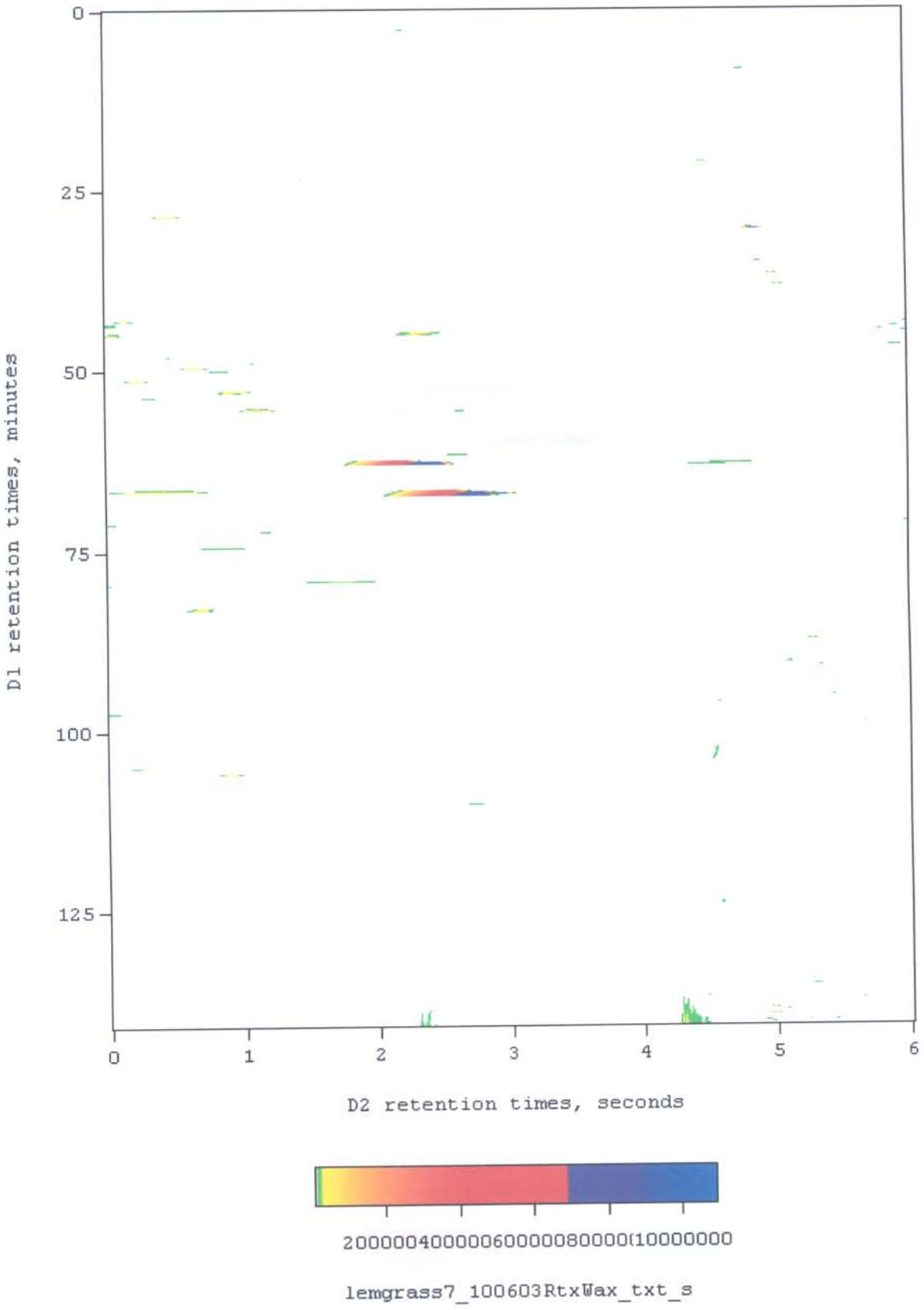
(1) lemgrass6090603RtxWaxbgs, (2) lemgrass6140603RtxWaxbgs, (3) lemgrass6170603RtxWaxb (4) lemgrass6210603RtxWaxbgs, and (5) lemgrass6210603RtxWaxbbgs

Peak	Mean D1 tr, minutes			Mean D2 tr, seconds			Mean D2 Wh, msec		
no.	Mean	STD	%RSD	Mean	STD	%RSD	Mean	STD	%RSD
1.	27.90	0.35	1.26	2.56	0.05	1.91	133.00	14.00	10.53
2.	30.14	0.38	1.25	0.92	0.10	10.65	108.00	17.20	15.93
3.	36.24	0.33	0.90	1.00	0.09	8.94	55.00	3.16	5.75
4.	37.60	0.31	0.82	1.08	0.07	6.93	57.00	4.00	7.02
5.	44.16	0.48	1.10	4.50	0.06	1.41	204.00	8.00	3.92
6.	49.04	0.17	0.36	2.70	0.06	2.34	125.00	6.32	5.06
7.	50.74	0.22	0.44	2.20	0.06	2.87	101.00	2.00	1.98
8.	52.34	0.17	0.33	2.96	0.05	1.66	133.00	11.66	8.77
9.	54.86	0.14	0.25	3.12	0.04	1.28	142.00	7.48	5.27
10.	70.96	0.14	0.19	2.00	0.00	0.00	84.00	4.90	5.83
11.	82.84	0.08	0.10	2.62	0.04	1.53	114.00	9.70	8.50
12.	97.12	0.10	0.10	1.96	0.05	2.50	86.00	10.20	11.86
	Mean		**0.59**			**3.50**			**7.53**

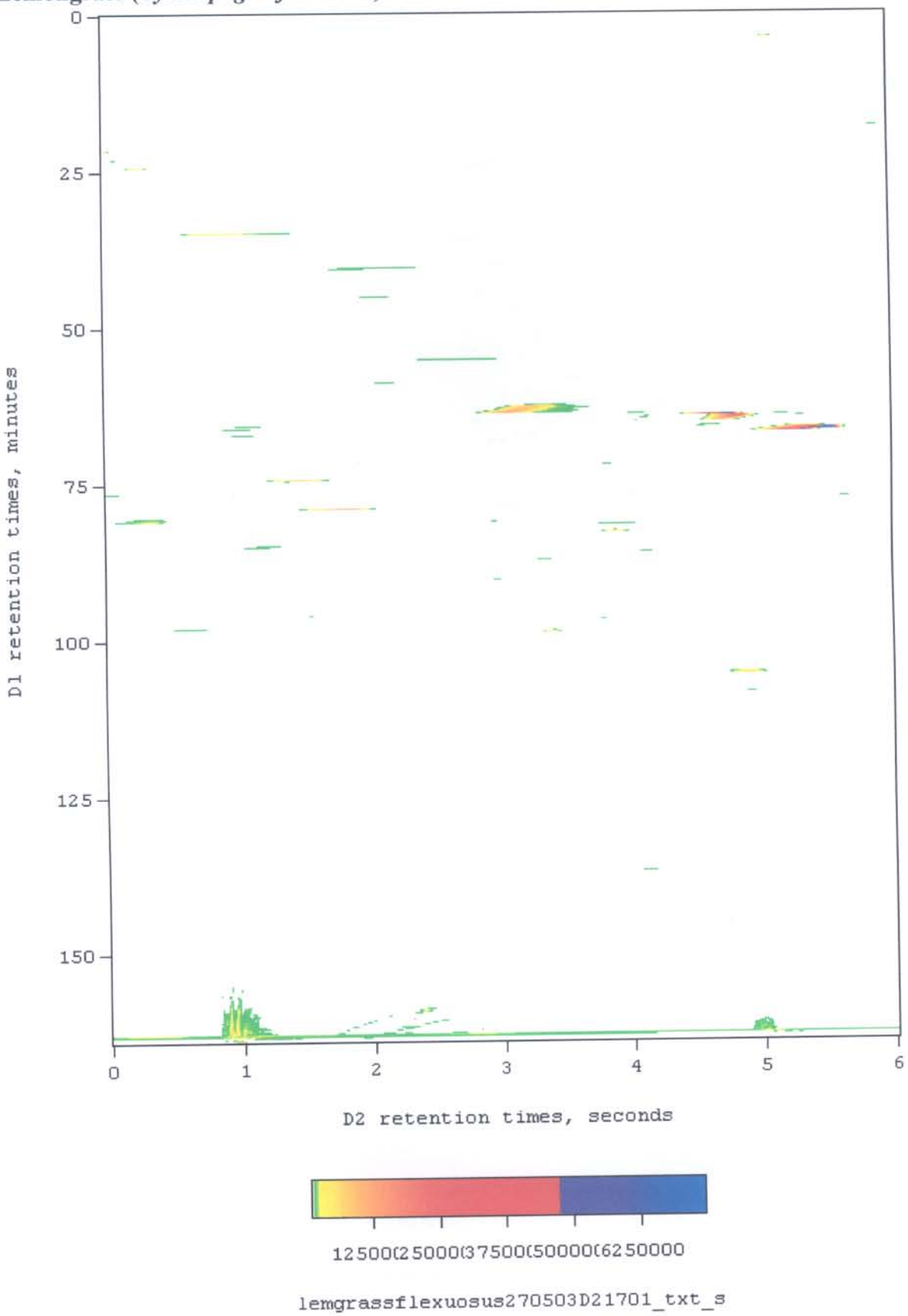
Lemongrass (*Cymbopogon citratus*) sample-7

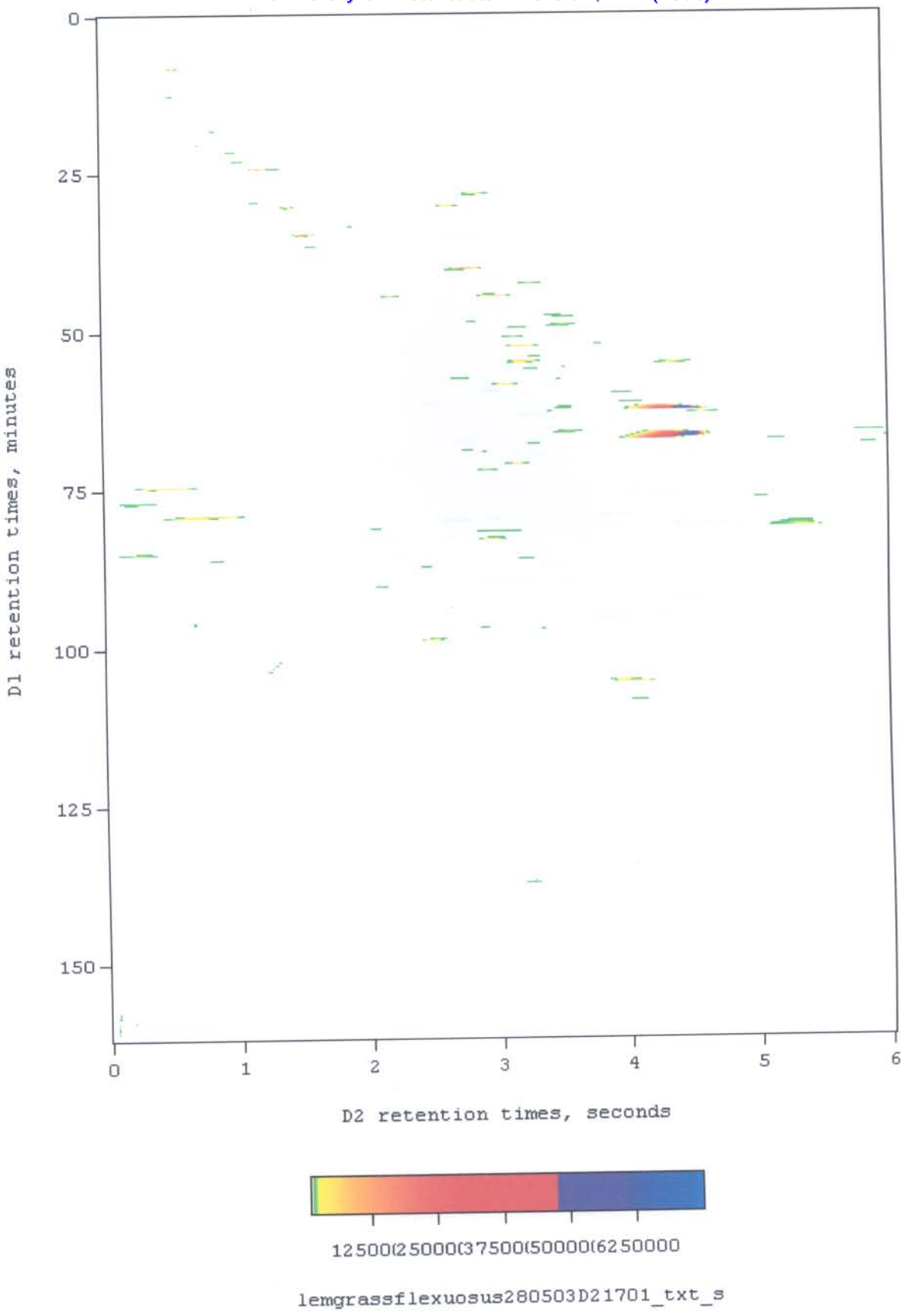


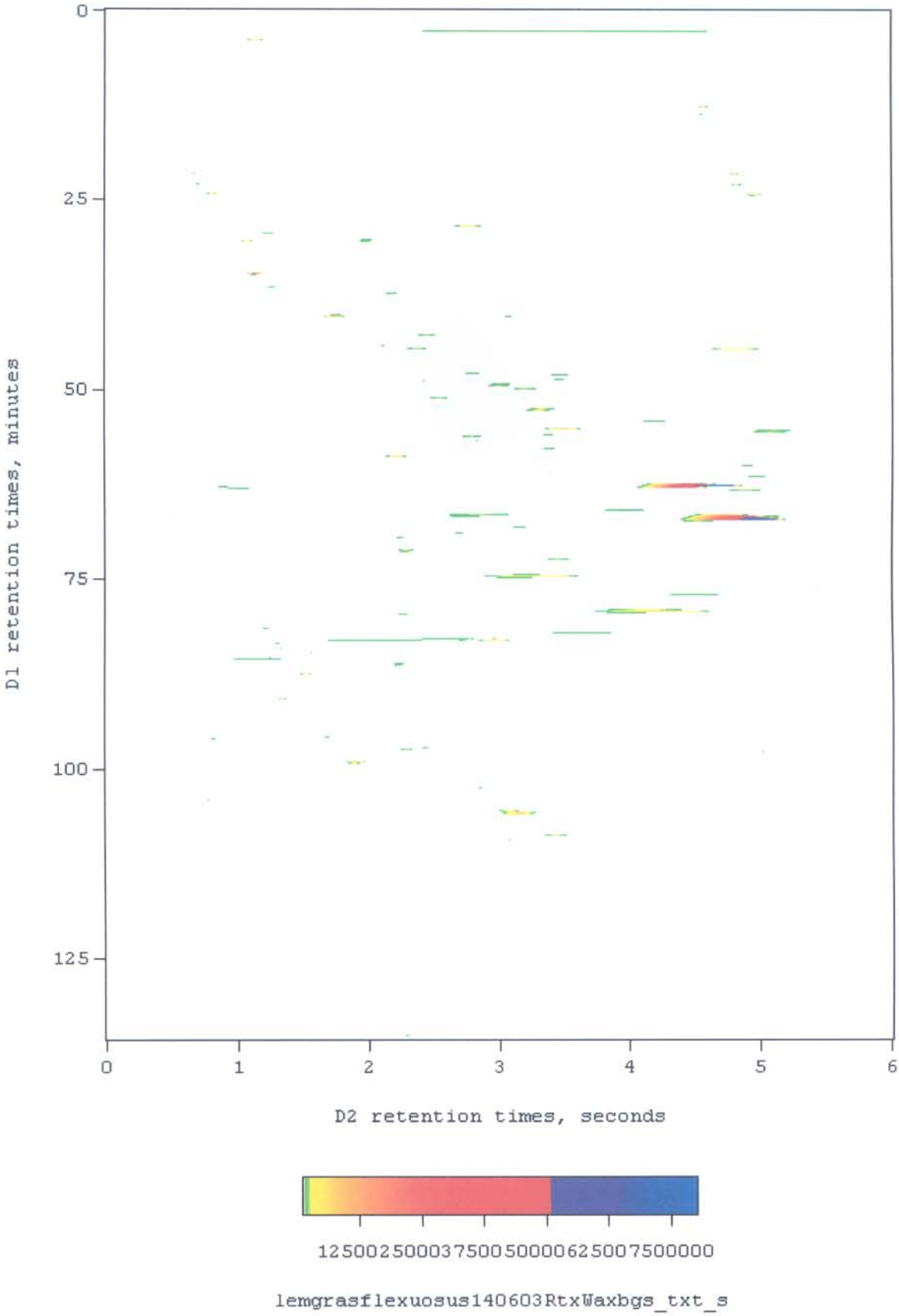


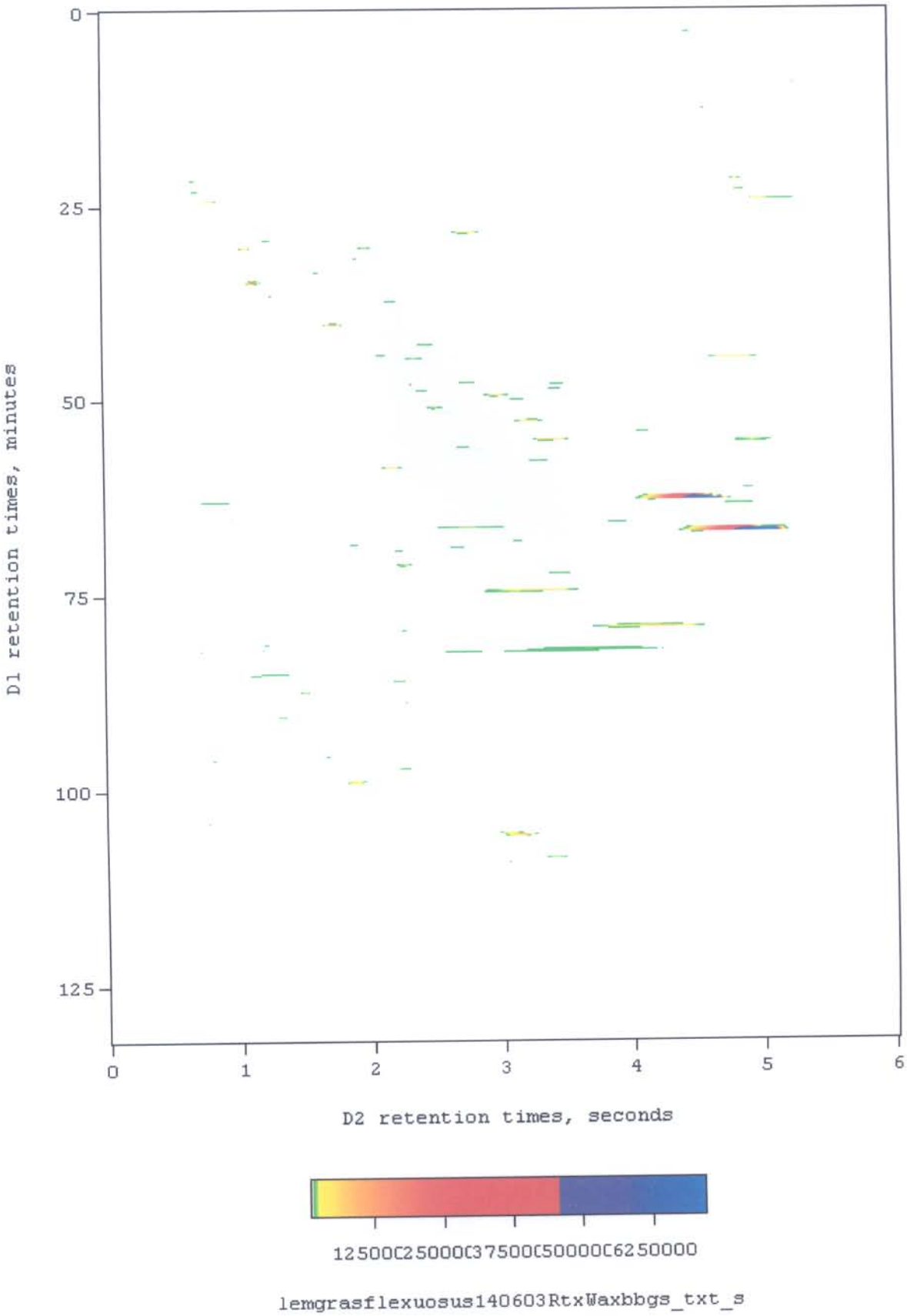


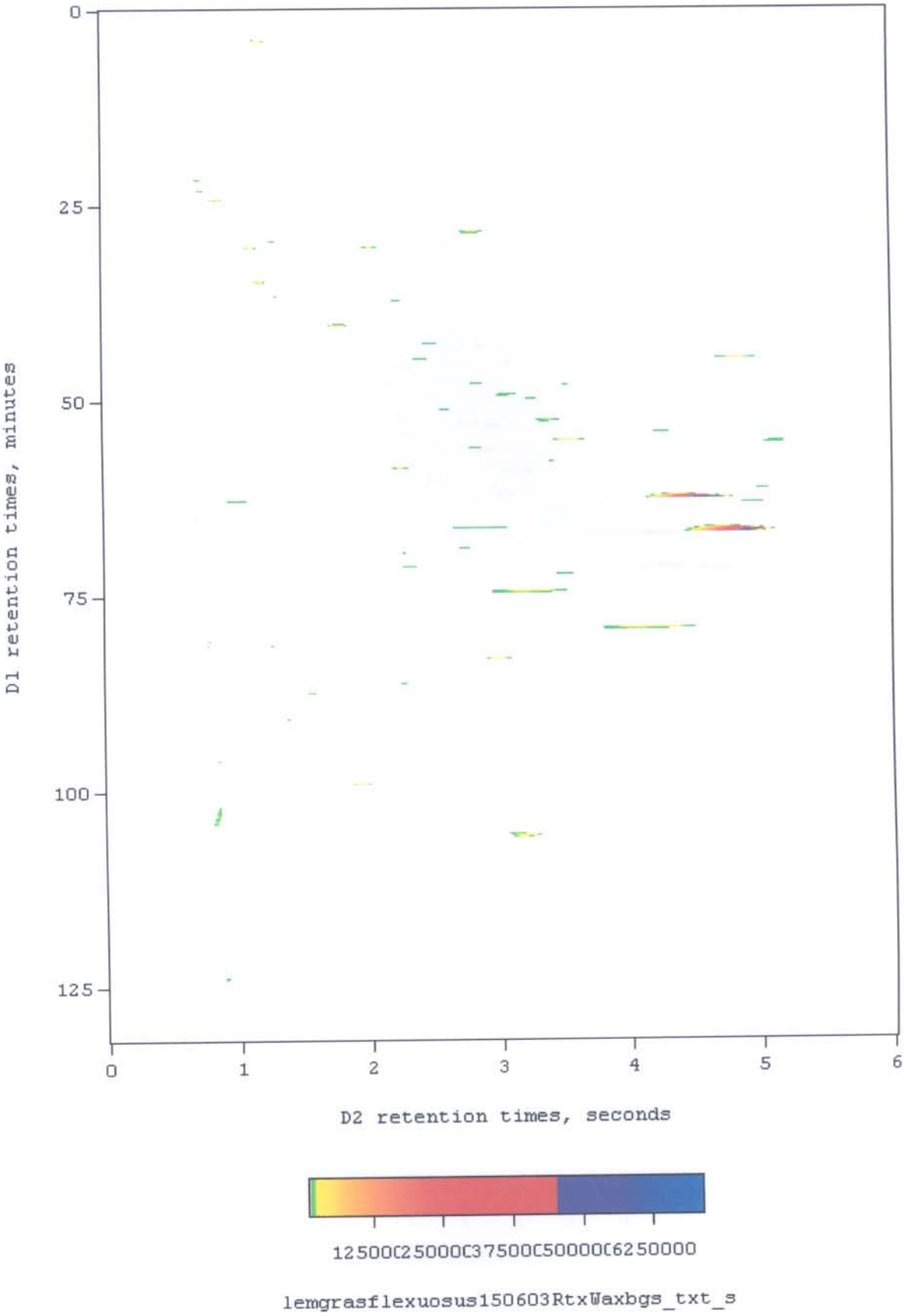
Lemongrass (*Cymbopogon flexuosus*)

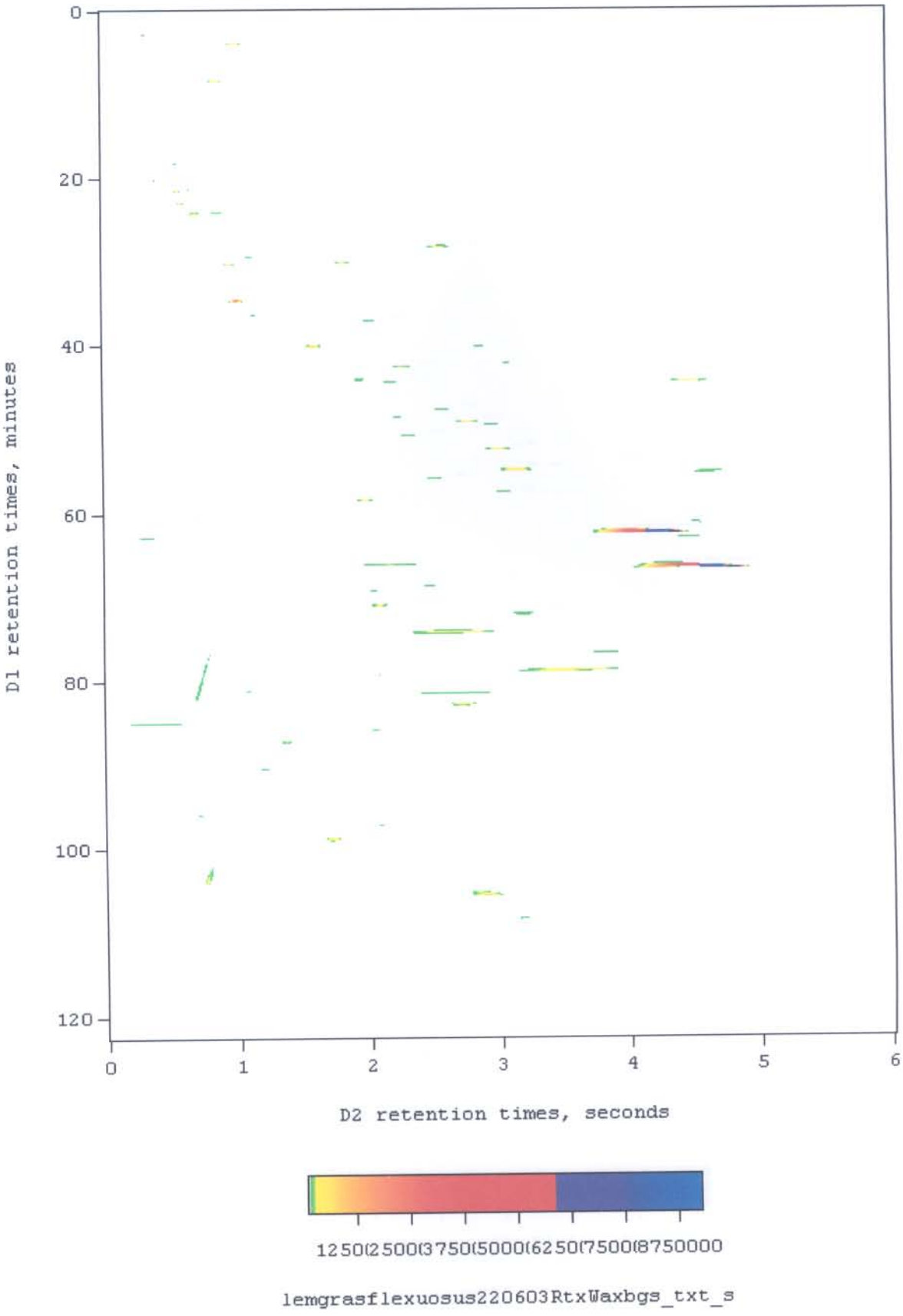


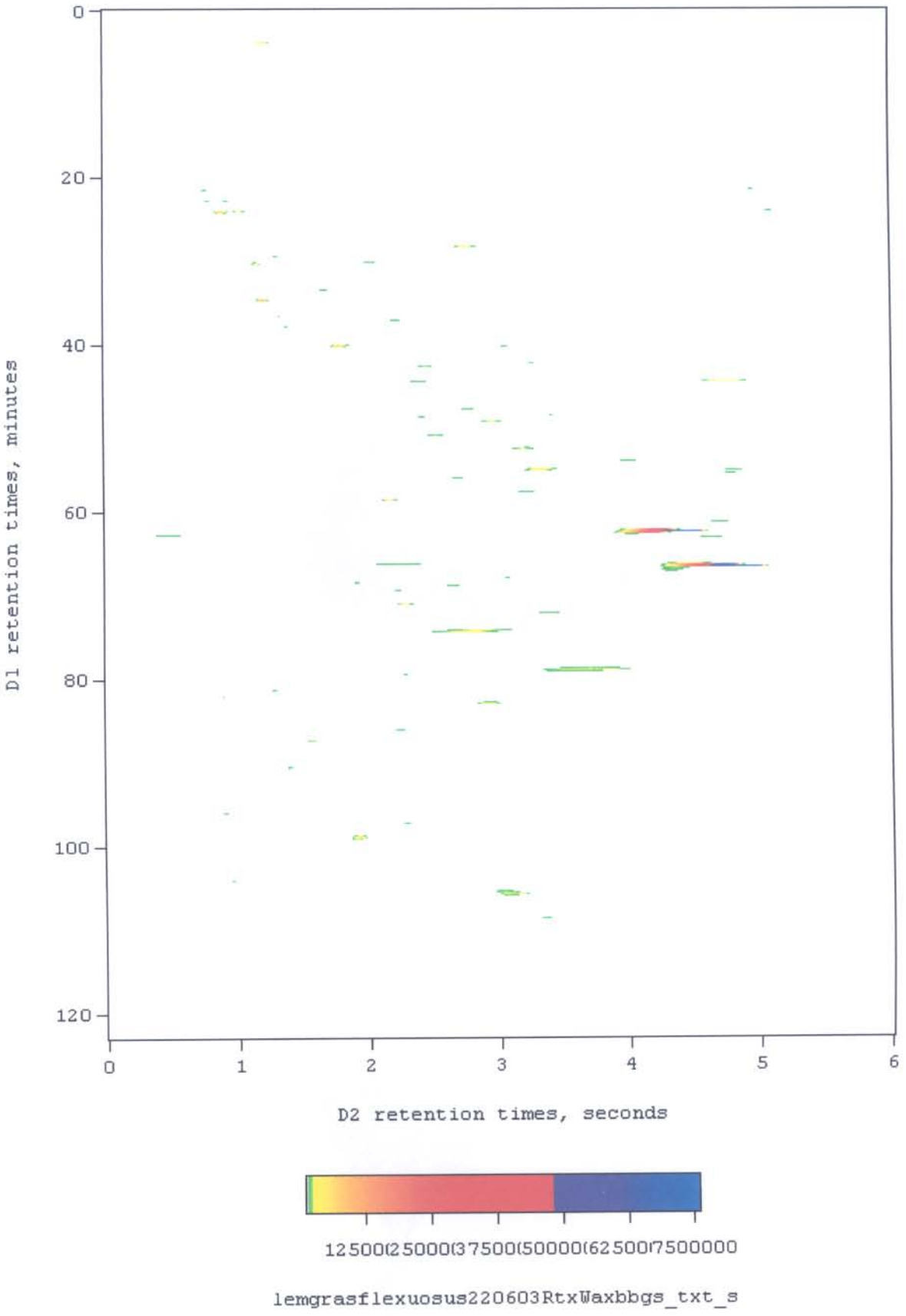


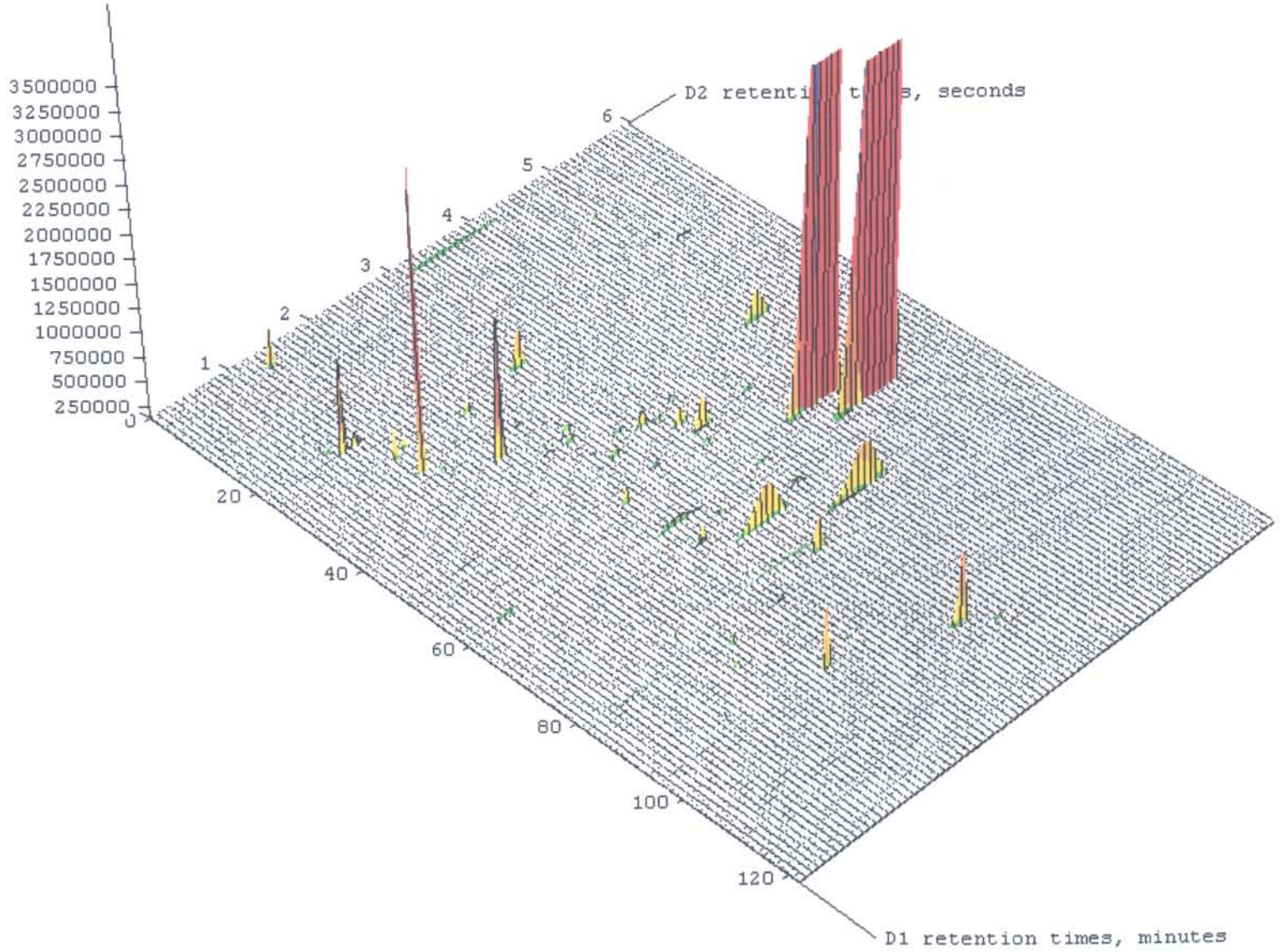








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1750000
1500000
1250000
1000000
750000
500000
250000
0
1
2
3
4
5
6
D2 retenti t s, seconds
20
40
60
80
100
120
D1 retention times, minutes

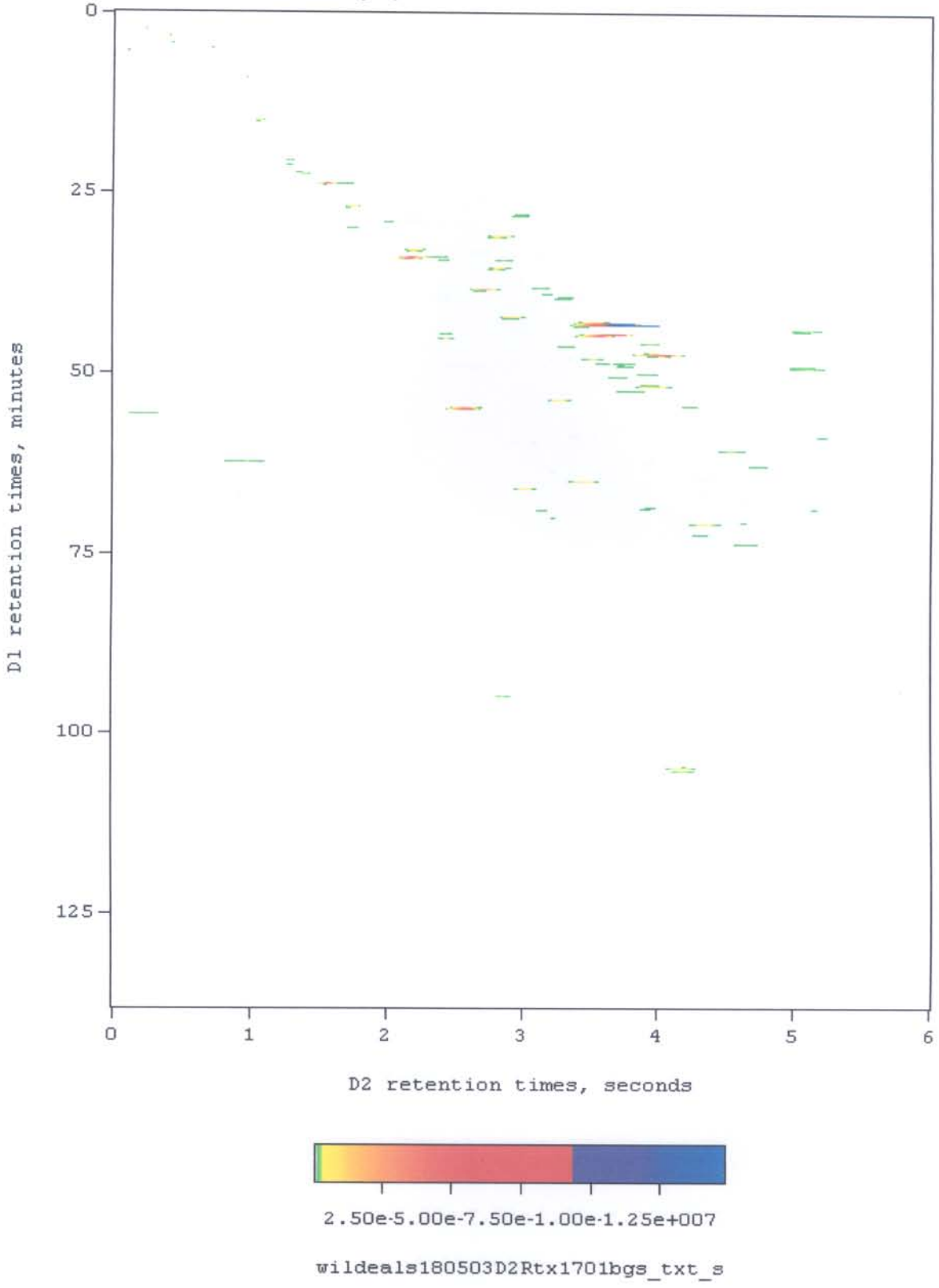
Lemongrass (*Cymbopogon flexuosus*) run-to-run peak reproducibility

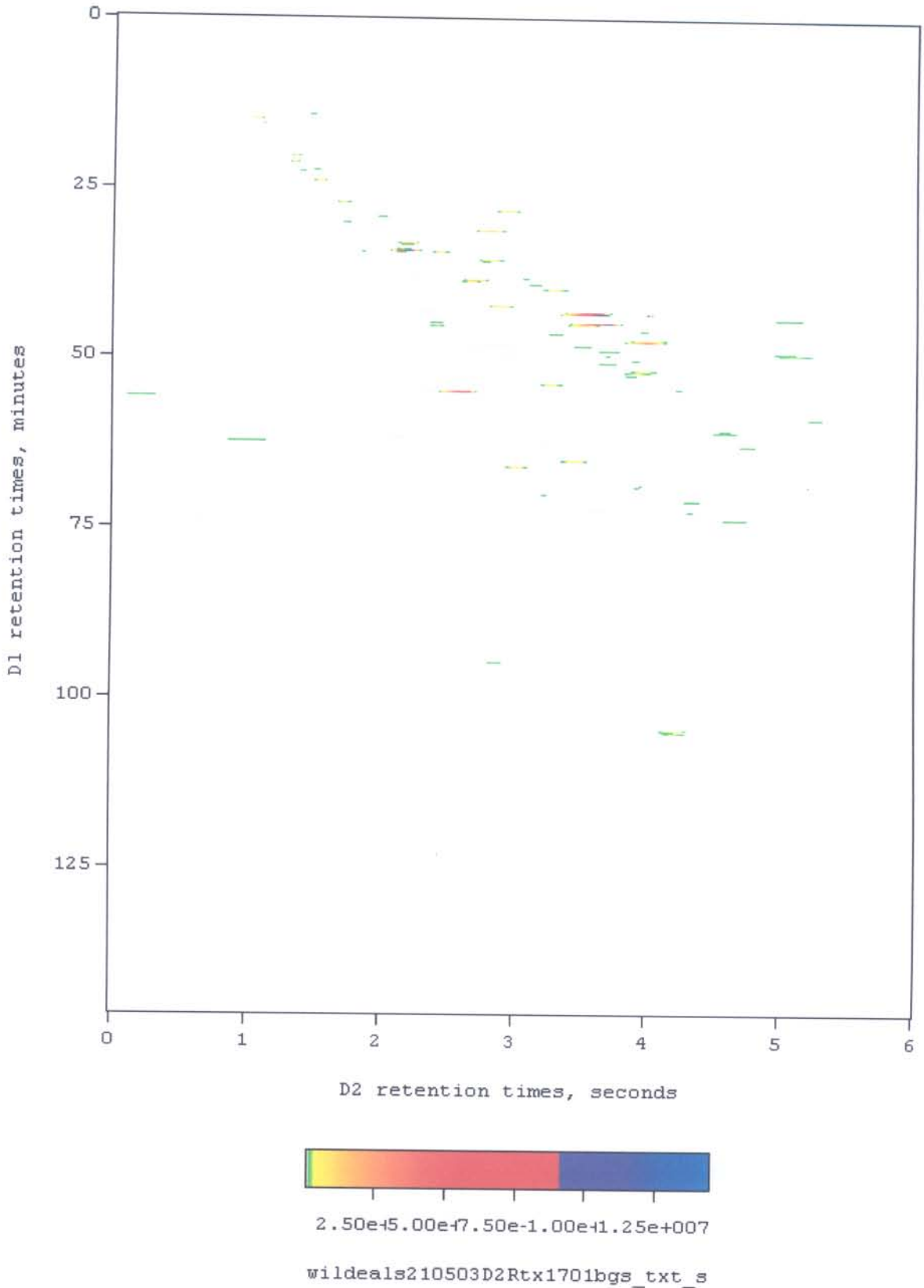
Peak	D1 retention times in minutes					D2 retention times in seconds					D2 Peak width at half height, msec				
no.	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1.	24.1	24.2	24.2	24.0	24.0	0.8	0.8	0.9	0.7	0.9	50	50	50	45	45
2.	28.2	28.3	28.4	28.0	28.1	2.8	2.8	2.8	2.6	2.8	125	120	125	110	120
3.	34.6	34.7	34.7	34.5	34.5	1.1	1.1	1.2	1.0	1.2	60	50	50	50	50
4.	40.1	40.1	40.2	39.9	40.0	1.7	1.7	1.8	1.6	1.8	70	70	80	70	70
5.	44.5	44.6	44.6	44.2	44.3	4.8	4.8	4.8	4.5	4.7	225	200	200	200	200
6.	52.5	52.6	52.6	52.3	52.2	3.3	3.2	2.4	3.0	3.2	150	140	150	140	140
7.	55.0	55.1	55.1	54.8	54.9	3.5	3.4	3.5	3.2	3.3	140	130	150	150	140
8.	82.9	81.9	83.0	82.7	82.8	3.0	3.6	3.0	2.7	2.9	125	---	125	110	120
9.	98.9	98.9	99.0	98.7	98.8	1.9	1.9	1.9	1.7	1.9	70	70	70	65	70
10.	105.7	105.7	105.8	105.5	105.5	3.2	3.1	3.2	2.9	3.1	125	120	125	110	120

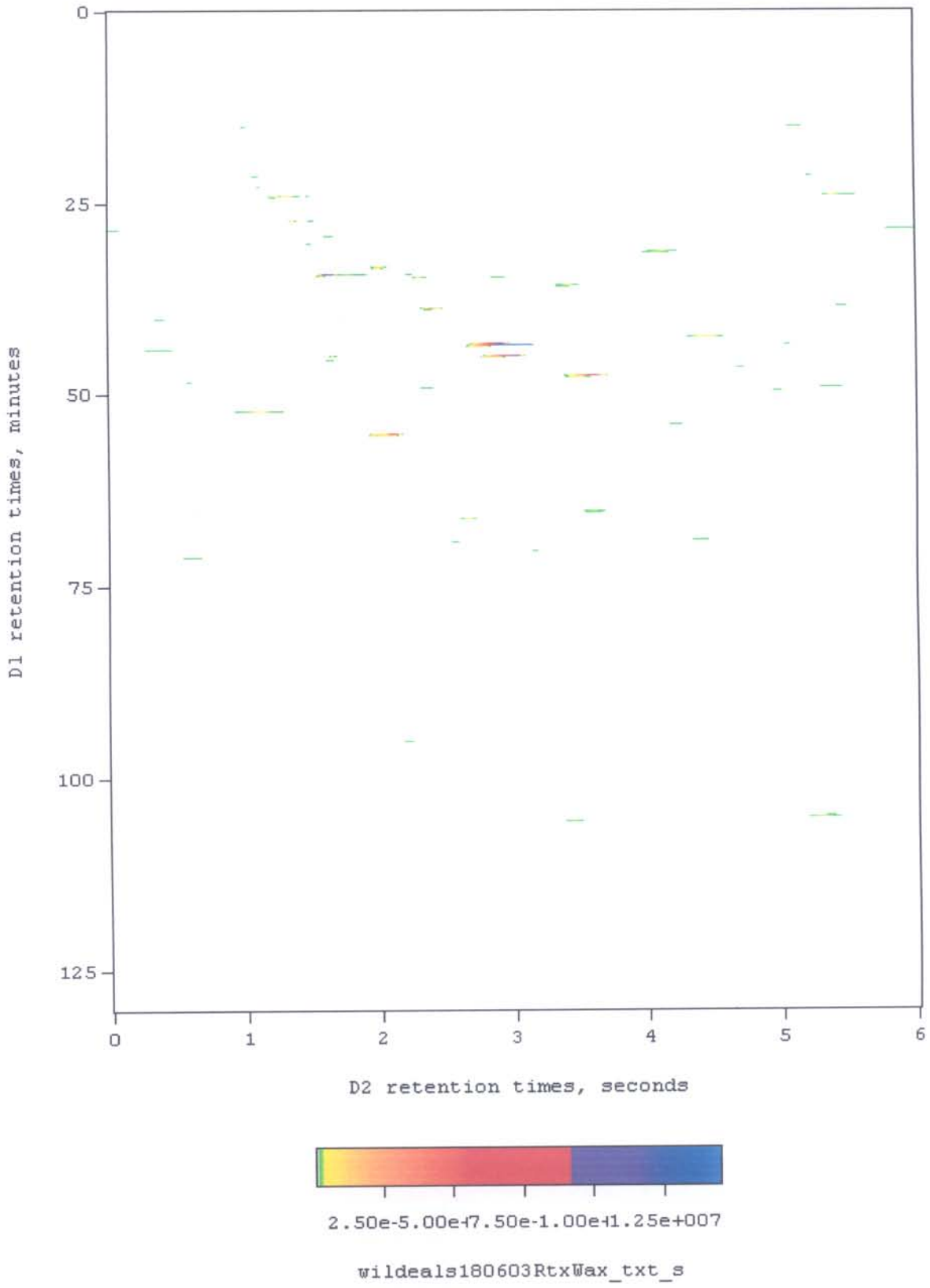
(1) lemgrassflexuosus140603RtxWax, (2) lemgrassflexuosus140603RtxWaxb, (3) lemgrassflexuosus150603RtxWax (4) lemgrassflexuosus220603RtxWax and (5) lemgrassflexuosus220603RtxWaxb

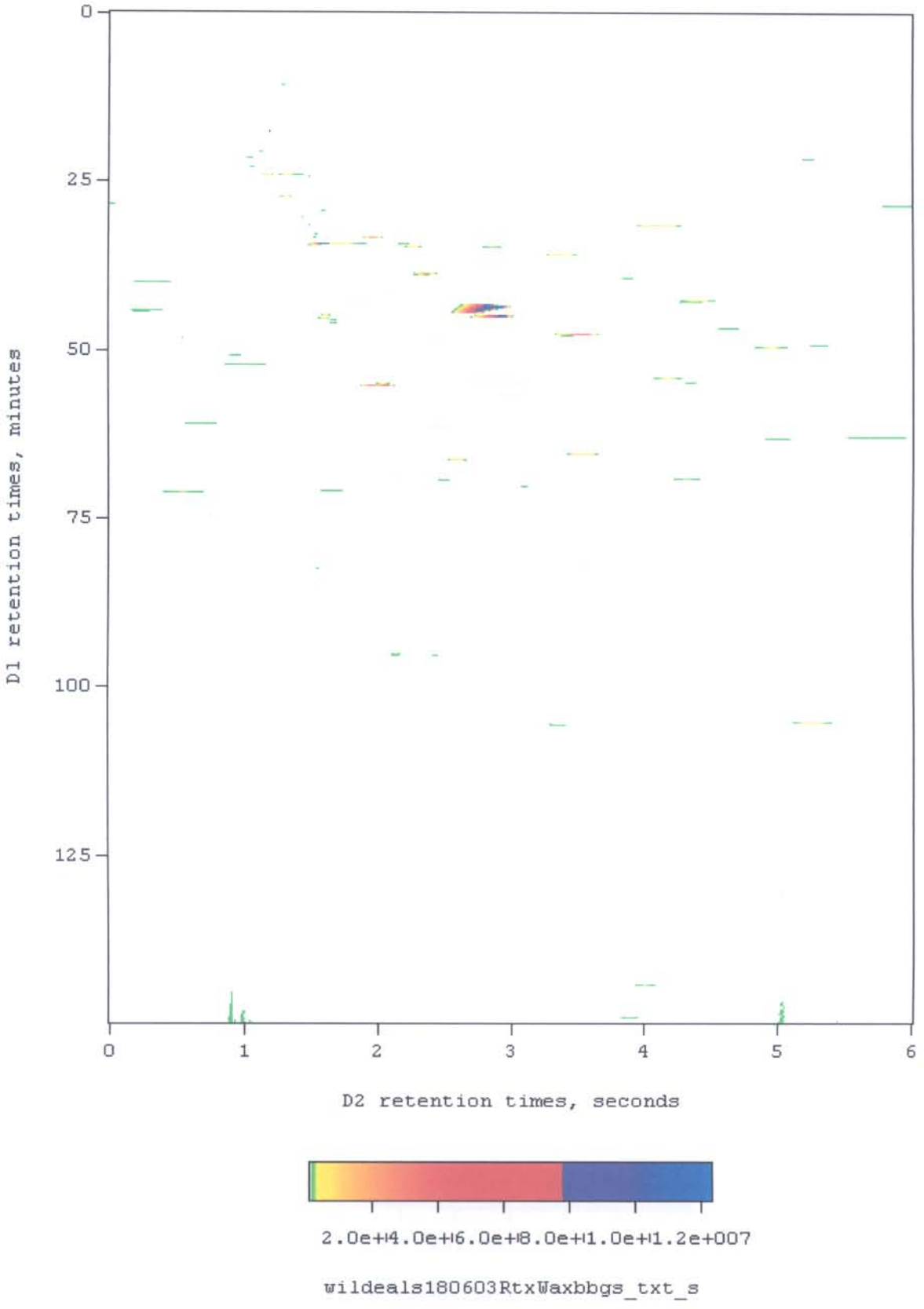
Peak	Mean D1 tr, minutes			Mean D2 tr, seconds			Mean D2 Wh, msec		
no.	Mean	STD	%RSD	Mean	STD	%RSD	Mean	STD	%RSD
1.	24.10	0.09	0.32	0.82	0.07	9.13	48.00	2.45	5.10
2.	28.20	0.14	0.41	2.76	0.08	2.90	120.00	5.48	4.56
3.	34.60	0.09	0.22	1.12	0.07	6.68	52.00	4.00	7.69
4.	40.06	0.10	0.23	1.72	0.07	4.35	72.00	4.00	5.56
5.	44.44	0.16	0.31	4.72	0.12	2.47	205.00	10.00	4.88
6.	52.44	0.16	0.30	3.02	0.32	10.76	144.00	4.90	3.40
7.	54.98	0.12	0.14	3.38	0.12	3.45	142.00	7.48	5.27
8.	82.66	0.39	0.40	3.04	0.30	9.89	120.00	6.12	5.10
9.	98.86	0.10	0.10	1.86	0.08	4.30	69.00	2.00	2.90
10.	105.64	0.12	0.11	3.10	0.11	3.53	120.00	5.48	4.56
	Mean		**0.25**			**5.75**			**4.90**

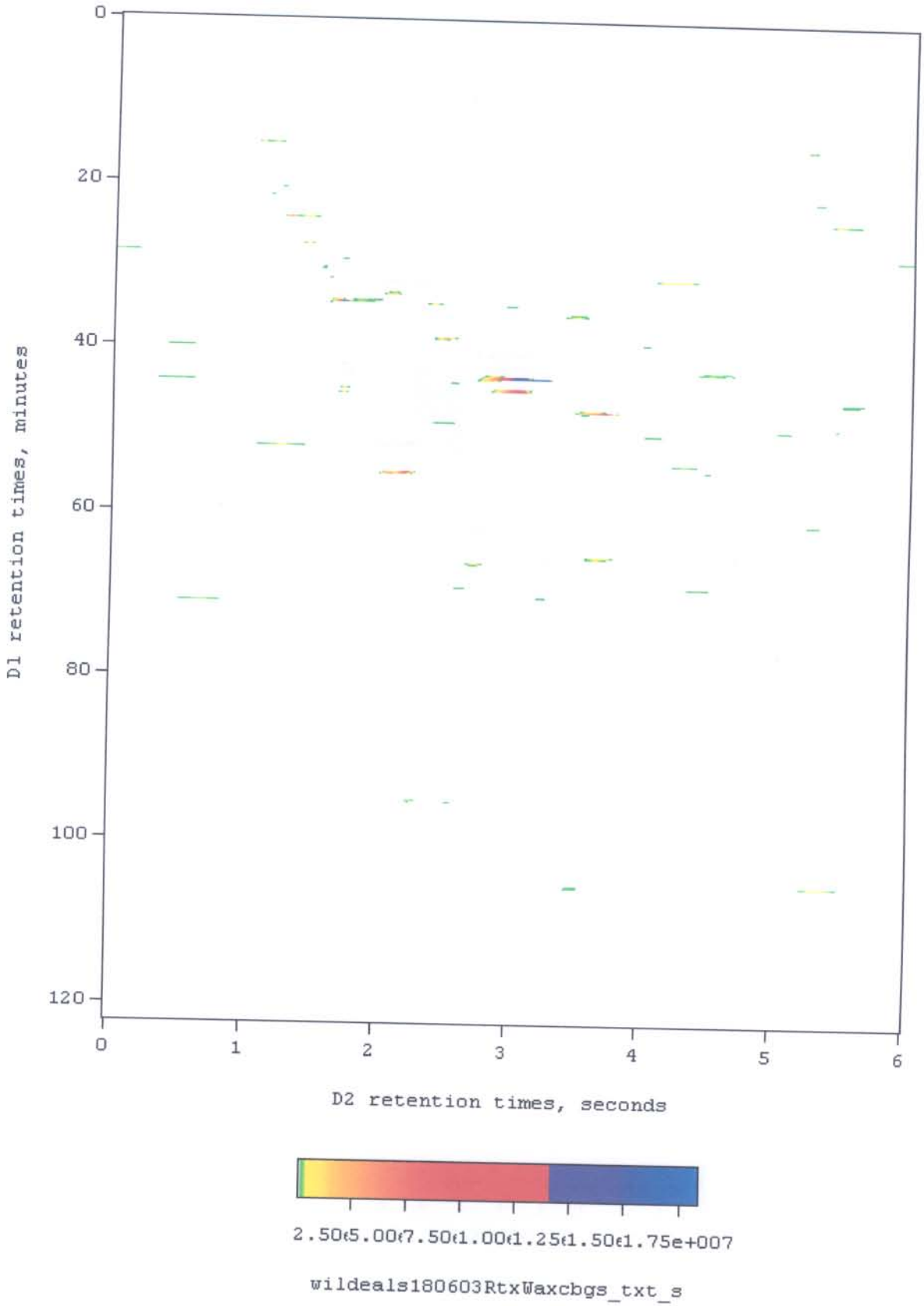
African Wormwood (*Artemisia afra*)

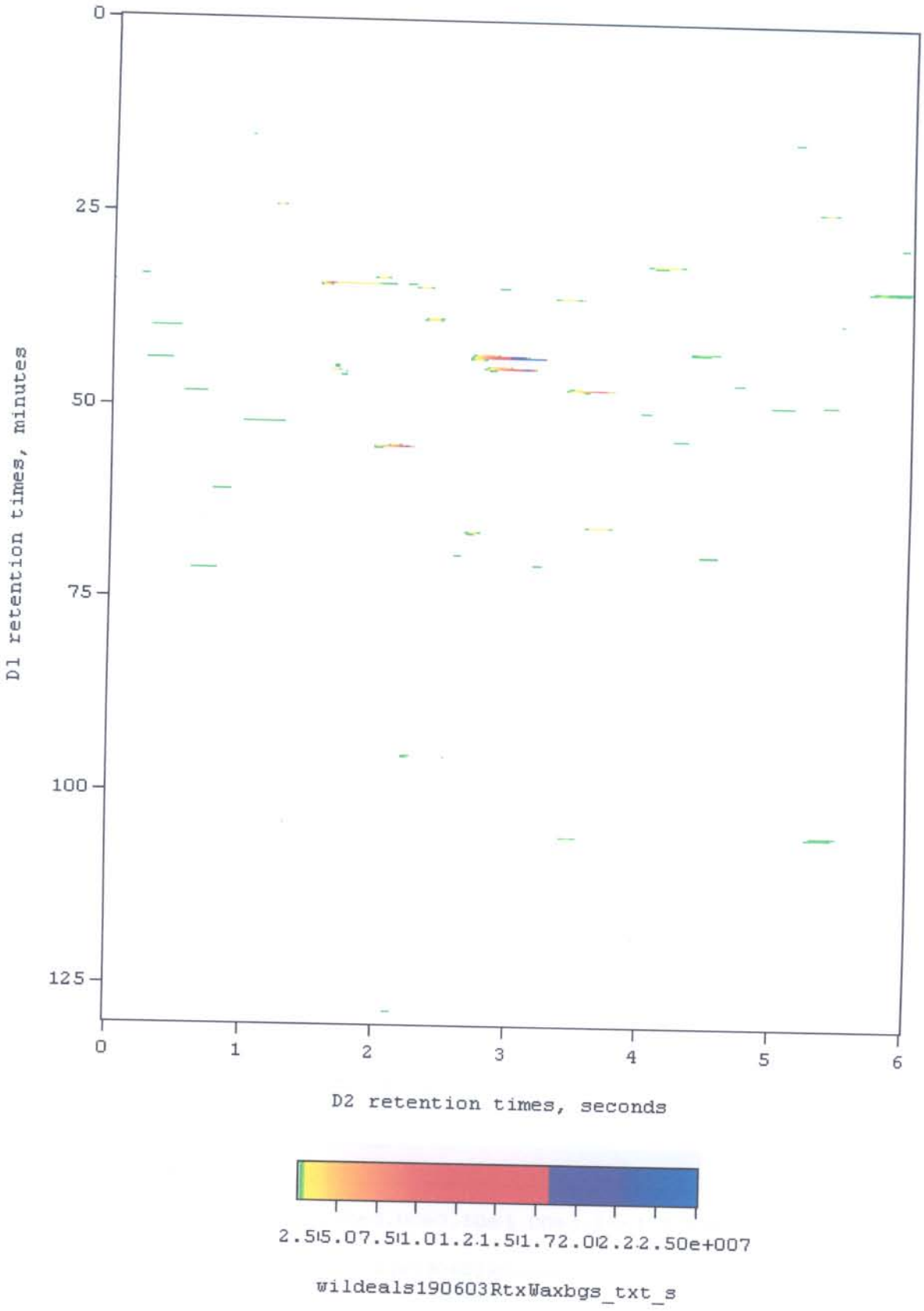


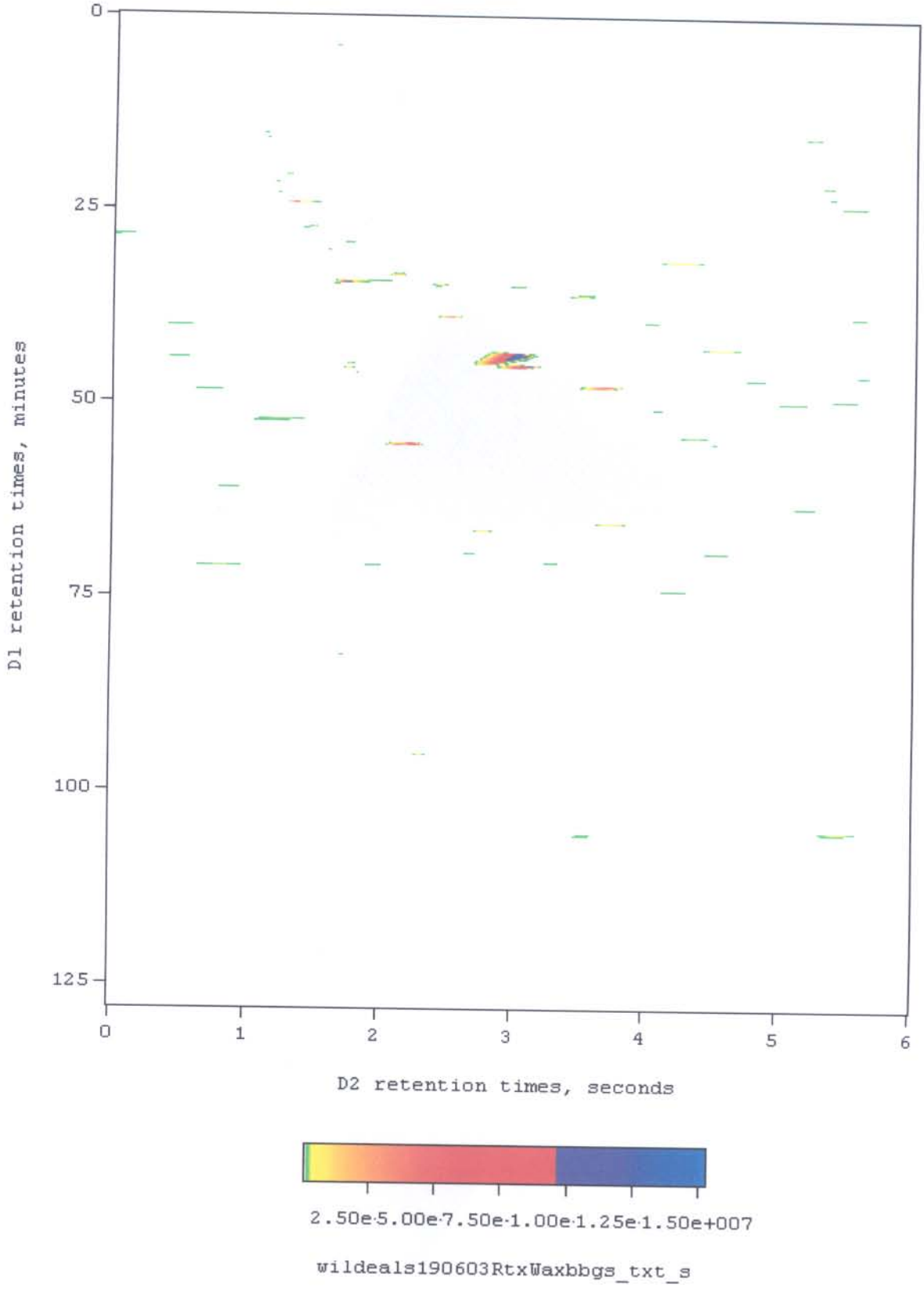


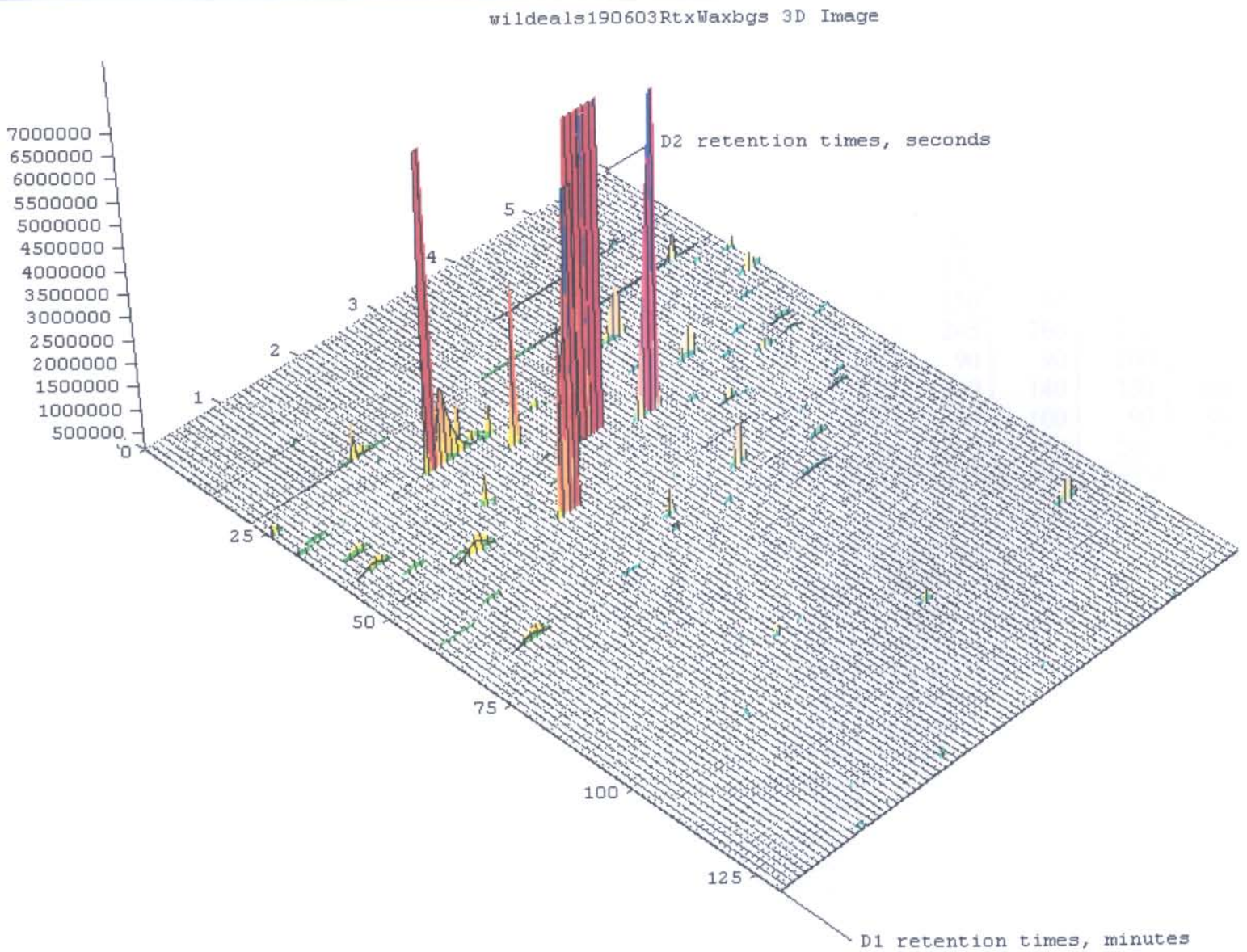











wildeals190603RtxWaxbgs 3D Image
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4
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D2 retention times, seconds
25
50
75
100
125
D1 retention times, minutes

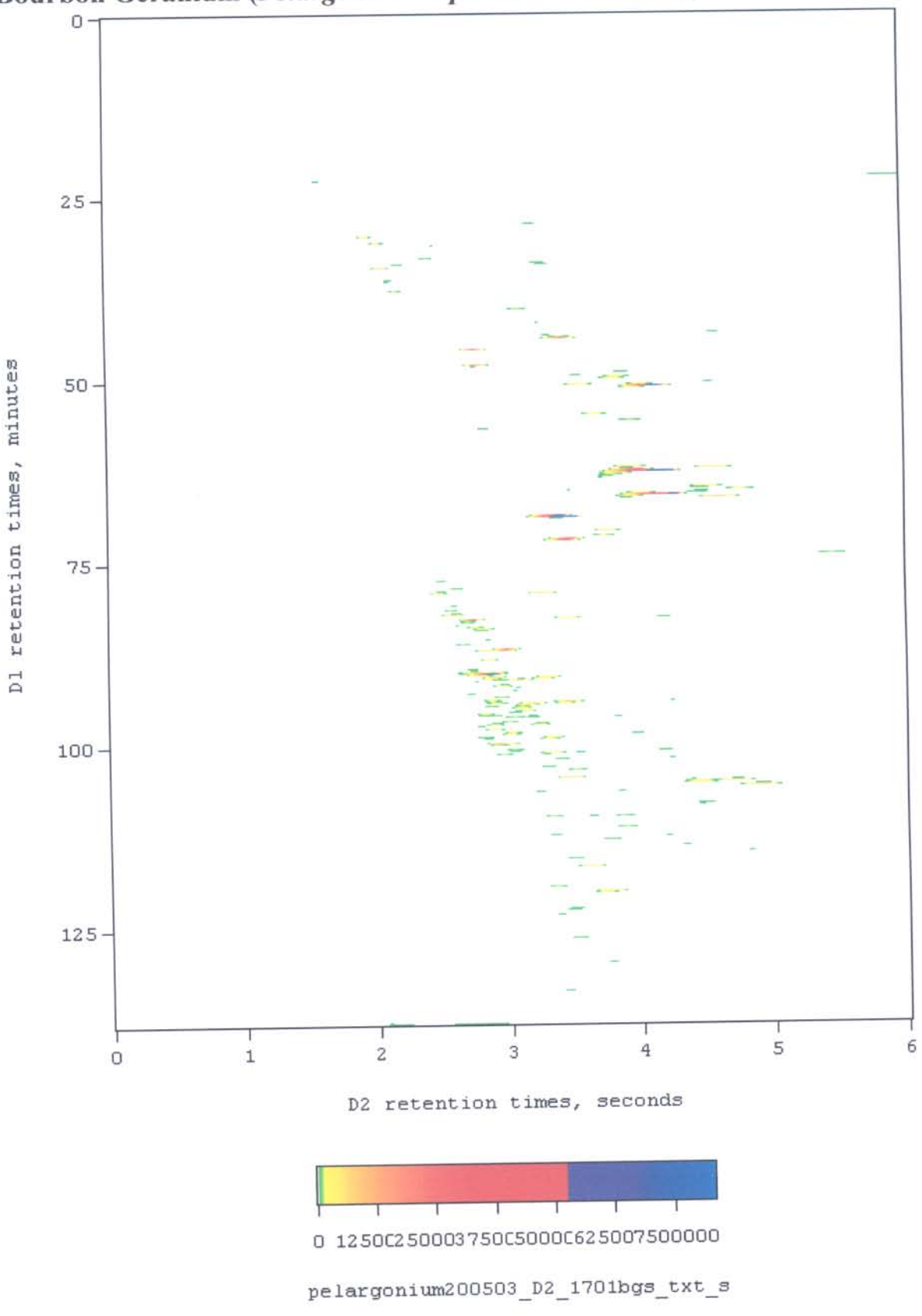
Artemisia afra run-to-run peak reproducibility

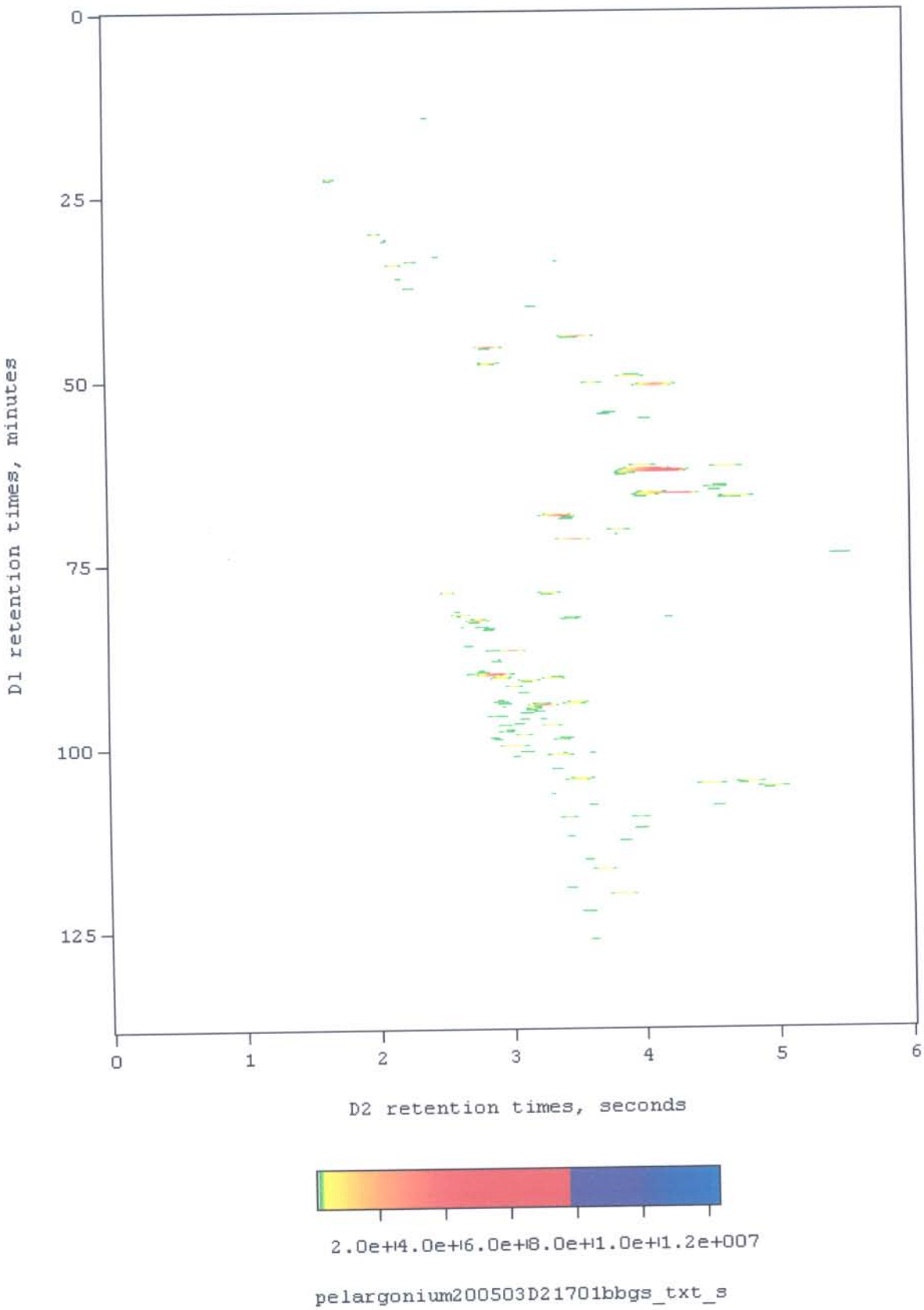
Peak	D1 retention times in minutes					D2 retention times in seconds					D2 Peak width at half height, msec				
no.	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1.	15.1	15.0	15.0	15.0	15.0	1.0	1.0	1.1	1.0	1.1	50	50	40	50	45
2.	24.1	24.0	24.0	23.9	24.0	1.2	1.2	1.3	1.2	1.3	50	40	50	50	50
3.	31.3	31.3	31.2	31.3	31.3	4.1	4.1	4.2	4.1	4.3	180	160	175	160	175
4.	33.3	33.2	33.2	33.2	33.2	2.0	2.0	2.1	2.0	2.1	70	70	70	70	80
5.	34.2	34.1	34.1	34.1	34.1	1.7	1.6	1.7	1.7	1.7	80	70	80	75	70
6.	35.7	35.7	35.6	35.5	35.7	3.4	3.4	3.5	3.4	3.5	150	135	130	135	125
7.	47.4	47.5	47.4	47.4	47.5	3.6	3.5	3.7	3.6	3.7	150	150	150	180	125
8.	52.1	52.0	52.0	51.9	52.1	1.1	1.1	1.2	1.2	1.2	245	260	275	275	250
9.	55.1	55.0	55.0	55.0	55.0	2.1	2.1	2.2	2.2	2.2	90	90	100	100	90
10.	65.2	65.2	65.1	65.2	65.2	3.6	3.5	3.7	3.6	3.7	140	140	130	130	125
11.	66.2	66.1	66.1	66.1	66.2	2.7	2.6	2.7	2.7	2.8	90	100	90	90	100
12.	105.2	105.1	105.1	105.1	105.1	5.3	5.2	5.3	5.4	5.5	200	180	200	175	245

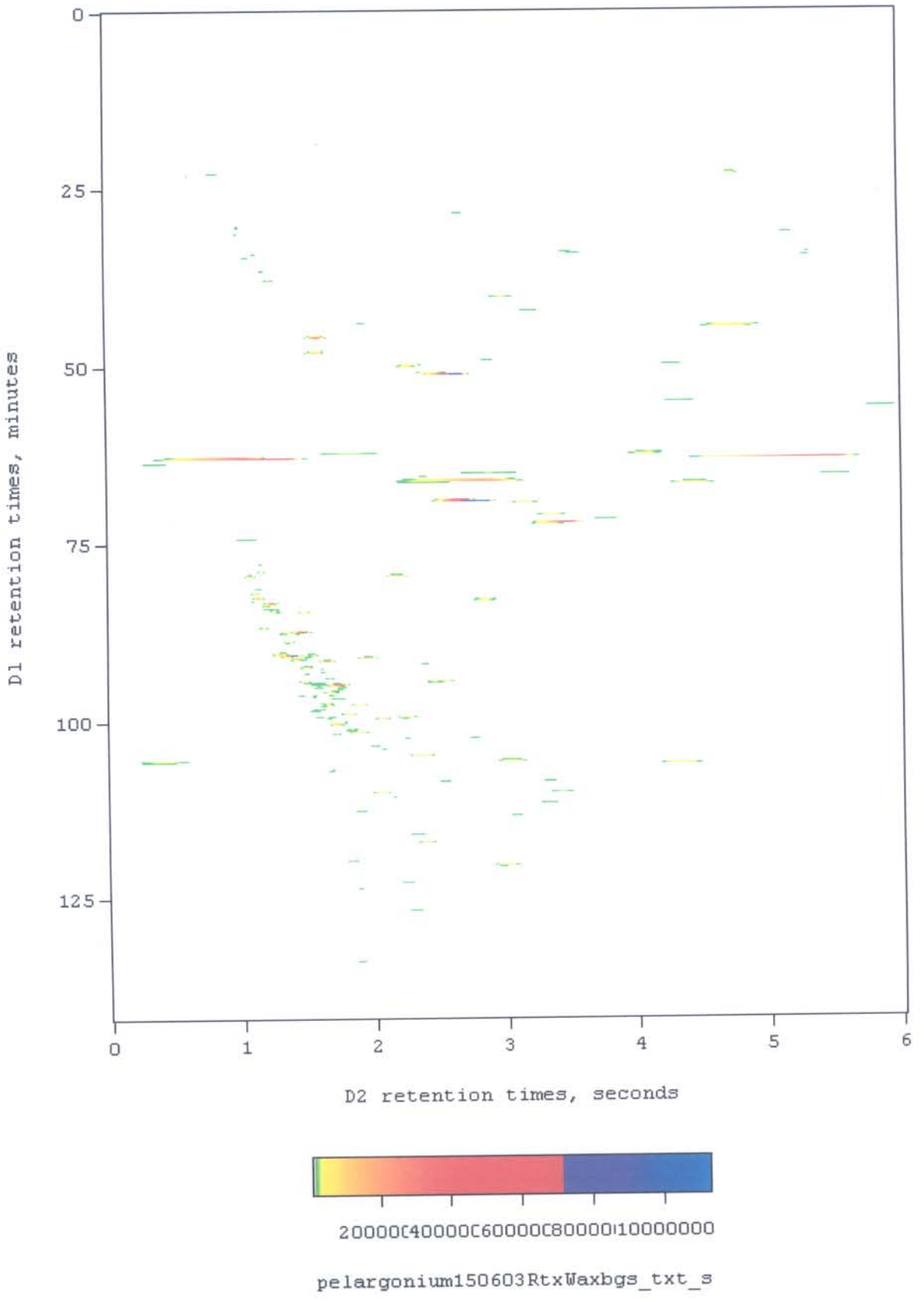
(1) wildeals180603RtxWax, (2) wildeals180603RtxWaxb, (3) wildeals180603RtxWaxc, (4) wildeals190603RtxWax and (5) wildeals190603RtxWaxb

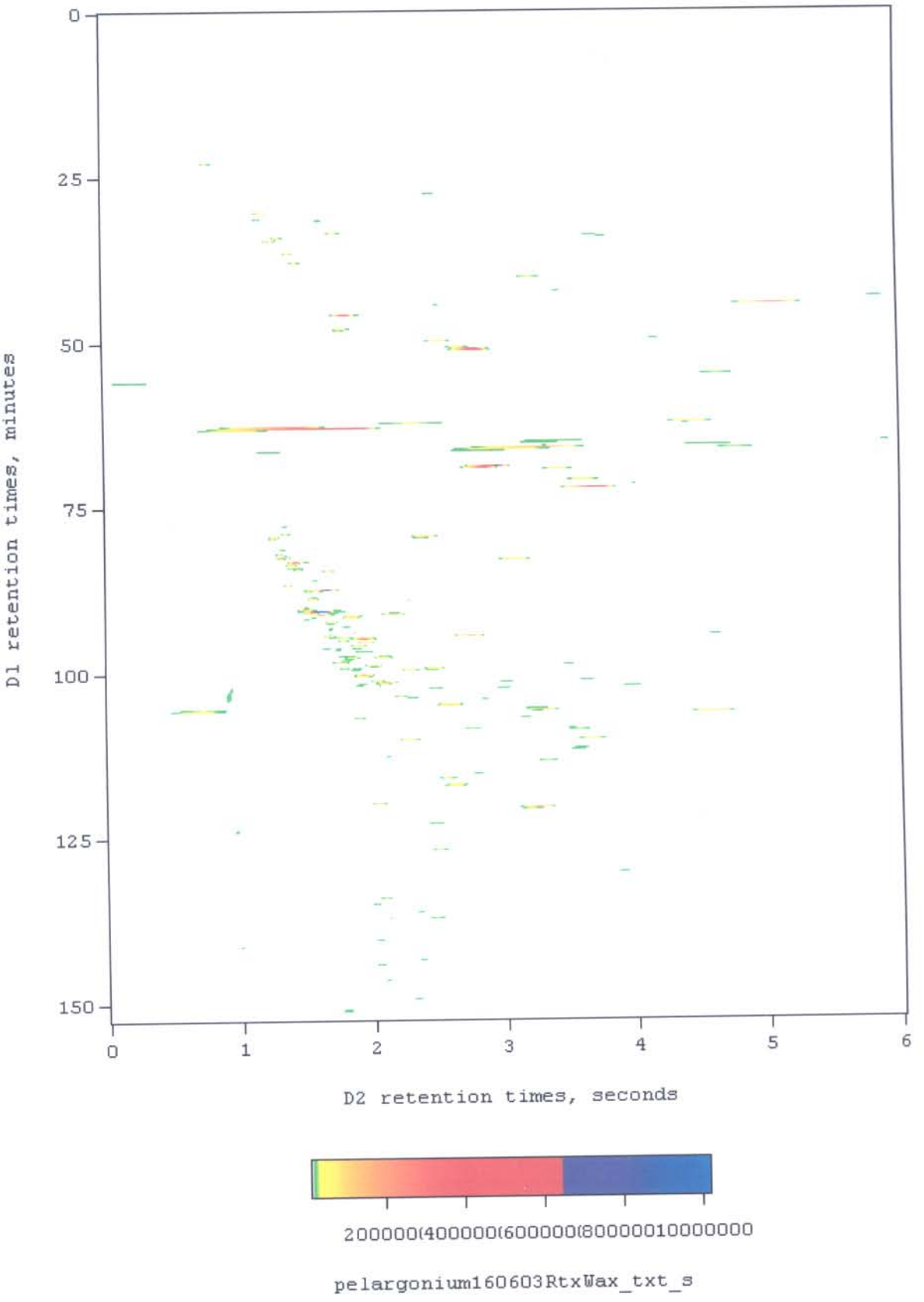
Peak	Mean D1 tr, minutes			Mean D2 tr, seconds			Mean D2 Wh, msec		
no.	Mean	STD	%RSD	Mean	STD	%RSD	Mean	STD	%RSD
1.	14.30	0.06	0.44	0.58	0.04	6.90	51.00	2.00	3.92
2.	16.80	0.06	0.38	0.88	0.04	4.55	59.00	2.00	3.39
3.	27.20	0.06	0.23	0.60	0.06	10.54	46.00	3.74	8.13
4.	34.52	0.07	0.22	0.80	0.06	7.91	70.00	7.07	10.10
5.	36.30	0.06	0.17	0.96	0.05	5.10	85.00	12.65	14.88
6.	37.40	0.00	0.00	1.46	0.05	3.36	124.00	18.55	14.96
7.	41.24	0.05	0.12	1.54	0.05	3.18	80.00	0.00	0.00
8.	44.02	0.04	0.09	1.72	0.07	4.35	86.00	8.00	9.30
9.	49.80	0.00	0.00	2.34	0.05	2.09	116.00	4.90	4.22
10.	154.72	0.04	0.03	4.28	0.04	0.93	178.00	12.88	7.24
11.	160.10	0.06	0.04	4.48	0.07	1.67	180.00	12.65	7.03
12.	164.62	0.04	0.02	4.96	0.05	0.99	195.00	10.00	5.13
	Mean		**0.15**			**4.30**			**7.36**

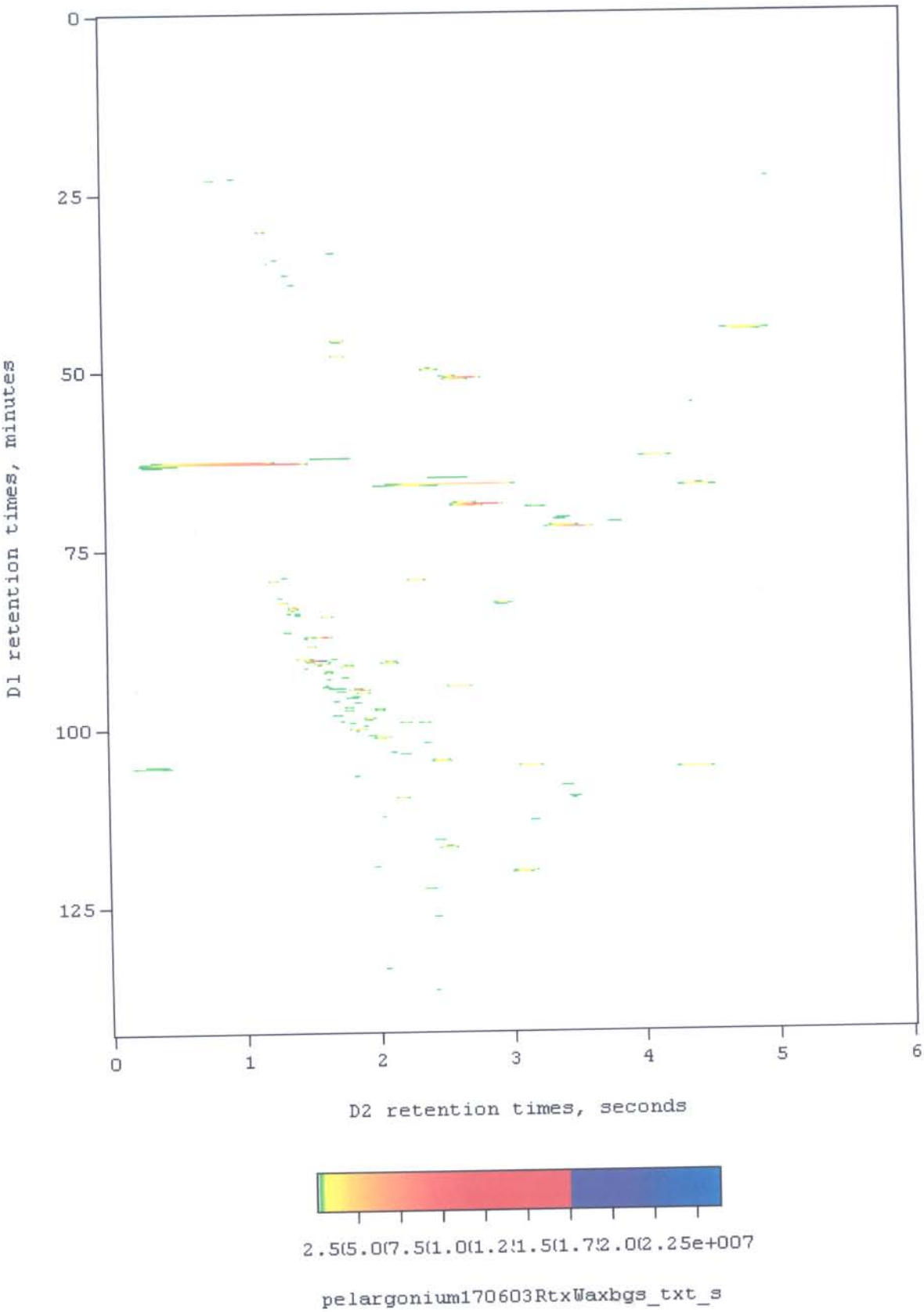
Bourbon Geranium (*Pelargonium capitatum* × *P. radens*)

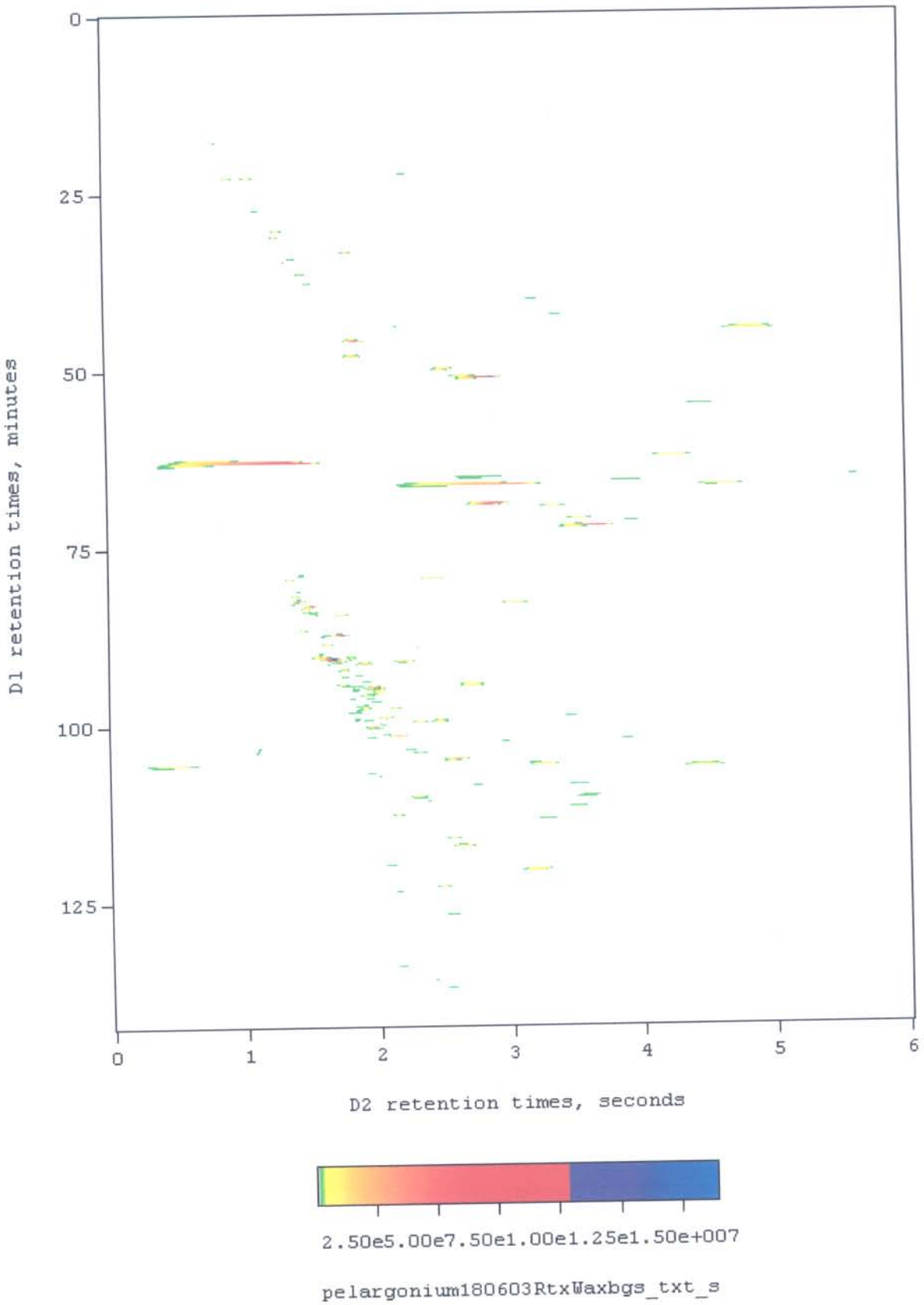


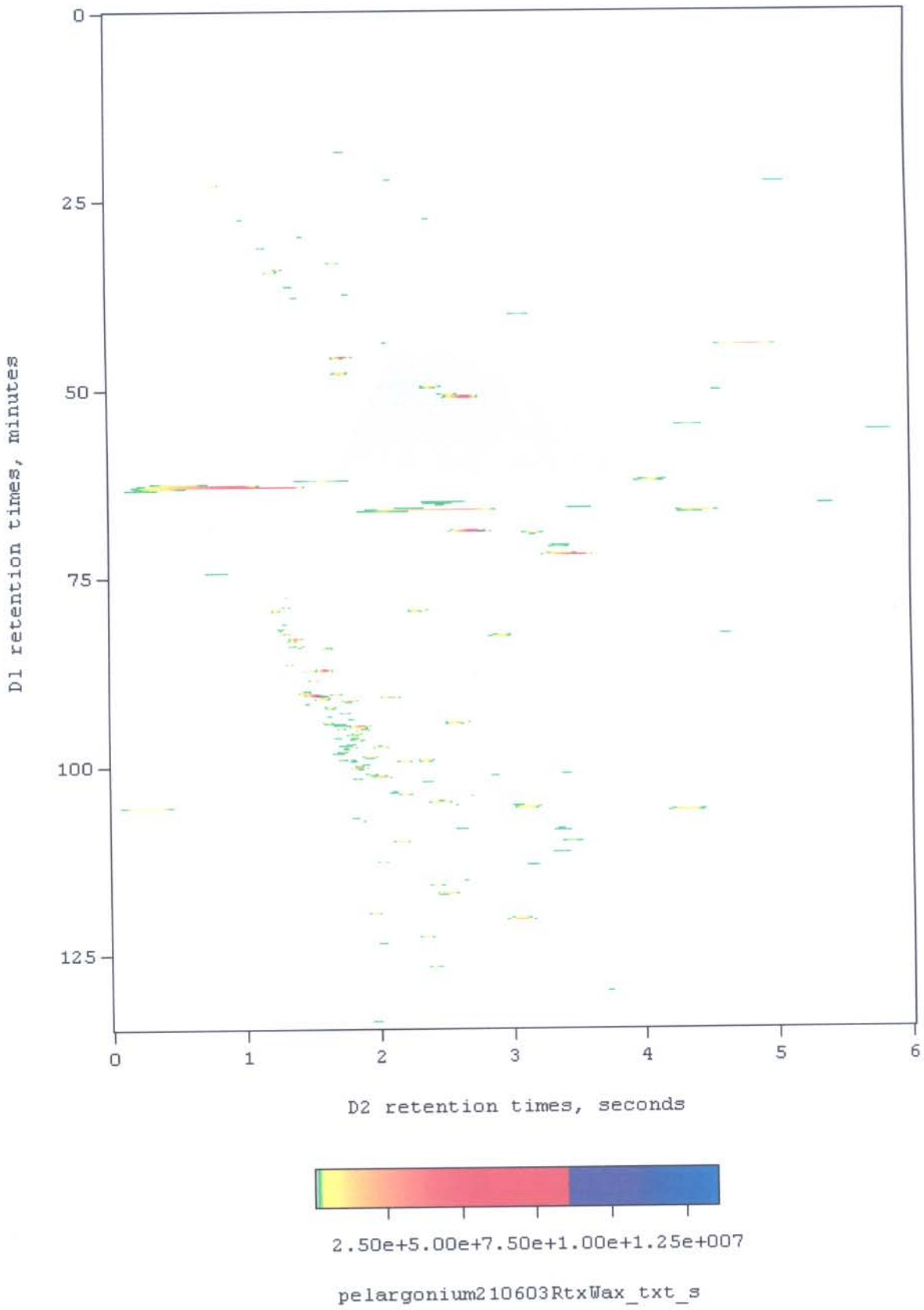


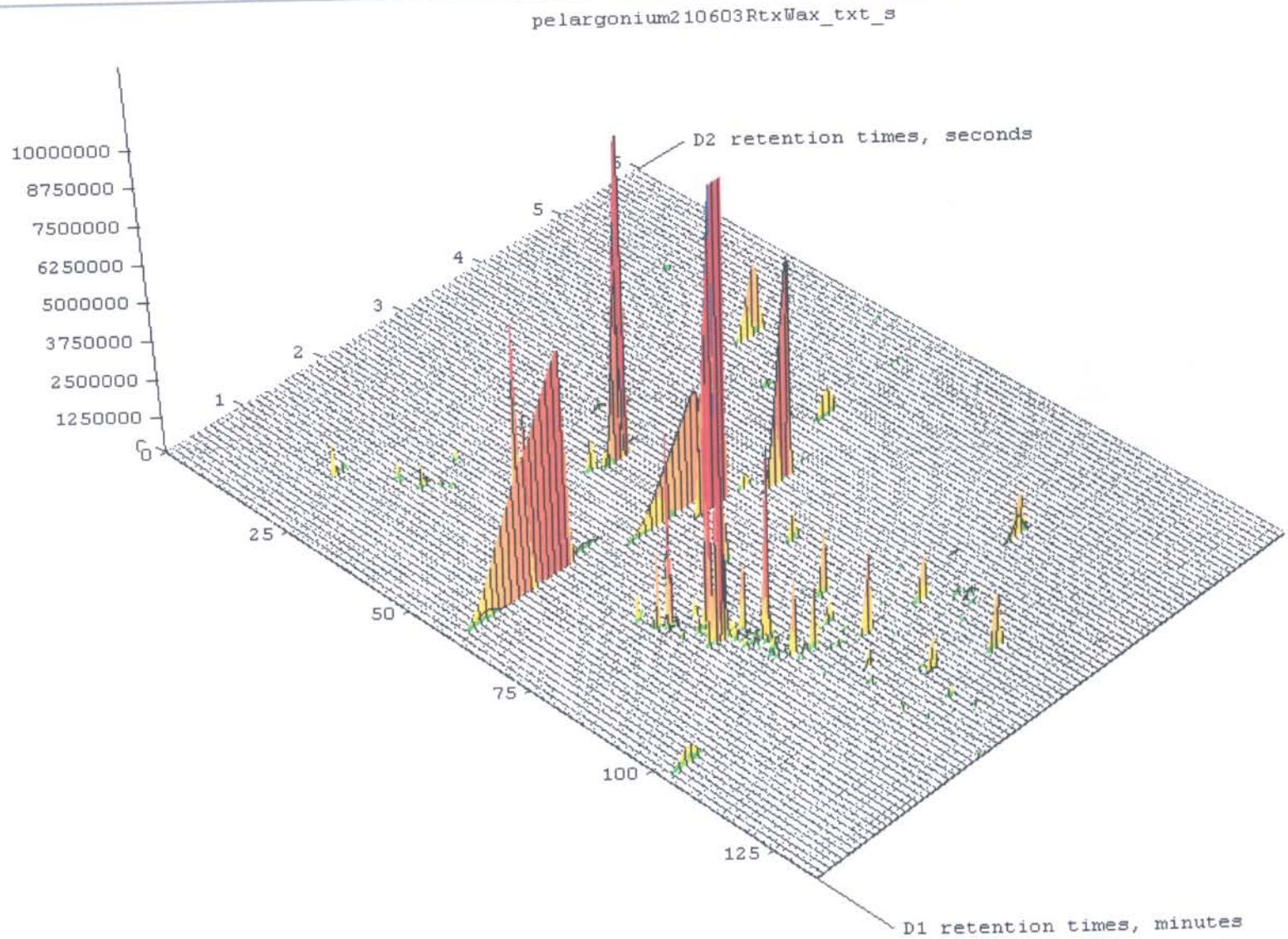












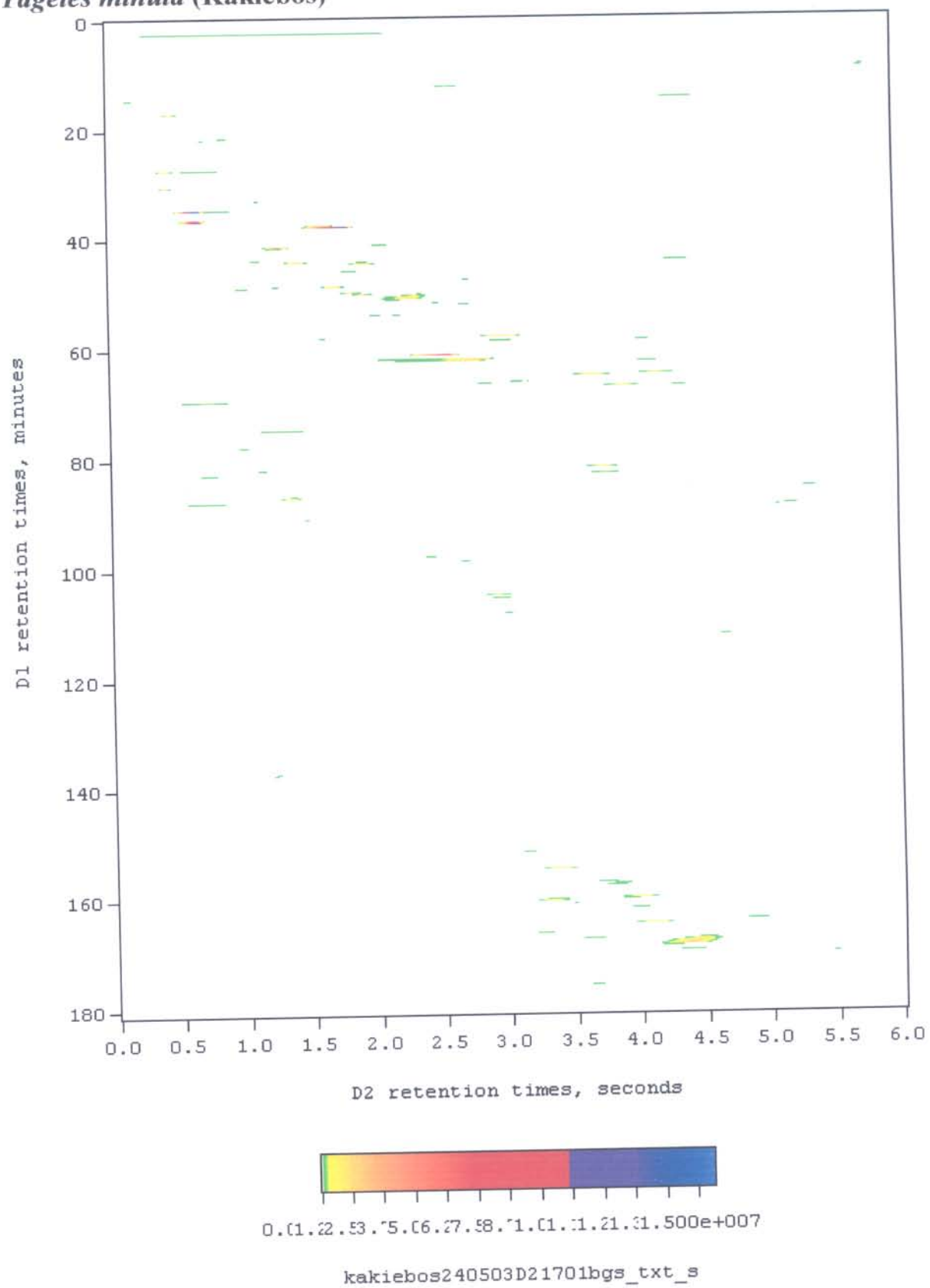
Bourbon Geranium run-to-run peak reproducibility

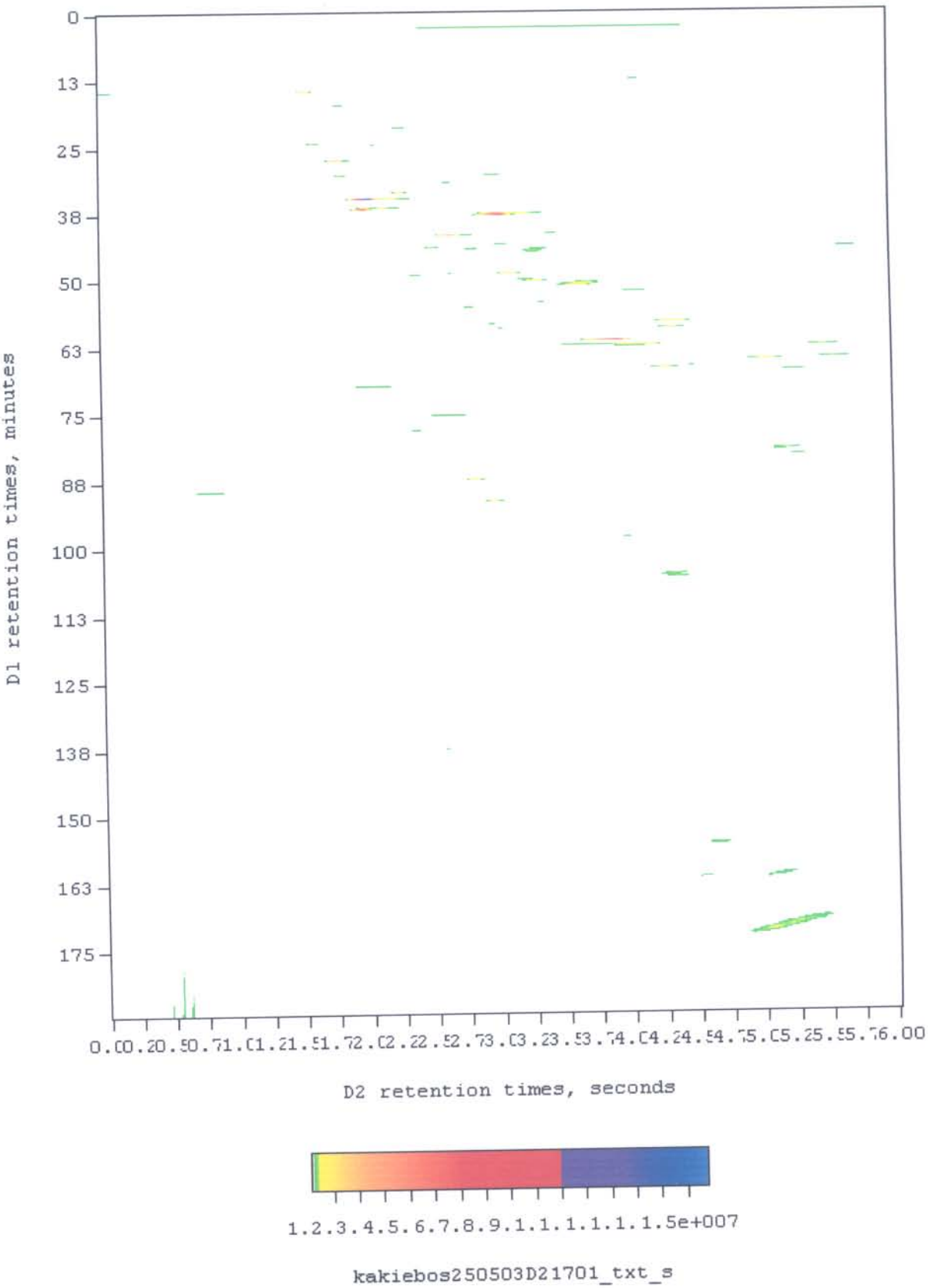
Peak	D1 retention times in minutes					D2 retention times in seconds					D2 Peak width at half height, msec				
no.	1	2	3	4	4	1	2	3	4	5	1	2	3	4	5
1.	44.4	44.3	44.4	44.3	44.2	4.8	5.1	4.9	4.9	4.8	200	225	200	225	200
2.	45.7	45.7	45.7	45.7	45.7	1.6	1.8	1.8	1.9	1.7	70	90	70	70	70
3.	47.9	47.9	47.9	47.8	47.8	1.6	1.8	1.8	1.9	1.7	70	80	70	70	60
4.	49.8	49.8	49.7	49.8	49.7	2.3	2.5	2.4	2.5	2.4	100	95	90	90	90
5.	79.4	79.4	79.2	79.3	79.3	2.2	2.4	2.3	2.4	2.3	90	90	80	90	90
6.	83.2	83.2	83.1	83.0	83.1	1.2	1.4	1.4	1.5	1.4	50	55	50	50	50
7.	87.3	87.3	87.1	87.1	87.4	1.5	1.7	1.6	1.7	1.6	60	65	60	70	60
8.	104.8	104.8	104.6	104.7	104.7	2.4	2.6	2.5	2.6	2.5	100	90	100	90	90
9.	105.6	105.6	105.6	105.5	105.5	3.1	3.3	3.2	3.3	3.1	110	120	125	110	100
10.	106.1	106.0	105.8	106.0	105.9	4.3	4.6	4.4	4.5	4.3	170	180	160	150	160
11.	116.9	117.0	116.8	116.9	116.9	2.4	2.6	2.5	2.6	2.5	90	100	90	90	80
12.	120.4	120.4	120.2	120.3	120.3	3.0	3.3	3.1	3.2	3.1	120	120	120	110	105

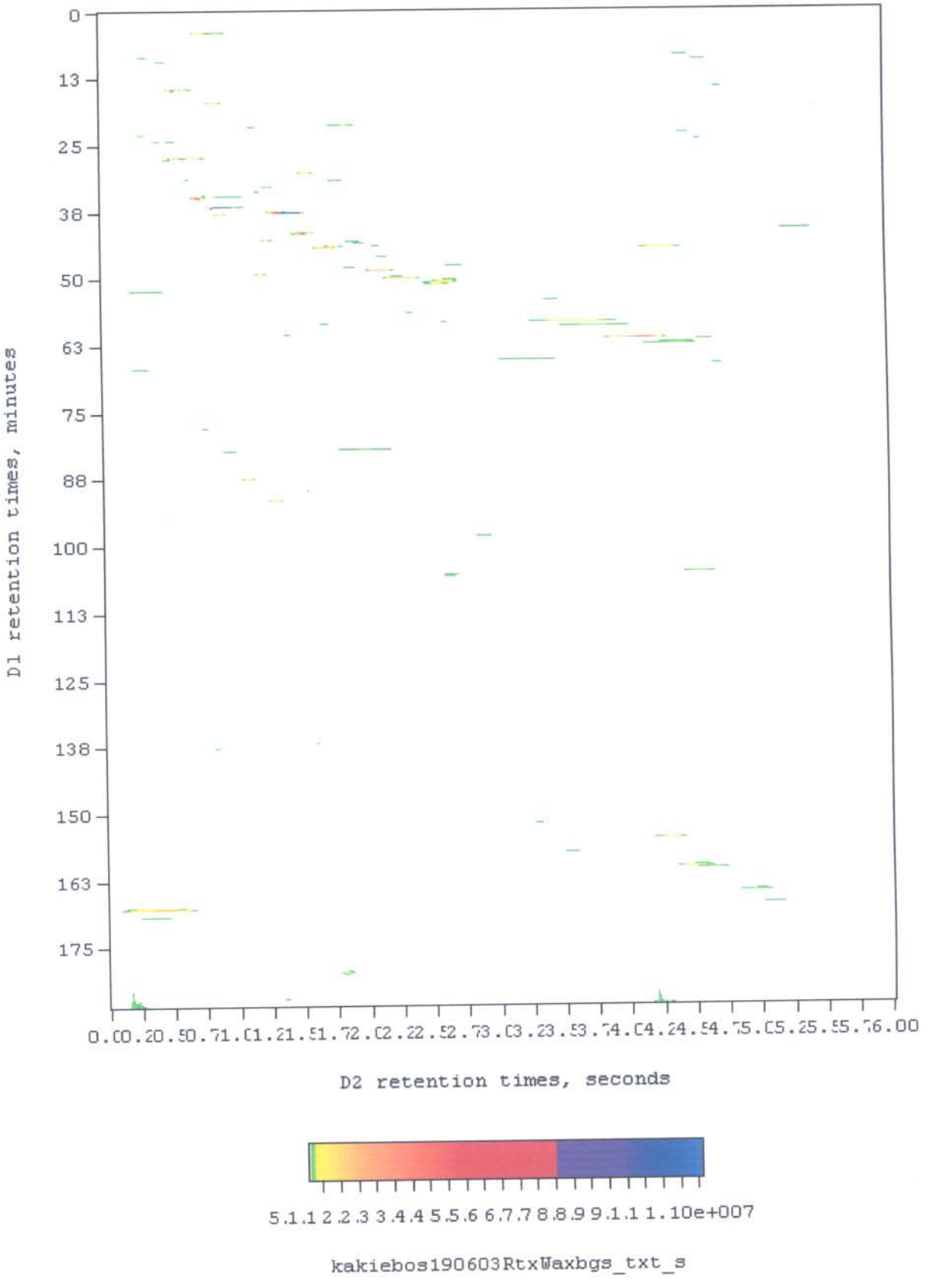
(1) pelargonium150603RtxWaxbgs, (2) pelargonium160603RtxWax, (3) pelargonium170603RtxWax, (4) pelargonium180603RtxWax and (5) pelargonium210603RtxWax

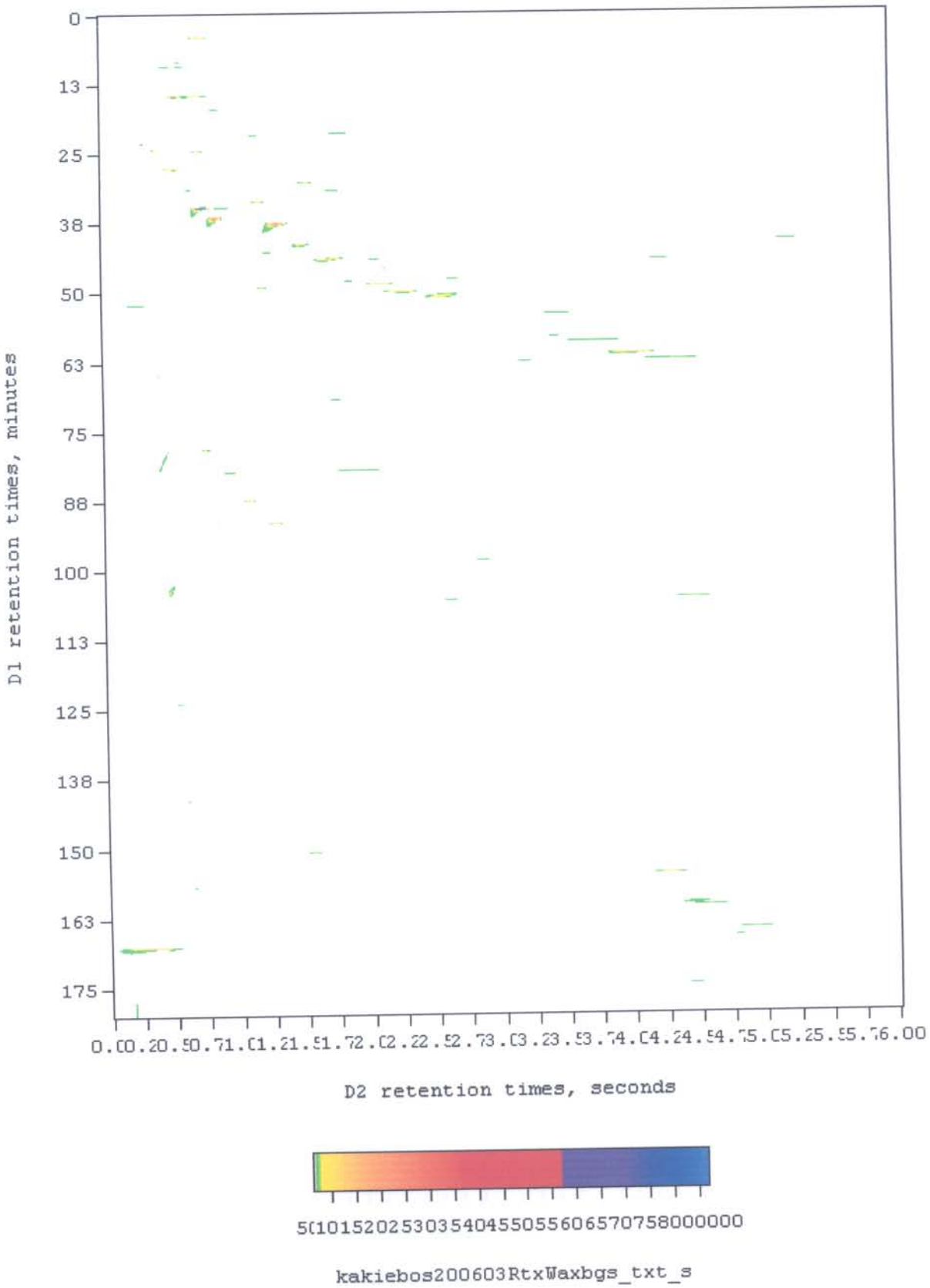
Peak	Mean D1 tr, minutes			Mean D2 tr, seconds			Mean D2 Wh, msec		
no.	Mean	STD	%RSD	Mean	STD	%RSD	Mean	STD	%RSD
1.	44.32	0.07	0.17	4.90	0.11	2.24	210.00	12.25	5.83
2.	45.70	0.00	0.00	1.76	0.10	5.79	74.00	8.00	10.81
3.	47.86	0.05	0.10	1.76	0.10	5.79	70.00	6.32	9.04
4.	49.76	0.05	0.10	2.42	0.07	3.09	93.00	4.00	4.30
5.	79.32	0.07	0.09	2.32	0.07	3.23	88.00	4.00	4.55
6.	83.12	0.07	0.09	1.38	0.10	7.10	51.00	2.00	3.92
7.	87.24	0.12	0.14	1.62	0.07	4.62	63.00	4.00	6.35
8.	104.72	0.07	0.07	2.52	0.07	2.97	94.00	4.90	5.21
9.	105.56	0.05	0.05	3.20	0.09	2.80	113.00	8.72	7.71
10.	105.96	0.10	0.10	4.42	0.12	2.64	164.00	10.20	6.22
11.	116.90	0.06	0.05	2.52	0.07	2.97	90.00	6.32	7.03
12.	120.32	0.07	0.06	3.14	0.10	3.25	115.00	6.32	5.50
	Mean		**0.09**			**3.87**			**6.37**

***Tagetes minuta* (Kakiebos)**








0
13
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163
175
D1 retention times, minutes
0.00 0.50 1.00 1.50 2.00 2.50 3.00 3.50 4.00 4.50 5.00 5.50 6.00
D2 retention times, seconds
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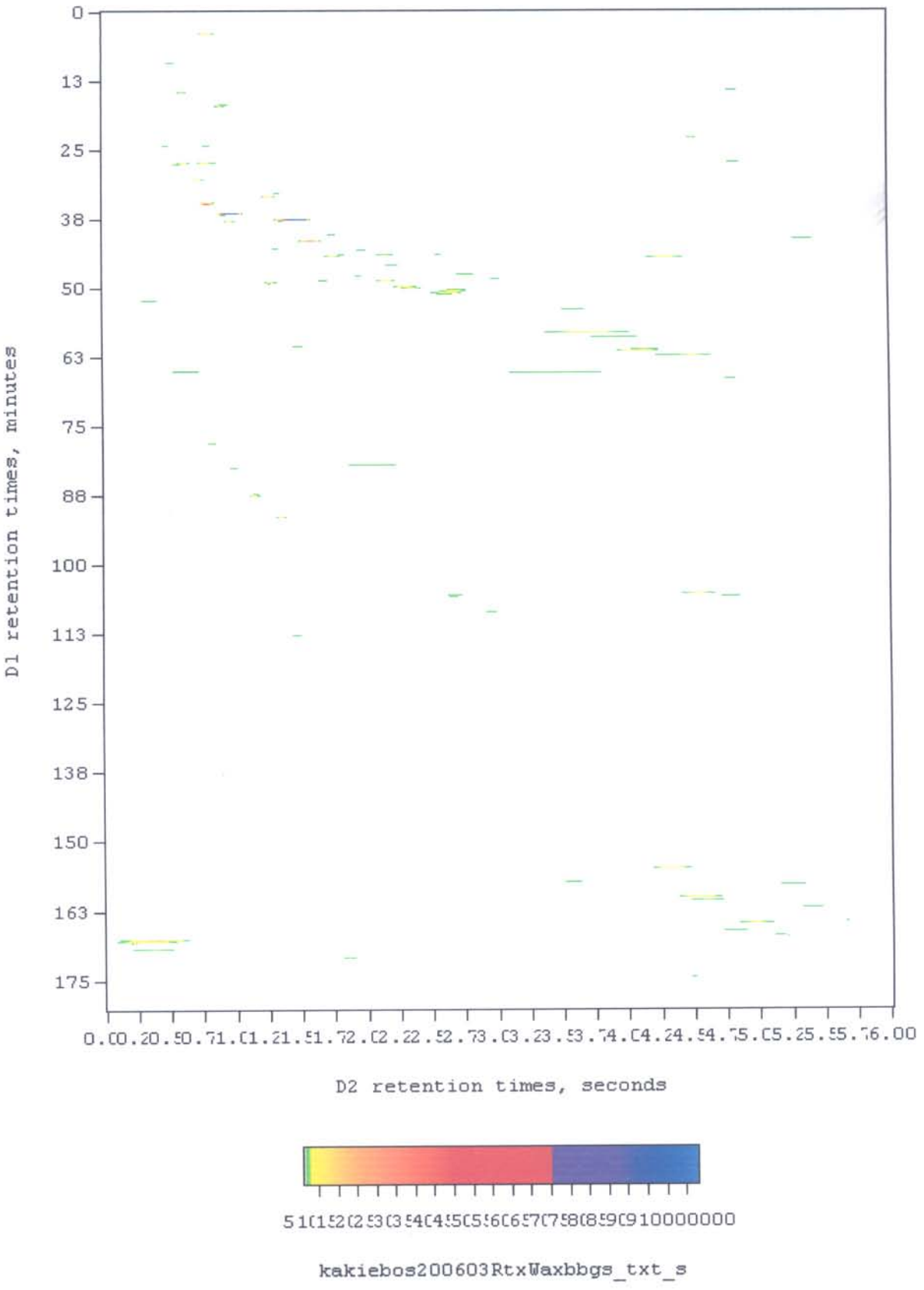
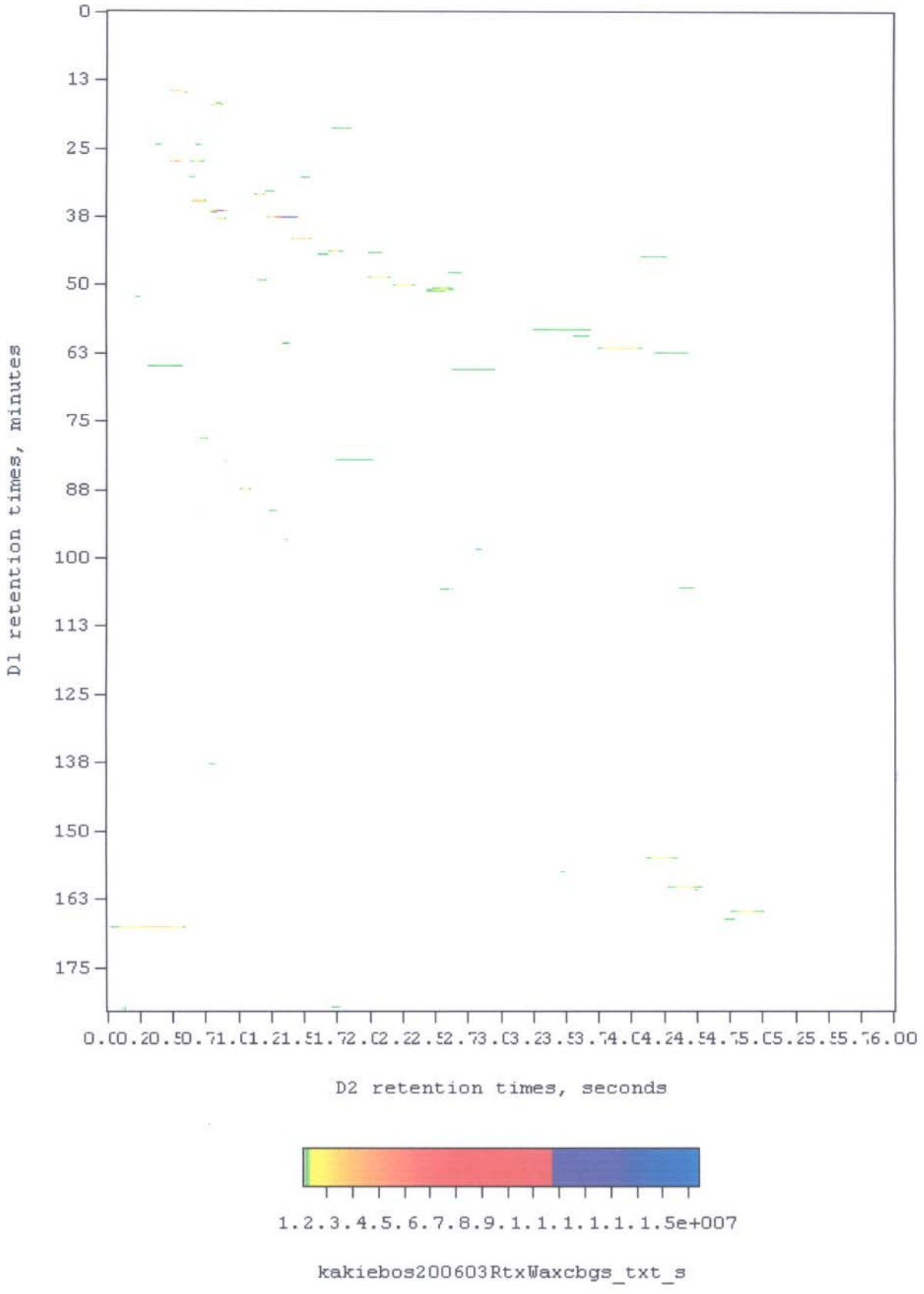
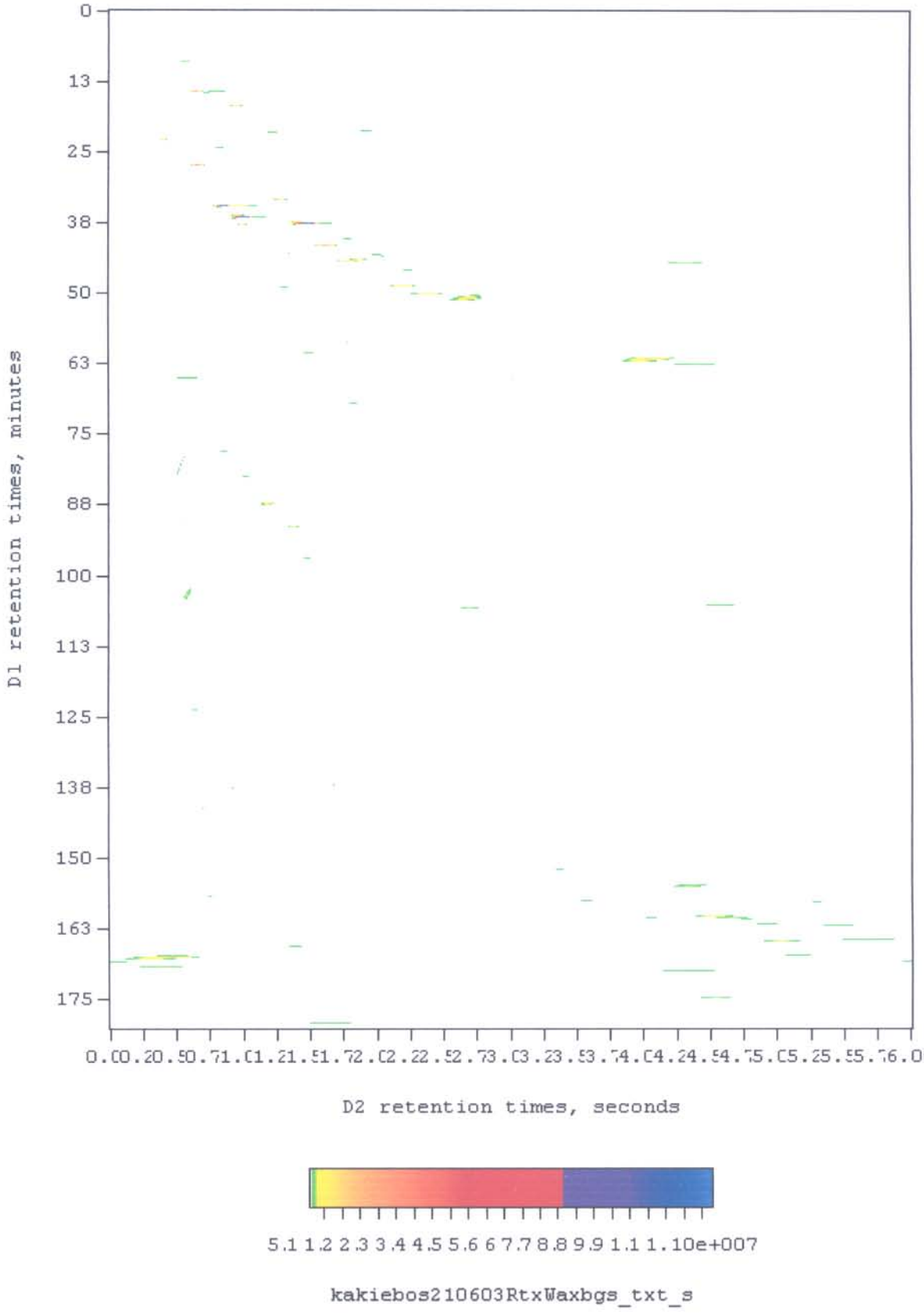
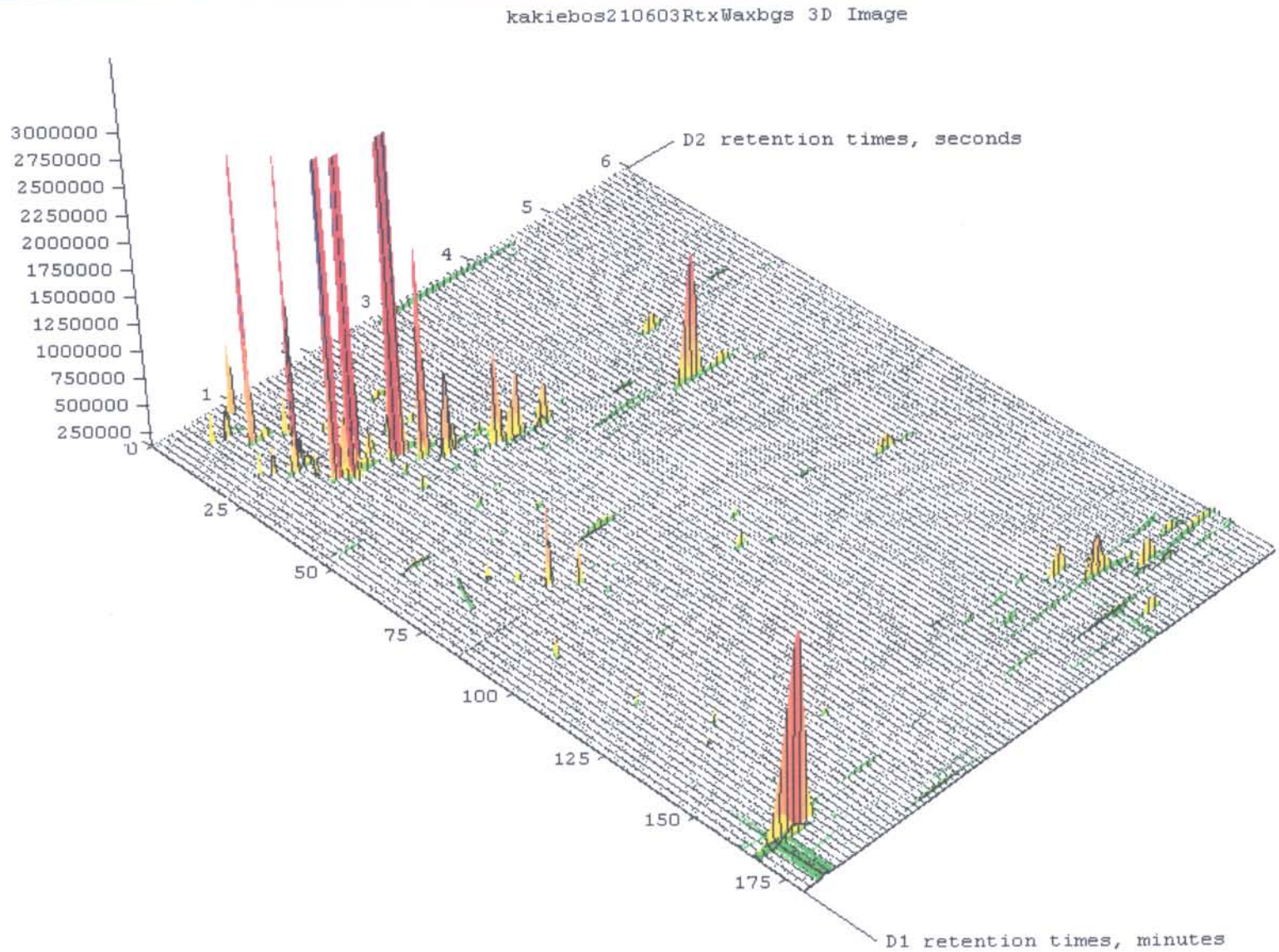
kakiebos200603RtxWaxbgs_txt_s









Tagetes minuta run-to-run peak reproducibility

Peak	D1 retention times in minutes					D2 retention times in seconds					D2 Peak width at half height, msec				
no.	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
1.	14.3	14.4	14.3	14.3	14.2	0.6	0.6	0.6	0.5	0.6	50	50	55	50	50
2.	16.8	16.9	16.8	16.8	16.7	0.9	0.9	0.9	0.8	0.9	60	60	60	55	60
3.	27.2	27.3	27.2	27.2	27.1	0.6	0.6	0.6	0.5	0.7	50	45	50	40	45
4.	34.6	34.6	34.5	34.5	34.4	0.8	0.8	0.8	0.7	0.9	80	60	75	65	70
5.	36.3	36.4	36.3	36.3	36.2	1.0	0.9	1.0	0.9	1.0	100	70	100	80	75
6.	37.4	37.4	37.4	37.4	37.4	1.5	1.4	1.5	1.4	1.5	140	100	150	110	120
7.	41.2	41.3	41.2	41.2	41.3	1.5	1.5	1.6	1.5	1.6	80	80	80	80	80
8.	44.0	44.1	44.0	44.0	44.0	1.7	1.7	1.8	1.6	1.8	100	90	80	80	80
9.	49.8	49.8	49.8	49.8	49.8	2.3	2.3	2.4	2.3	2.4	120	120	120	110	110
10.	154.8	154.7	154.7	154.7	154.7	4.3	4.3	4.3	4.2	4.3	200	180	175	175	160
11.	160.2	160.1	160.1	160.1	160.0	4.5	4.4	4.5	4.4	4.6	200	180	180	180	160
12.	164.7	164.6	164.6	164.6	164.6	5.0	4.9	5.0	4.9	5.0	200	200	200	200	175

(1) kakiebos190603RtxWax, (2) kakiebos200603RtxWax, (3) kakiebos200603RtxWaxb (4) kakiebos190603RtxWax and (5) kakiebos190603RtxWax

Peak	Mean D1 tr, minutes			Mean D2 tr, seconds			Mean D2 Wh, msec		
no.	Mean	STD	%RSD	Mean	STD	%RSD	Mean	STD	%RSD
1.	14.30	0.06	0.44	0.58	0.04	6.90	51.00	2.00	3.92
2.	16.80	0.06	0.38	0.88	0.04	4.55	59.00	2.00	3.39
3.	27.20	0.06	0.23	0.60	0.06	10.54	46.00	3.74	8.13
4.	34.52	0.07	0.22	0.80	0.06	7.91	70.00	7.07	10.10
5.	36.30	0.06	0.17	0.96	0.05	5.10	85.00	12.65	14.88
6.	37.40	0.00	0.00	1.46	0.05	3.36	124.00	18.55	14.96
7.	41.24	0.05	0.12	1.54	0.05	3.18	80.00	0.00	0.00
8.	44.02	0.04	0.09	1.72	0.07	4.35	86.00	8.00	9.30
9.	49.80	0.00	0.00	2.34	0.05	2.09	116.00	4.90	4.22
10.	154.72	0.04	0.03	4.28	0.04	0.93	178.00	12.88	7.24
11.	160.10	0.06	0.04	4.48	0.07	1.67	180.00	12.65	7.03
12.	164.62	0.04	0.02	4.96	0.05	0.99	195.00	10.00	5.13
	Mean		**0.15**			**4.30**			**7.36**