Extraction, identification of active constituents from Gliricidia sepium and development of a bait to be as a Rodenticide

Submitted

By

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Gliricidia sepium

Gliricidia sepium, often simply referred to as **Gliricidia** (common names: **quickstick**, *mata ratón*; *cacao de nance*, *cachanance*, it is commonly known as *piñon Cubano* in the Dominican Republic; *madreado* in Honduras; *kakawate* in the Philippines; *madre xacao* or *madre de cacao* in the Philippines and Guatemala; and *madero negro* in Nicaragua), is a medium size leguminous tree belonging to the family Fabaceae. It is considered as the second most important multi-purpose legume tree, surpassed only by *Leucaena leucocephala*.

Scientific classification

Kingdom:	Plantae
(unranked):	Angiosperms
(unranked):	Eudicots
(unranked):	Rosids
Order:	Fabales
Family:	Fabaceae
Genus:	Gliricidia
Species:	G. sepium

Description

Flower of Gliricidia sepium in West Timor

Gliricidia sepium is a medium-sized tree and can grow to from 10 to 12 meters high. The bark is smooth and its color can range from a whitish gray to deep red-brown. It has composite leaves that can be 30 cm long. Each leaf is composed of leaflets that are about 2 to 7 cm long and 1 to 3 cm wide. The flowers are located on the end of branches that have no leaves. These flowers have a bright pink to lilac color that is tinged with white. A pale yellow spot is usually at the flower's base. The tree's fruit is

a poc which is about 10 to 15 cm in length. It is green when unripe and becomes yellow-brown when it reaches maturity. The pod produces 4 to 10 round brown seeds. The tree grows well in acidic soils with a pH of 4.5-6.2. The tree is found on volcanic soils in its native range in Central America and Mexico. However, it can also grow on sandy, clay and limestone soils.

Uses

Gliricidia sepium in Agoo, La Union(foreground tree, with numerous other examples behind it), used as a landscaping/reforesting tool. In this case the main means of preventing erosion and a quick way of providing shade.

The tree is used in many tropical and sub-tropical countries for various purposes such as live fencing, fodder, coffee shade, firewood,[5] green manure and rat poison.[6] Live fences can be grown from 1.5 m to 2.0 m stakes of *Gliricidia sepium* in just a month. Gliricidia can be intercropped with maize. Its effect is that of a potent fertilizer.[7][6]
G. sepium is also used for its medicinal and insect repellent properties. Farmers in Latin Americaoften wash their livestock with a paste made of crushed *G. sepium* leaves to ward off torsalos. In the Philippines, the extract obtained from its leaves is used to remove external parasites.[3]

G. sepium is a fast-growing ruderal species that takes advantage of slash and burn practices in its native range. Its swift propagation has caused it to be considered as a weed in Jamaica.[4]Because it is easily propagated and grows quickly, it has also been suggested that this species may be planted to reduce topsoil erosion in the initial stages of reforesting denuded areas, an intermediate step to be taken before introducing species that take longer to grow.

According to World Agroforestry Centre, this species is becoming an important part of farming practices in Africa. *G. sepium* has a combination of desirable properties. Because it fixes nitrogen in the soil, it boosts crop yields significantly without the expense of chemical fertilizers. In addition, it tolerates being cut back to crop height year after year. The trees go into a dormant state when they are cut back, so the root system is not competing straight away for the nutrients, and the crop is free to become established. The trees only really start to come out of the dormant phase when the crop is already tall.

Other chemical poisons include:

- ANTU (α-naphthylthiourea; specific against Brown rat, Rattus norvegicus)
- Arsenic trioxide
- Barium carbonate
- Chloralose (a narcotic prodrug)
- Crimidine (inhibits metabolism of vitamin B6)
- 1,3-Difluoro-2-propanol ("Gliftor")
- Endrin (organochlorine insecticide, used in the past for extermination of voles in fields)
- Fluoroacetamide ("1081")
- Phosacetim (a delayed-action organophosphate)
- White phosphorus
- Pyrinuron (an urea derivative)
- Scilliroside
- Sodium fluoroacetate ("1080")
- Strychnine (A naturally occurring convulsant and stimulant)
- Tetramethylenedisulfotetramine ("tetramine")

- Thallium chloride
- Nitrophenols like bromethalin and 2,4-dinitrophenol (cause high fever and brain swelling, no known antidote)
- Zyklon B/Uragan D2 (hydrogen cyanide gas absorbed in an inert carrier)

Combinations

In some countries, fixed three-component rodenticides, i.e., anticoagulant + antibiotic + vitamin D, are used. Associations of a second-generation anticoagulant with an antibiotic and/or vitamin D are considered to be effective even against most resistant strains of rodents, though some second generation anticoagulants (namely brodifacoum and difethialone), in bait concentrations of 0.0025% to 0.005% are so toxic that resistance is unknown, and even rodents resistant to other rodenticides are reliably exterminated by application of these most toxic anticoagulants.

Alternatives

More environmentally-safe preparations, using natural ingredients such as powdered corn cob, have been developed and were approved in the EU and patented in the US in 2013. These preparations rely on lethal dehydration to cause death.

Non-target issues

Secondary poisoning and risks to wildlife

One of the potential problems when using rodenticides is that dead or weakened rodents may be eaten by other wildlife, either predators or scavengers. Members of the public deploying rodenticides may not be aware of this or may not follow the product's instructions closely enough.

The faster a rodenticide acts, the more critical this problem may be. For the fast-acting rodenticide bromethalin, for example, there is no diagnostic test or antidote.

This has led environmental researchers to conclude that low strength, long duration rodenticides (generally first generation anticoagulants) are the best balance between maximum effect and minimum risk.

Proposed US legislation change

In 2008, after assessing human health and ecological effects, as well as benefits, the US Environmental Protection Agency (EPA) announced measures to reduce risks associated with ten rodenticides. New restrictions by sale and distribution restrictions, minimum package size requirements, use site restriction, and tamper resistant products would have taken effect in 2011. The regulations were delayed pending a legal challenge by manufacturer Reckitt-Benkiser.

Notable rat eradications

The entire rat populations of several islands have been eradicated, most notably Campbell Island, New Zealand (11,300 ha),Hawadax Island, Alaska (formerly known as Rat Island, 2,670 ha) and Canna, Scotland (1,030 ha, declared rat-free in 2008).

Alberta, Canada, through a combination of climate and control, is believed to be rat-free.

Rodenticides, colloquially **rat poison**, are typically non-specific pest control chemicals made and sold for the purpose of killing rodents.

Some rodenticides are lethal after one exposure while others require more than one. Rodents are disinclined to gorge on an unknown food (perhaps reflecting an adaptation to their inability to vomit), preferring to sample, wait and observe whether it makes them or other rats sick.[1][2] This phenomenon ofbait shyness or poison shyness is the rationale for poisons that kill only after multiple doses.

Besides being directly toxic to the mammals that ingest them, including dogs, cats and people, many rodenticides present a secondary poisoning risk to animals that hunt or scavenge the dead corpses of rats.

Chemical preparations

Anticoagulants

Anticoagulants are defined as chronic (death occurs one to two weeks after ingestion of the lethal dose, rarely sooner), single-dose (second generation) or multiple-dose (first generation) rodenticides, acting by effective blocking of the vitamin K cycle, resulting in inability to produce essential blood-clotting factors — mainly coagulation factors II (prothrombin) and VII (proconvertin).

In addition to this specific metabolic disruption, massive toxic doses of 4-hydroxycoumarin, 4-thiochromenone and indandione anticoagulants cause damage to tiny blood vessels (capillaries), increasing their permeability, causing diffuse internal bleeding. These effects are gradual, developing over several days. In the final phase of the intoxication, the exhausted rodent collapses due to hemorrhagic shock or severe anemia and dies calmly. The question of whether the use of these rodenticides can be considered humane has been raised.[3]

The main benefit of anticoagulants over other poisons is that the time taken for the poison to induce death means that the rats do not associate the damage with their feeding habits.

First generation rodenticidal anticoagulants generally have shorter elimination half-lives,[4] require higher concentrations (usually between 0.005% and 0.1%) and consecutive intake over days in order to accumulate the lethal dose, and are less toxic than second generation agents.

Second generation agents are far more toxic than first generation. They are generally applied in lower concentrations in baits — usually on the order of 0.001% to 0.005% — are lethal after a single ingestion of bait and are also effective against strains of rodents that became resistant to first generation anticoagulants; thus, the second generation anticoagulants are sometimes referred to as "superwarfarins".[5]

Class	Examples
Coumarins/4-hydroxycoumarins	First generation: warfarin, coumatetralyl Second generation: difenacoum, brodifacoum,[6] flocoumafen and bromadiolone.
1,3-indandiones	diphacinone, chlorophacinone,[7] pindone These are harder to group by generation. According to some sources, the indandiones are considered second generation.[8] However, according to theU.S. Environmental Protection Agency, examples of first generation agents include chlorophacinone and diphacinone.[6]
4-thiochromenones	Difethialone is considered a second generation anticoagulant rodenticide .[9]
Indirect	Sometimes, anticoagulant rodenticides are potentiated by an antibiotic or bacteriostatic agent, most commonly sulfaquinoxaline. The aim of this association is that the antibiotic suppresses intestinal symbiotic microflora, which are a source of vitamin K. Diminished production of vitamin K by the intestinal microflora contributes to the action of anticoagulants. Added vitamin D also has a synergistic effect with anticoagulants.

Vitamin K1 has been suggested, and successfully used, as antidote for pets or humans accidentally or intentionally exposed to anticoagulant poisons. Some of these poisons act by inhibiting liver functions and in advanced stages of poisoning, several blood-clotting factors are absent, and the volume of circulating blood is diminished, so that a blood transfusion (optionally with the clotting factors present) can save a person who has been poisoned, an advantage over some older poisons.

Metal phosphides

Rat poison vendor's stall at a market in Linxia City, China

Metal phosphides have been used as a means of killing rodents and are considered single-dose fast acting rodenticides (death occurs commonly within 1–3 days after single bait ingestion). A bait consisting of food and a phosphide (usually zinc phosphide) is left where the rodents can eat it. The acid in the digestive system of the rodent reacts with the phosphide to generate the toxic phosphine gas. This method of vermin control has possible use in places where rodents are resistant to some of the anticoagulants, particularly for control of house and field mice; zinc phosphide baits are also cheaper than most second-generation anticoagulants, so that sometimes, in the case of large infestation by rodents, their population is initially reduced by copious amounts of zinc phosphide bait applied, and the rest of population that survived the initial fast-acting poison is then eradicated by prolonged feeding on anticoagulant bait. Inversely, the individual rodents, that survived anticoagulant bait poisoning (rest population) can be eradicated by pre-baiting them with nontoxic bait for a week or two (this is important to overcome bait shyness, and to get rodents used to feeding in specific areas by specific food, especially in eradicating rats) and subsequently applying poisoned bait of the same sort as used for pre-baiting until all consumption of the bait ceases (usually within 2–4 days). These methods of alternating rodenticides with different modes of action gives actual or almost 100% eradications of the rodent population in the area, if the acceptance/palatability of baits are good (i.e., rodents feed on it readily).

Zinc phosphide is typically added to rodent baits in a concentration of 0.75% to 2.0%. The baits have strong, pungent garlic-like odor due to the phosphine liberated by hydrolysis. The odor attracts (or, at least, does not repel) rodents, but has an

repulsive effect on other mammals. Birds, notably wild turkeys, are not sensitive to the smell, and will feed on the bait, and thus become collateral damage.

The tablets or pellets (usually aluminium, calcium or magnesium phosphide for fumigation/gassing) may also contain other chemicals which evolve ammonia, which helps to reduce the potential for spontaneous combustion or explosion of the phosphine gas.

Metal phosphides do not accumulate in the tissues of poisoned animals, so the risk of secondary poisoning is low.

Before the advent of anticoagulants, phosphides were the favored kind of rat poison. During World War II, they came into use in United States because of shortage of strychninedue to the Japanese occupation of the territories where the strychnine tree is grown. Phosphides are rather fast-acting rat poisons, resulting in the rats dying usually in open areas, instead of in the affected buildings.

Phosphides used as rodenticides include:

- aluminium phosphide (fumigant only)
- calcium phosphide (fumigant only)
- magnesium phosphide (fumigant only)
- zinc phosphide (bait only)

Hypercalcemia

Calciferols (vitamins D), cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) are used as rodenticides. They are toxic to rodents for the same reason they are important to humans: they affect calcium and phosphate homeostasis in the body. Vitamins D are essential in minute quantities (few IUs per kilogram body weight daily, only a fraction of a milligram), and like most fat soluble vitamins, they are toxic in larger doses, causing hypervitaminosis. If the poisoning is severe enough (that is, if the dose of the toxin is high enough), it leads to death. In rodents that consume the rodenticidal bait, it causes hypercalcemia, raising the calcium level, mainly by increasing calcium absorption from food, mobilising bone-matrix-fixed calcium

into ionised form (mainly monohydrogencarbonate calcium cation, partially bound to plasma proteins, [CaHCO3]+), which circulates dissolved in the blood plasma. After ingestion of a lethal dose, the free calcium levels are raised sufficiently that blood vessels, kidneys, the stomach wall and lungs are mineralised/calcificated (formation of calcificates, crystals of calcium salts/complexes in the tissues, damaging them), leading further to heart problems (myocardial tissue is sensitive to variations of free calcium levels, affecting both myocardial contractibility and excitation propagation between atrias and ventricules), bleeding (due to capillary damage) and possibly kidney failure. It is considered to be single-dose, cumulative (depending on concentration used; the common 0.075% bait concentration is lethal to most rodents after a single intake of larger portions of the bait) or sub-chronic (death occurring usually within days to one week after ingestion of the bait). Applied concentrations are 0.075% cholecalciferol and 0.1% ergocalciferol when used alone, wihich can kill a rodent or a rat.

There is an important feature of calciferols toxicology, that they are synergistic with anticoagulant toxicants, that means, that mixtures of anticoagulants and calciferols in same bait are more toxic than a sum of toxicities of the anticoagulant and the calciferol in the bait, so that a massive hypercalcemic effect can be achieved by a substantially lower calciferol content in the bait, and vice versa, a more pronounced anticoagulant/hemorrhagic effects are observed if the calciferol is present. This synergism is mostly used in calciferol low concentration baits, because effective concentrations of calciferols are more expensive than effective concentrations of most anticoagulants.

The first application of a calciferol in rodenticidal bait was in the Sorex product Sorexa D (with a different formula than today's Sorexa D), back in the early 1970s, which contained 0.025% warfarin and 0.1% ergocalciferol. Today, Sorexa CD contains a 0.0025% difenacoum and 0.075% cholecalciferol combination. Numerous other brand products containing either 0.075-0.1% calciferols (e.g. Quintox) alone or alongside an anticoagulant are marketed.

Althcugh this rodenticide [cholecalciferol] was introduced with claims that it was less toxic to nontarget species than to rodents, clinical experience has shown that

rodenticides containing cholecalciferol are a significant health threat to dogs and cats. Cholecalciferol produces hypercalcemia, which results in systemic calcification of soft tissue, leading to renal failure, cardiac abnormalities, hypertension, CNS depression and GI upset. Signs generally develop within 18-36 hours of ingestion and can include depression, anorexia, polyuria and polydipsia. As serum calcium concentrations increase, clinical signs become more severe. GI smooth muscle excitability decreases and is manifest by anorexia, vomiting and constipation. ... Loss of renal concentrating ability is a direct result of hypercalcemia. As hypercalcemia persists, mineralization of the kidneys results in progressive renal insufficiency."[10] Additional anticoagulant renders the bait more toxic to pets as well as human. Upon single ingestion, solely calciferol-based baits are considered generally safer to birds than second generation anticoagulants or acute toxicants. A specific antidote for calciferol intoxication is calcitonin, a hormone that lowers the blood levels of calcium. The therapy with commercially available calcitonin preparations is, however, expensive.

Rat Bait

The effectiveness of any rat bait depends upon environment and the habits of the rat in question. Rats tend to avoid new objects, so traps take some time to be effective. Traps can be made more effective if an attractant, sometimes called a bait, is used. Because rats are scavengers, the most effective rat attractant is food. Fruits and vegetables are attractive to roof rats. Meats or fish are more attractive to Norway rats. However, it is best to utilize food baits that are not likely to spoil in unreachable locations. Peanut butter should not be used since some people are allergic to the smell of peanuts. Another possible attractant would be nest materials such as bits of string, yarn or even cotton balls.

Rodenticide baits are engineered to kill rodents, rather than to catch them. These baits can be harmful to humans, house pets and wildlife if misapplied or ingested. Bait stations are available to prevent children and pets from accessing these baits.

Nevertheless, it is recommended that a pest control professional be consulted prior to using any bait around a home. Proper pest identification is necessary prior to attempting any pest eradication methods, as different measures are effective with each

species. Pest control professionals are trained to identify specimens and to determine and carefully administer the most effective and lasting treatment.

Natural Rat Deterrent

Peppermint oil is said to deter rats from entering treated areas. In high concentrations, peppermint oil may exhibit some repellancy. The challenge with using essential oils as a repellant is they often are required to be maintained at such high concentrations as to be unrealistic or unpleasant for the homeowner to smell. Once the odor becomes acceptable to the person, it no longer repels the rodents. Secondly, a determined rodent will not be deterred by the odor.

The best natural rat deterrent is cleanliness. Food scraps are highly attractive to rats and garbage areas are often infested. Keeping trash sealed tightly and ensuring that no food crumbs or residue are available around your home can prevent rat infestations.
The idea behind this research project is to develop bait from natural source by extraction and

Identification of active constituents from *Gliricidia sepilim* and development of a bait to be used as a Rodenticide.

Origin of research problem:

Rats are some of the most troublesome and damaging rodents They consume and contaminate food, damage structures and property and transmit parasites and diseases to other animals and humans. There are three main reasons why rats and mice are considered pests: They consume and damage human foods in field and stores. Through their gnawing and burrowing habit they destroy many articles which include packaging, clothes, furniture, floors, buildings, electrical cables etc. They are responsible for transmitting diseases dangerous to man. There are economic and sanitary reasons why rats need to be controlled.

Rodenticides are a diverse group of chemically and strcturally unrelated compounds. Over $ I . I billion of commercial, industrial and household rodenticide containing products are produced and consumed yearly. Acute and chronic profiles of rodenticides differ significantly from each other in severity and mechanism. Gliricidia sepium is a nitrogen fixing tree. It is a leguminous tree belonging to family Fabaceae.

It is used as a fence for fields and can grow on soil with medium to low fertility. A large number of compounds have been reported from Gliricidia. The most researched are the tannins. There are reports of the plants ability to decrease soil nematode populations. The name of the plant literally means "rat-killer' however its potential has not been exploited and there are various theories regarding its efficacy and the part of the plant to be used as a rodenticide. The research that is proposed here will elucidate the issue of extraction and identification of the component with rodenticide activity and determination of optimal bait for practical usage.

Pest control is an important environmental issue and there is a surge in research activity for natural products which can be effectively used for pest control. Integrated pest management is the need of the day. In this search for naturals for control of rodent (rat and mice) population in urban city like Mumhai there is need for concerted effort among plant scientists, chemists and biochemist to develop an effective bait for controlling the population of rats which is proving to be a menace in more than one ways. The incidence of leptosporosis has been increasing at an alarming rate. One may not be aware of the fact that rats can excrete 100 million bacteria per milliliter of urine. Its possible for a single pair to 2000 offspring per year. The damage caused by rodents worldwide is so enormous that it cannot be calculated. Many groups are involved in developing an effective Rodenticide which are mainly synthetic. These are associated with pest resistance and toxicity to other mammals and associated issues of safety. These units are spread globally. However the search for a natural rodenticide continues.

National status: The National laboratories which are working on natural products are Mainly interested in secondary metabolites with pharmaceutical values. Agrichemicals of plant origin have mainly focused on antiftingal and antimicrobial agents like neem based and Pongamia based pesticides. Large animals are still being controlled using synthetic pesticides. Our group in the institution has been working on secondary metabolites from plants and we would like to extend this work to isolation of specific molecules which can be used as an effective tool for control of rodent population specially in urban areas.

As mentioned above rats are a major pest in rural and urban areas and the extent of damage caused by them is enormous. The damage caused by rats include crop damage, grain damage in storage and structural damage in house, shops and commercial areas. In addition they transmit diseases to humans and livestock many of them are fatal. This study would help in developing a natural rodenticide which could be helpful in controlling the rodent population in a locality.

Objectives of the project

a) Collection of the leaves, bark, flowers of Gliricidia sepium from three different locations in Murnbai and Thane district.
b) Standardisation of protccol for extraction and identification of major chemical constituents from the above mentioned plant parts.
c) Identification of active constituents from dry plant parts and fermented plant parts of Gliricidia sepium.
d) Testing the toxicity of the various fractions to ascertain the exact chemical constituent which is potent as a rodenticide.
e) Preparation of an optimal bait to used as a rodenticide.

Methodology:

Collection of plant material of Gliricidia sepium:

Field trips will be carried out to collect the leaves, stem bark and flowers from selected trees localized at Veermata Jijabai Udyan Byculla, National Park, Borivili, Yeor village, Thane this will ensure that the source material is well identified and whenever needed plant material will be procured from the same tree to avoid chemotypic variability.

Extraction:

Extraction of active constituents will be carried out by Physical and Chemical fractionation. The active constituents will be extracted both from dry plant material and fermented plant material (leaves/bark/flowers will be soaked plant along with maize grains). The duration of fermentation will range from 24-72 hours and constituents will be analysed from each of these treatments. Extracts will be separated by preparative TLC (Wagner and Bladt, 1995) for purification and quantified by HPTLC (available at our Institution).

Identification of active constituent from *Gliricidia sepium* using High-performance liquid chromatography method and High-performance thin liquid chromatography:

High-performance liquid chromatography (sometimes referred to as high-pressure liquid / chromatography), HPLC, is a chromatographic technique that can separate a mixture of compounds and is used in biochemistry and analytical chemistry to identify, quantify and purify the individual components of the mixture.

HPLC typically utilizes different types of stationary phases, a pump that moves the mobile phase(s) and analyte through the column, and a detector to provide a characteristic retention time for the analyte. The detector may also provide additional information related to the analyte, (i.e. UV/Vis spectroscopic data for analyte if so equipped). Analyte retention time varies depending on the strength of its interactions with the stationary phase, the ratio/composition of solvent(s) used, and the flow rate of the mobile phase. It is a form of liquid chromatography that utilizes smaller column size, smaller media inside the column, and higher mobile phase pressures.

With HPLC, a pump (rather than gravity) provides the higher pressure required to move the mobile phase and analyte through the densely packed column. The increased density arises from smaller particle sizes. This allows for a better separation on columns of shorter length when compared to ordinary column chromatography.

The sample to be analyzed is introduced, in small volumes, into the stream of mobile phase. The solution moved through the column is slowed by specific chemical or physical interactions with the stationary phase present within the column. The velocity of the solution depends on the nature of the sample and on the compositions of the stationary (column) phase. The time at which a specific sample elutes (comes out of the end of the column) is called the retention time. The retention time under particular conditions is considered an identifying characteristic of a given sample. The use of smaller particle size column packing (which creates higher backpressure) increases the linear velocity giving the components less time to diffuse within the column, improving the chromatogram resolution. Common solvents used include any miscible combination of water or various organic liquids (the most common are methanol and acetonitrile). Water may contain buffers or salts to assist in the

separation of the sample components, or compounds such as trifluoroacetic acid which acts as an ion pairing agent.

A further refinement of HPLC is to vary the mobile phase composition during the analysis; gradient elution. A normal gradient for reversed phase chromatography might start at 5% methanol and progress linearly to 50% methanol over 25 minutes; the gradient depends on how hydrophobic the sample is. The gradient separates the sample mixtures as a function of the affinity. This partitioning process is similar to that which occurs during a liquid-liquid extraction but is continuous, not step-wise. In this example, using a water/methanol gradient, more hydrophobic components will elute (come off the column) when the mobile phase consists mostly of methanol (giving a relatively hydrophobic mobile phase).

The choice of solvents, additives and gradient depend on the nature of the column and sample. Often a series of tests are performed on the sample together with a number of trial runs in order to find the HPLC method which gives the best peak separation.

A modem self contained HPLC: Partition chromatography was the first kind of chromatography that chemists developed. The partition coefficient principle has been applied in paper chromatography, thin layer chromatography, gas phase and liquid-liquid applications. The 1952 Nobel Prize in chemistry was earned by Archer John Porter Martin and Richard Laurence Millington Synge for their development of the technique, which was used for their separation of amino acids. Partition chromatography uses a retained solvent, on the surface or within the grains or fibres of an "inert" solid supporting matrix as with paper chromatography; or takes advantage of some coulombic and/or hydrogen donor interaction with the solid support. Molecules equilibrate (partition) between a liquid stationary phase and the eluent. Known as Hydrophilic Interaction Chromatography (HILIC) in **HPLC,** this method separates analytes based on polar differences.

HPTLC is one type of planar chromatography and most advanced form of instrumental TLC. Now a day, HPTLC is more useful than TLC and HPLC. Because HPTLC is independent of sample application, chromatogram development, detection,

etc. it is not only instrumenta TLC but entire concept that include widely standardize methodology based on validated method. It is instrument controlled by software.

Planar Chromatography as opposed to column chromatography (e.g. GC, HPLC) utilizes a flat (planar) stationary phase for separation. In Thin-Layer Chromatography (TLC) this stationary phase is supported by a glass plate or a foil (plastic or aluminum). Again unlike column separations, the TLC plate constitutes an open system, which passes through the individual steps of the TLC analysis in an off-line mode.

The relative independence of sample application, chromatogram development, detection, etc. in time and location makes possible the parallel analysis of many samples on the same plate. The most advanced form of instrumental TLC is commonly called high performance thin-layer chromatography (HPTLC), but the term does not simply imply instrumental TLC on special high performance layers. HPTLC is an entire concept that includes a widely standardized methodology based on scientific facts as well as the use of validated methods for qualitative and quantitative analysis. Sophisticated instruments, controlled by an integrated software platform ensure to the highest possible degree the usefulness, reliability, and reproducibility of generated data. HPTLC is therefore the term for a method that meets all quality requirements of today's analytical labs even in a fully regulated environment.

Initial costs for an HPTLC system as well as maintenance, and cost per sample still remain comparatively low and all advantages derived from the planar separation principle are certainly maintained, the possibility of visual evaluation of separated samples on the plate is one of the most valuable aspects of TLC. It reaches a completely new dimension in HPTLC through the use of modern techniques for generating and evaluating

FEATURES OF HPTLC:'

The advantages of this off-line arrangement as compared with an on-line process, such as column high-performance liquid chromatography (HPLC), have been outlined and include the following:

- Technically, it is simple to learn and operate.

- Several analysts work simultaneously on the system.
- Lower analysis time and less cost per analysis.
- Low maintenance cost.
- Visual detection possible - as it is an open system.
- Availability of a great range of stationary phases with unique selectivity for mixture components.
- Chromatographic layer (sorbent) requires no regeneration as TLC/HPTLC plates are disposable.
- Ability to choose solvents for the mobile phase is not restricted by low UV transparency or the need for ultra-high purity. Corrosive and UV-absorbing mobile phases can be employed.
- No prior treatment for solvents like filtration and degassing.
- There is no possibility of interference from previous analysis as fresh stationary and mobile phases are used for each analysis. No carry over, hence no contamination.
- Repetition of densitometric evaluation of the same sample can be achieved under different conditions without repeating the chromatography to optimize quantification, since all sample fractions are stored on the TLC/HPTLC plate.
- Samples rarely require cleanup.
- High sample throughput since several samples can be chromatographed simultaneously.
- Lower expenditure of solvent purchase and disposal since the required amount of mobile phase per sample is small. In addition, it minimizes exposure risks of toxic organic effluents and reduces possibilities of environment pollution.
- Accuracy and precision of quantification is high because samples and standards are chromatographed and measured under the identical experimental conditions on a single TLC/HPTLC plate.
- Sensitivity limits of analysis are typically at nanogram (ng) to pictogram (pg) levels.
- Use of different universal and selective detection methods.3

HPTLC is a modern adaptation of TLC with better and advanced separation efficiency and detection limits.

HPTLC METHODOLOGY:

Set the analytical objective first that may be quantification or qualitative identification or separation of two components/multicomponent mixtures or optimization of analysis time before starting HPTLC. Method for analyzing drugs in multicomponent dosage forms by HPTLC demands primary knowledge about the nature of the sample, namely, structure, polarity, volatility, stability, and the solubility parameter. Method development involves considerable trial and error procedures. The most difficult problem usually is where to start, with what kind of mobile phase.

Selection of stationary phase is quite easy, that is, to start with silica gel which is reasonable and nearly suits all kind of drugs. Mobile phase optimization is carried out by using three level techniques. First level involves use of neat solvents and then by finding some such solvents which can have average separation power for the desired drugs. Second level involves decreasing or increasing solvent strength using hexane or water for respective purposes. Third level involves trying of mixtures instead of neat solvents from the selected solvents of first and second level which can further be optimized by the use of modifier like acids or bases.

Analytes are detected using fluorescence mode or absorbance mode. But, if the analytes are not detected perfectly than it needs change of stationary phase or mobile phase or need the help of pre or post chromatographic derivatization. Optimization can be started only after a reasonable chromatogram which can be done by slight change in mobile-phase composition. This leads to a reasonable chromatogram which has all the desired peaks in symmetry and well separated.

Procedure for HPTLC method development:

STATIONARY PHASE: HPTLC can be regarded as the most advanced form of modern TLC. It uses HPTLC plates featuring small particles with a narrow size distribution. As a result, homogenous layers with a smooth surface can be obtained. HPTLC uses smaller plates (10 x 10 or 10 x 20 cm) with significantly decreased development distance (typically 6 cm) and analysis time (7-20 mm). HPTLC plates

provide improved resolution, higher detection sensitivity, and improved in situ quantification and are used for industrial pharmaceutical densitornetric quantitative analysis.

Normal phase adsorption TLC on silica gel with a less polar mobile phase, such as chloroform-methanol, has been used for more than 90% of reported analysis of pharmaceuticals and drugs. Lipophilic C-18, C-8, C-2; phenyl chemically-modified silica gel phases; and hydrocarbon-impregnated silica gel plates developed with a more polar aqueous mobile phase, such as methanol-water or dioxane-water, are used for reversed-phase TLC.

Other precoated layers that are used include aluminum oxide, magnesium silicate, magnesium oxide, polyamide, cellulose, kieselguhr, ion exchangers, and polar modified silica gel layers that contain bonded amino, cyano, diol, and thiol groups.

Optical isomer separations that are carried out on a chiral layer produced from C-18 modified silica gel impregnated with a Cu (II) salt and an optically active enantiomerically pure hydroxyproline derivative, on a silica layer impregnated with a chiral selector such as brucine, on molecularly imprinted polymers of a-agonists, or on cellulose with mobile phases having added chiral selectors such as cyclodextrins have been reported mostly for amino acids and their derivatives.

HPTLC plates need to be stored under appropriate conditions. Before use, plates should be inspected under white and UV light to detect damage and impurities in the adsorbent. It is advisable to prewash the plates to improve the reproducibility and robustness of the results.3

MOBILE PHASE: The selection of mobile phase is based on adsorbent material used as stationary phase and physical and chemical properties of analyte.

General mobile-phase systems that are used based on their diverse selectivity properties are diethyl ether, methylene chloride, and chloroform combined individually or together with hexane as the strength-adjusting solvent for normal-

phase TLC and methanol, acetonitrile, and tetrahydrofuran mixed with water for strength adjustment in reversed-phase TLC.

Separations by ion pairing on C-18 layers are done with a mobile phase such as methanol-0.1 M acetate buffer (pH 3.5) containing 25 mM sodium pentanesulfonate (15.5:4.5).

Accurate volumetric measurements of the components of the mobile phase must be performed separately and precisely in adequate volumetric glassware and shaken to ensure proper mixing of the content.

Volumes smaller than 1 ml are measured with a suitable micropipette. Volumes up to 20 ml are measured with a graduated volumetric pipette of suitable size. Volumes larger than 20 ml are measured with a graduated cylinder of appropriate size. To minimize volume errors, developing solvents are prepared in a volume that is sufficient for one working day.

Gliricidia sepium is an exotic plant belonging to the family Fabaceae. Gliricidia which literally means "Rat Poison" originated in Central America and widely neutralised in tropical America, Africa and Asia. It is a medium size tree to10m height. It grows in warm, seasonally dry climate.

A large number of compounds have been reported from Gliricidia. One of the reported compound is Coumarin. Coumarins are the most important group of rodenticides.

Coumarin is a chemical compound, a toxin found in many plants. It has clinical medical value as the precursor for several anticoagulants. The name comes from a french word, 'coumarou'. Coumarin has apetite supressing properties, suggesting its widespread occurance in plants, especially grass. Coumarin is moderately toxic, it is a potent rodenticide : rats and other rodents can cause internal hemorrhage and death. Rodenticides are chemicals that prevent, inhibit, destroy, kill or migrate rodent. More than 750 species of rats live throughout the world and their population estimated to exceed 3 billions, constitutes a permanent menace through diseases and dsetruction.

There are three main reasons to develop an effective rodenticide from natural product : the rats and mice consume and damage human foods in field and stores. Through their gnawing and burrowing habit they destroy many articles which include packaging, clothes, furniture, floors, electrical cables etc. They are responsible for transmitting diseases dangerous to man. There are economic and sanitary reasons why rats need to be controlled using natural rodenticide.

Generally rodenticides are applied as baits to control rodent pets. For effective control, the bait must satisfy the folllowing criteria : the bait should have no repellent action or odour, bait should be attractive, clean and fresh. It must not induce bait shyness. It should have much lower toxicity to domestic animals especially cats and dogs, which eat the poisoned rats. Natural rodenticide are those which consists of food grade cellulose with molasses as a sweet attractant.The aim of this project is to develop optimum bait from natural product to be used as a rodent.

Plant material of Gliricidia sepium were collected from Veermata Jijabai Udyan Byculla,National Park, Borivili &Yeor Village,Thane and authenticated.The leaves,bark and flowers were separeted.Some material was dried,ground and then used for further studies To identify the chemical constituents of plant parts.fresh bark(250g), leaves (250g)and flowers (100g) were cut into small pieces and ground into paste using grinder.This material was subjected to steam distillation for about three hours. About 2 litres of distillate were collected and extracted with diethyl ether ($3x100cm^3$) and evapoured to dryness. The dry ether extracts were used for HPTLC and HPLC. The major 19 compounds have been already reported from bark. (coumarin 8.07% from bark)(Indian J.of pharmacy&pharmaceuticals sciences,vol.2 Issue 3,2010, ISSN 0975-1491). Using HPLC major compounds have been reported already, and we are interested in compound like coumarin which will be used in preparation of bait, therefore HPTLC technique was adopted first to separate and identify the active constituents in the extract.

HPTLC-High performance thin-layer chromatography is a recent development and can be thought of in simple terms as a new improved version of conventional Thin layer chromatography (TLC). Some of the advantages offered by HPTLC over TLC are: Separation of compounds and there analyses are faster and more distinct; HPTLC

chromatographic plates are easier to handle. HPTLC is more suitable for further analysis..TLC& HPTLC techniques have long been used in drug abuse screening programmes.They are often regarded as the most suitable technique for the rapid detection of drugs because they meet the criteria of: (1) Minimal instrumentation. (2)Low cost (3)Simplicity (4)Minimum Laboratory space & Maintenance (5)Rapidity of analysis (6)Specificity & resolution that is superior to non – choromatographic techniques. (7)Ease of interpretation of results. In the present study HPTLC has been chosen as a method of analysis.A standard procedure was developed for evaluation of active coustituents from extracts of bark, leaves & flowers of Gliricidia sepia. HPTLC was used for only identification of coumarion in extracts of sample ,quaytification was done with the help of HPLC techique.

A CAMAG HPTLC systems was used to analyse the samples.
No of tracks ; 4 (standred coumarin & 3 samples)
Max wavelength:254nm. Lamp:D2. Scanning speed 20 mm/s.
Mobile phase: Toluene:Diethyl ether.
All samples &std. dissolved in methanol 10mg/ cm^3. Sample injected :20 micro l.

Results obtained from HPTLC reveals that all the 3 sample countains coumarin and the amount of coumarin found in leaves was maximum & in flower it was minimum. Quantification was done using HPLC results.Fig.1,2,3,4,
(1)Coumarin Std (2) Chromatogram for coumarin in Gliricidia sepia fresh Leaves (3) Chromatogram for coumarin in Gliricidia sepia Fresh bark (4) Chromatogram for coumarin in Gliricidia sepia Fresh flower (5) TLC.

HPLC-High pressure or performance liquid chromatography Technique that can separate a mixture of compounds and is used to identify and quantify the individual components of the mixture .HPLC typically utilizes different types of stationary phases,a pump that moves the mobile phases andanalyse through the column and a defector to provide a characteristic retention time for the analyte. Analyte retention time varies depending on the strength its interaction with the stationery phase, the ratio /composition of solvent used and the flow rate of the mobile phase. It is a form of liquid chromatography that uitizes smaller column size, smaller media inside the column and higher mobile phase pressure.`

In the present study coumarin is identified in the samples with the help of HPTLC.HPLC is carried out to quantify the coumarin in samples. The chromatography conditious for HPLC: (1)Instrument : Agilent 1200 series HPLC.

(2)HPLC Column : edipse X DB (15cm X 4.6mm,5µm)

(3)λ max : 280nm

(4)Detector :Diode array detector

(5)Injection Volume :10λl, Flow rate : 1ml/min

(6) Gradient Distilled water : Acefonitnile

0min	90 : 10
5 min	80 : 20
10min	40 : 60
15min	30 : 70
20min	20 : 80
30min	90 :10

Preprtion of standard: Weighed accurately 27.5mg of coumarin& dissolved in 50cm3 acetonitrile. 0.3 cm^3 of this was further diluted to 10cm^3& Injected 10 cm^3 (165ppm) . Prepration of sample solutions: (1) weighed accurately 70 mg of flower extract &dissolved in 50 cm^3 of acetonitnile injected 10 cm^3 (1400ppm). (2)Weighed accurately 42mg of leaves extract & dissolved in 100$cm^3$of acetouitrile 5cm^3 of this was further diluted to 10cm^3 &injected to 10 cm^3l (210ppm) . (3) weighed accurately 70mg of bark extract & dissolved in 50cm^3 of acetonitrile& injected 10cm^3 (1400ppm)

Coumarin content (% w/w) is calculated as

$$\frac{M1 \times A2}{M2 \times A1} \times P$$

M1= Mass in mg of coumarin in standard

M2= Mass in mg Technical sample taken for test

A2=Peak area of coumarin in sample solution

A1=Peak area of coumarin in std solution

P=%Purity of coumarin

Retetion time of coumarin =10.90

Fig 1 2 3 4

Std L B F

Results obtained from HPLC

% of coumarin in flower 1.22%

Bark no peak observed

Leaves 73.5%

Toxicity study was carried out by using albina rats as the experimental model . The study was carried out to determine the optimum does of bait to be prepared .To assess the acute toxicity of the plant materials on oral administation the study was carried out.

Protocol:

Animal species	: Albino rats
Sex	: Male& Female
Body Weight	: Approx 250g
Animal procured from	: Bharat serum,Thane from does
No of groups	: 3
Animals per groups	: 6
Route of administration	: Oral / via a gavage
Vehicle for adminstartion	: DW & CMC (Carboxy Methyl Cellulose)
Volume for administration	: 1cm^3 as a combined volume of sample+vehicle
Dosing details	: as given in table .
Observation period	: 1 – To - 8 days.

More clinical observation such as condition of fur swelling, food & water intake etc. recorded as an indication for toxicity.

Animal maintenance: The animal rooms were maintained on a cycle of twelve hours light & 12 hours darkness. The relative humidity was maintained at 70+5%. The animals were housed in polyurethane mice cages. The cages were provided with rice husk bedding for the animals& were cleaned daily. The animals were provided with drinking water & were fed on commercially available mice fed supllied by Amrut feed or maize/corn which is available in the market . The specification of the commercial fed are listed below:

Pellet Size 12mm

Crude Portion 20-21% minimum

------''---Fibere 4% ----ting ---''-------

Ether Extractive 4-5% -------''---

Ash 8% maximum

Calcium 1.2

Phosphorus 0.6% minimum

ME Kcal/kg 3600

Experimental design:

Normal albino rats were first accumatized for 2days:60 normal healthy rats of both the sex were included for the study. Rats were divided in 4 groups. Each consisting of 6 animals of each. Overnight fasted normal rats were randomly divided in to different groups .each consisting of 6 animals each. The vehical & drugs were administred only by an intragastric tube.

Group I served as normal control , which received only 0.5% CMC 1 cm^3 Daily .

Group	Sex	Dose g/kg	No of animals used	Administred per day	
II	M/F	3	6	1cm^3	
III	M/F	2	6	1cm^3	
IV	M/F	1	6	1cm^3	

Each gp is further classified as

A) CMC with fresh leaves

B) CMC with dry leaves.

C) CMC wiyh fresh bark

D) CMC with dry bark

During this period commercial feed was suplied 50gm/day No mortality was recorded for with the peranimal above mentioned doses during the span of the study for Gliricichia sepia. But the fur became dull, food, &water intake reduseed in the span of 6 days.

The second set of experiment was carried out by fermenting plant materials with corn /maize for 48-72hrs. The fermented material was stained and that extract was feed to animals.

Group I Served as normal control which recived only corn 50gm perday per animal

Group	Sex	Dose	No of animals used	Per animals administred per day/animal
II	M/F	3%	6	1gm
III	M/F	2%	6	1gm
IV	M/F	1%	6	1gm

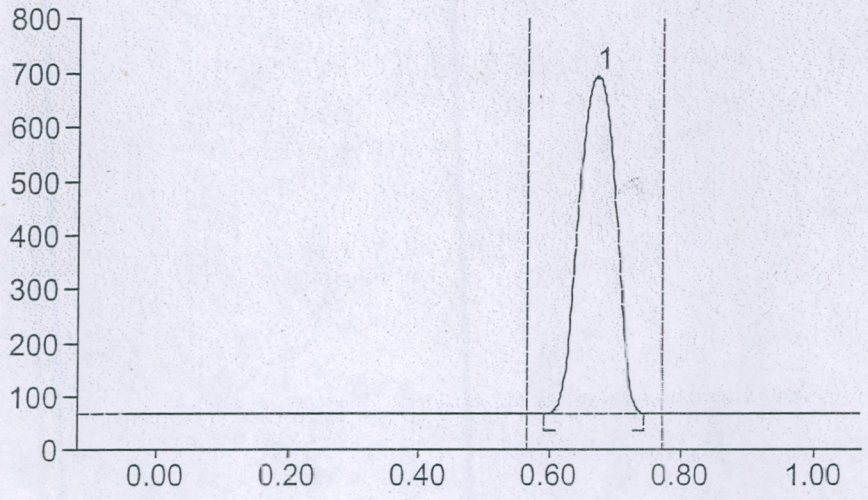
Each gp was further classified as

A) Maize with fresh leaves 3%, 2%, 1%
B) Maize with dry leaves 3%, 2%, 1%.
C) Maize with Fresh bark 3%, 2%, 1%.
D) M aize with dry bark 3%, 2%, 1%.

Extracts of each 1cm3/day / animal was tried but again no mortality was recorded Then molasses of 1gm each were given to animal per day After eating second doses mortality was observed for 3% fermented leaves & bark with corn .Mortality is the main criterion in assesing the toxicity of rodenticide .40% mortality obtained with 3% fermented dose for effective control the bait must satisfy the following criteria :

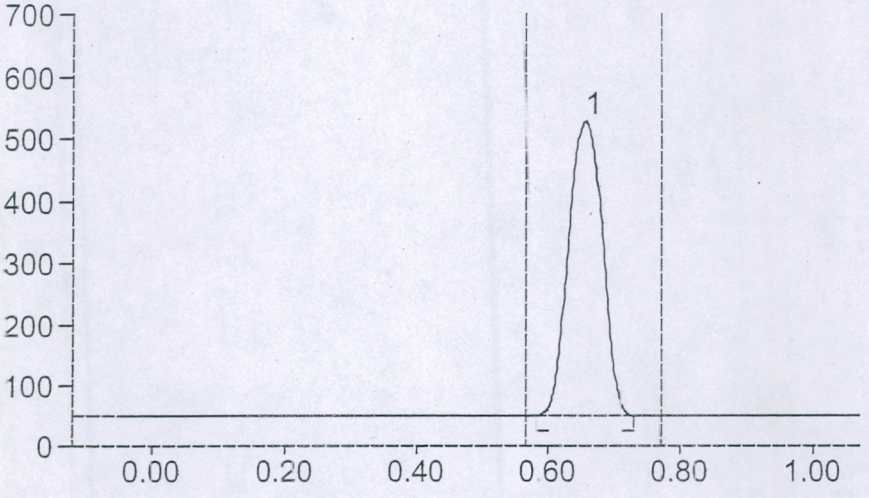
The bait should have no repellent action or odour bait should be attractive ,clean & fresh . It was observed that the animals were readily eating molasses because of corns sweet smell.

Track 1, ID: Std



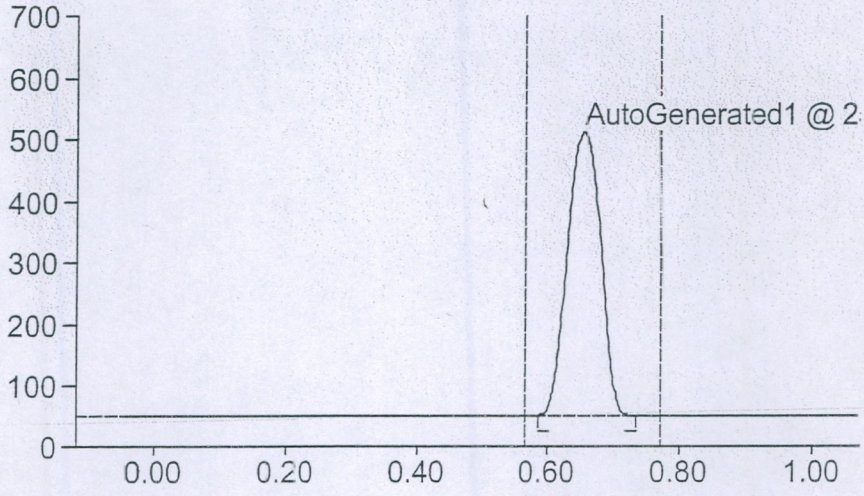
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.59	0.4	0.67	628.0	100.00	0.75	1.3	27669.2	100.00	unknown *

Track 2, ID: Std



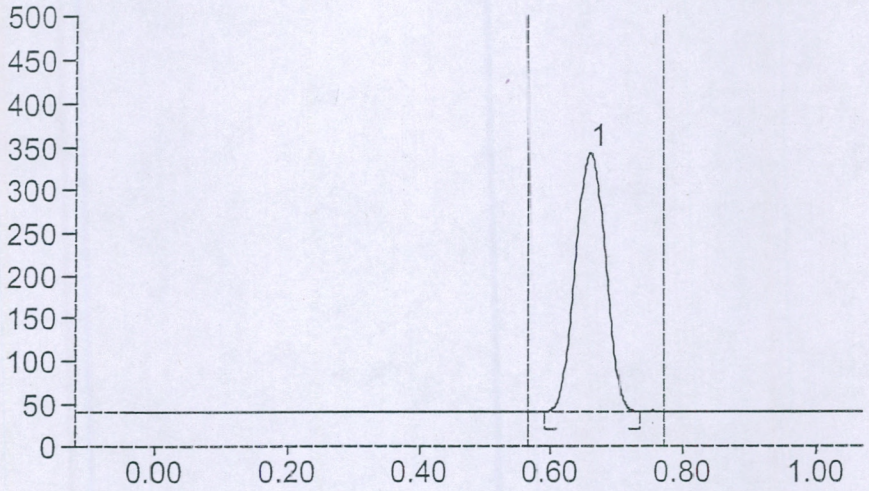
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.59	1.1	0.66	480.1	100.00	0.73	1.9	19258.9	100.00	unknown *

Track 3, ID: Std

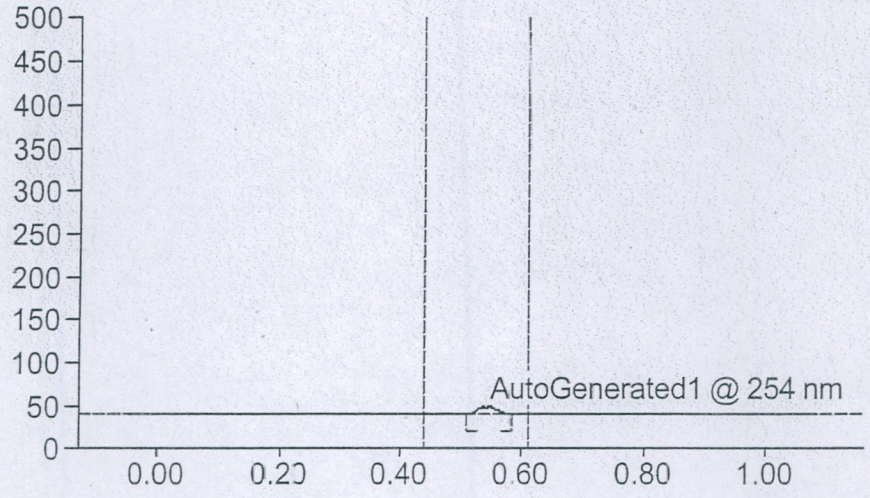


Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.59	2.9	0.66	464.4	100.00	0.74	0.2	18311.8	100.00	AutoGenerated1

Track 4, ID: Std

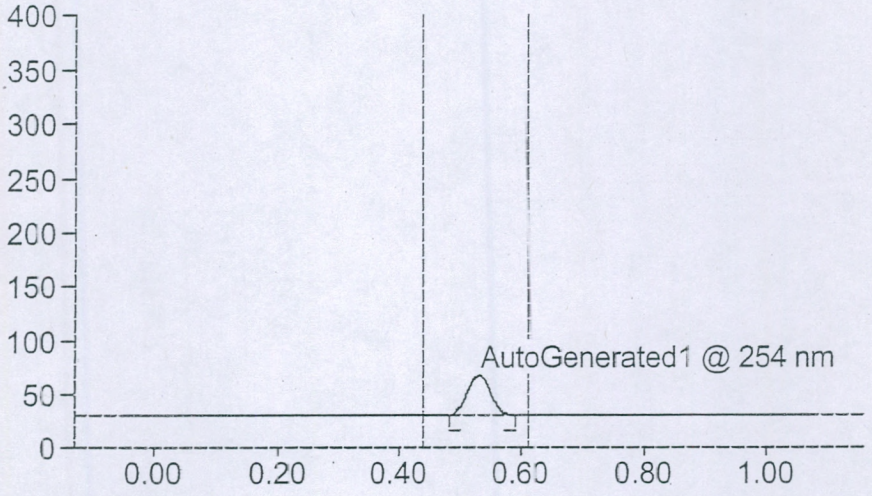


Track 2, ID: Bark (CHCl3)



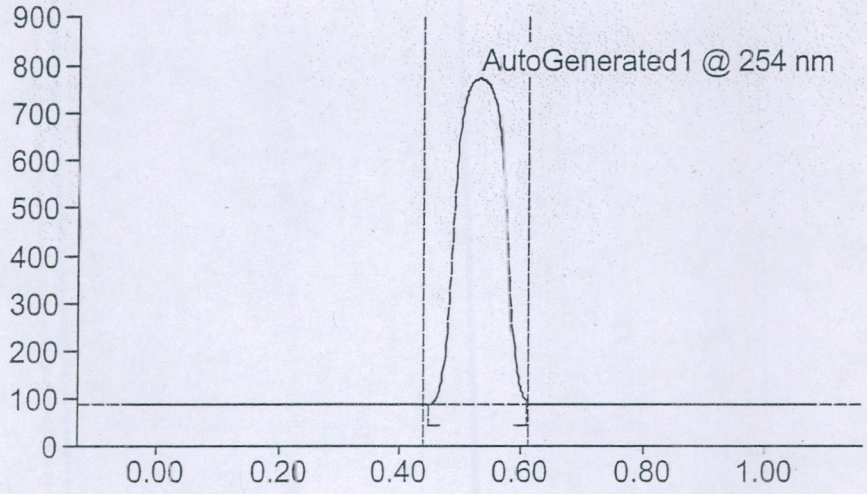
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.51	0.3	0.55	9.5	100.00	0.59	0.5	223.3	100.00	AutoGenerated1

Track 3, ID: Flower



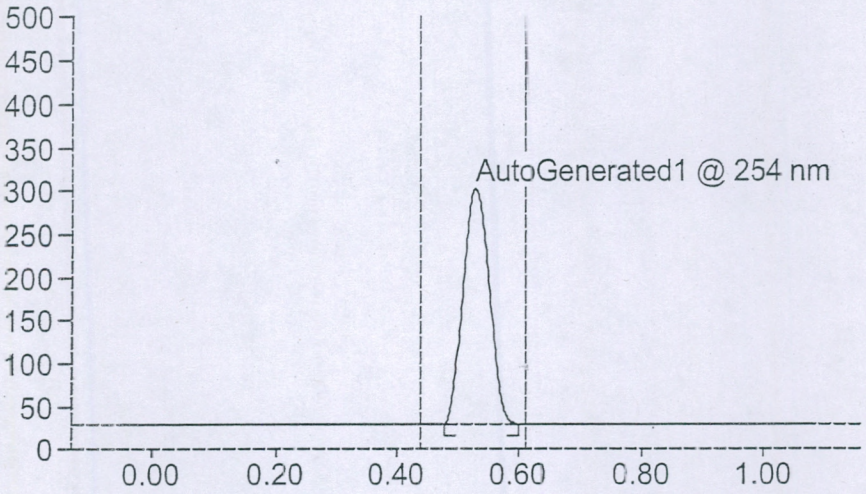
Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.48	0.2	0.53	37.7	100.00	0.59	0.8	1078.0	100.00	AutoGenerated1

Track 4, ID: Leaf



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.45	0.3	0.53	686.1	100.00	0.6	5.8	38639.9	100.00	AutoGenerated1

Track 5, ID: Std



Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %	Assigned substance
1	0.48	0.4	0.53	273.4	100.00	0.60	0.1	8673.1	100.00	AutoGenerated1

Spectrum scan

Executed by	Anchrom Test Lab Pvt Ltd. Friday, April 22, 2011 1:56:22 PM
Mode	All detected peaks
Slit dimensions	6.00 x 0.45 mm, Micro
Optimize optical system	Resolution
Scanning speed	100 nm/s
Data resolution	10 nm/step
Reference spectrum, pos X	10.0 mm
Reference spectrum, pos Y	10.0 mm

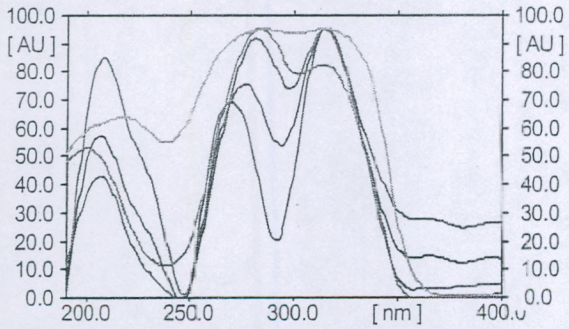
Measurement Table

Lamp	D2
Start wavelength	190 nm
End wavelength	400 nm
Measurement type	Remission
Measurement Mode	Absorption
Optical filter	Second order
Detector Mode	Automatic

Detector properties

Y-position for 0 adjust	0.0 mm
Track # for 0 adjust	0
Analog Offset	10%
Sensitivity	Automatic (22)

AutoGenerated1 on all Tracks

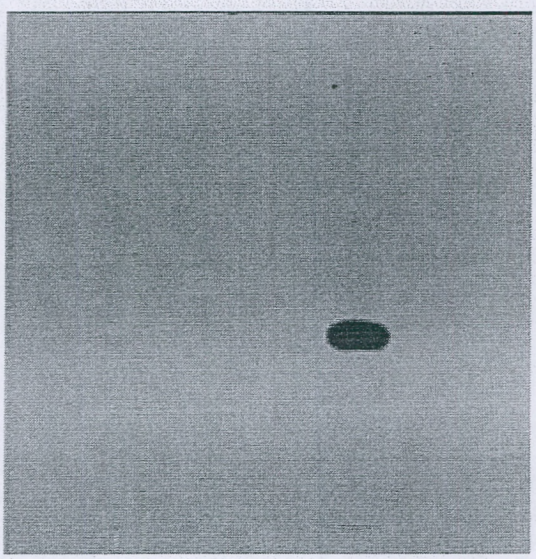


T	Rf	Substance	Max. @
1	0.56 Rf	AutoGenerated1	314 nm
2	0.55 Rf	AutoGenerated1	315 nm
3	0.53 Rf	AutoGenerated1	315 nm
4	0.53 Rf	AutoGenerated1	314 nm
5	0.53 Rf	AutoGenerated1	283 nm

Visualizer Document - Plate state Developed

Image information - 254 nm - Image1

Illumination instrument	CAMAG Visualizer : 150503 (Visualizer_150503)
Digital camera type : snr & Lens	DXA252 : 306921208, Computar, 16 mm, f4.0
Created by : on	Anchrom Testlab (I) Pvt.Ltd. : Friday, April 22, 2011 12:55:09 PM
Resolution	Full
Plate border size	-2 mm
Automatic capture	Off
Save mode	Lossy (JPG)
Exposure mode	Automatic, digital level: 80 %, Band
Capture settings:	
Image size:	952 Pxl x 952 Pxl (0.10 mm/Pxl)
Exposure :	140.76 ms gain: 1.00
White balance	R: 1.40, G: 1.00, B: 1.20
Illumination type / correction type :	254 nm remission : Default correction
Display settings:	
White balance:	R: 1.00 G: 1.00 B: 1.00
Contrast enhancement:	1.00
Brightness:	0.00
Accentuation:	0.80
Color saturation:	1.30 *
Blank plate compensation :	N/A

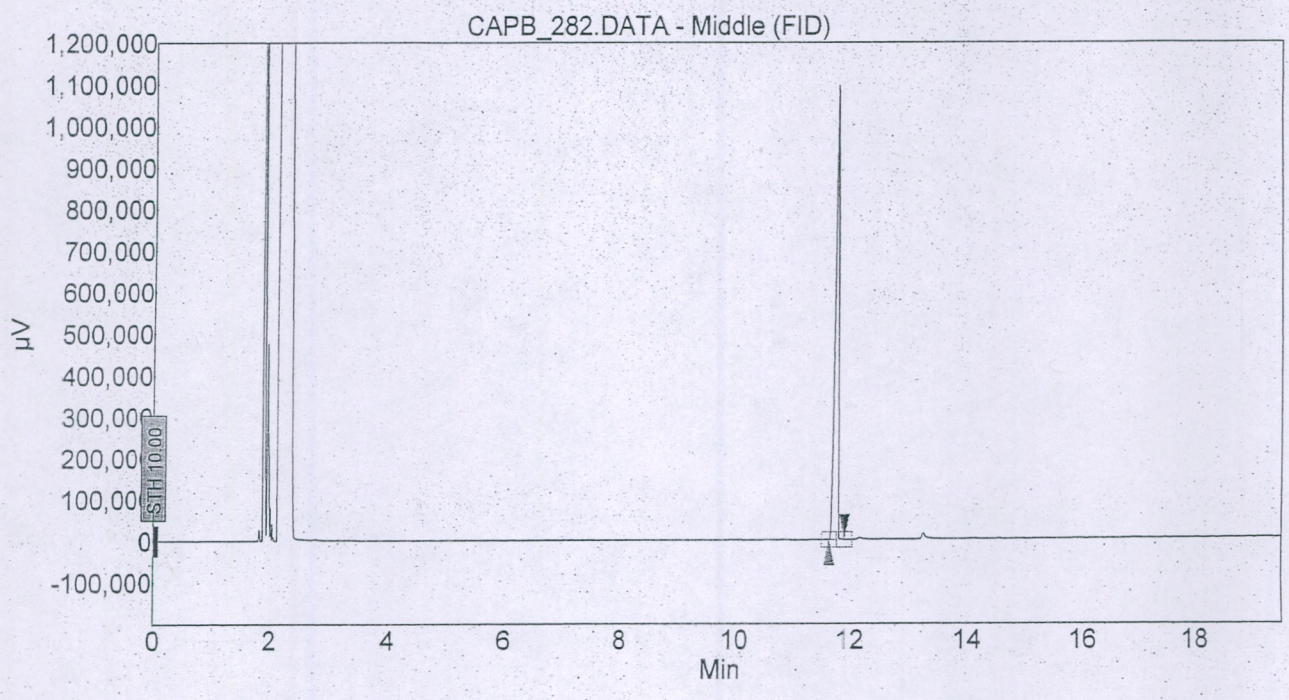


35.6 mg / 10 ml $CHCl_3$ → 9 µl injected
Coumarin

Chromatogram : CAPB_282_channel1

System : CP-3800
Method : C8-18 -DB-5
User : Bharat Parab

Acquired : 4/14/2011 7:30:28 PM
Processed : 4/14/2011 7:50:16 PM
Printed : 4/14/2011 7:51:30 PM
Sample Name : N.A.



Peak results :

Index	Name	Time [Min]	Quantity [% Area]	Height [µV]	Area [µV.Min]	Area % [%]
1	UNKNOWN	11.79	100.00	1095038.8	73576.2	100.000
Total			100.00	1095038.8	73576.2	100.000

58.4 mg (F) in 3 ml $CHCl_3$

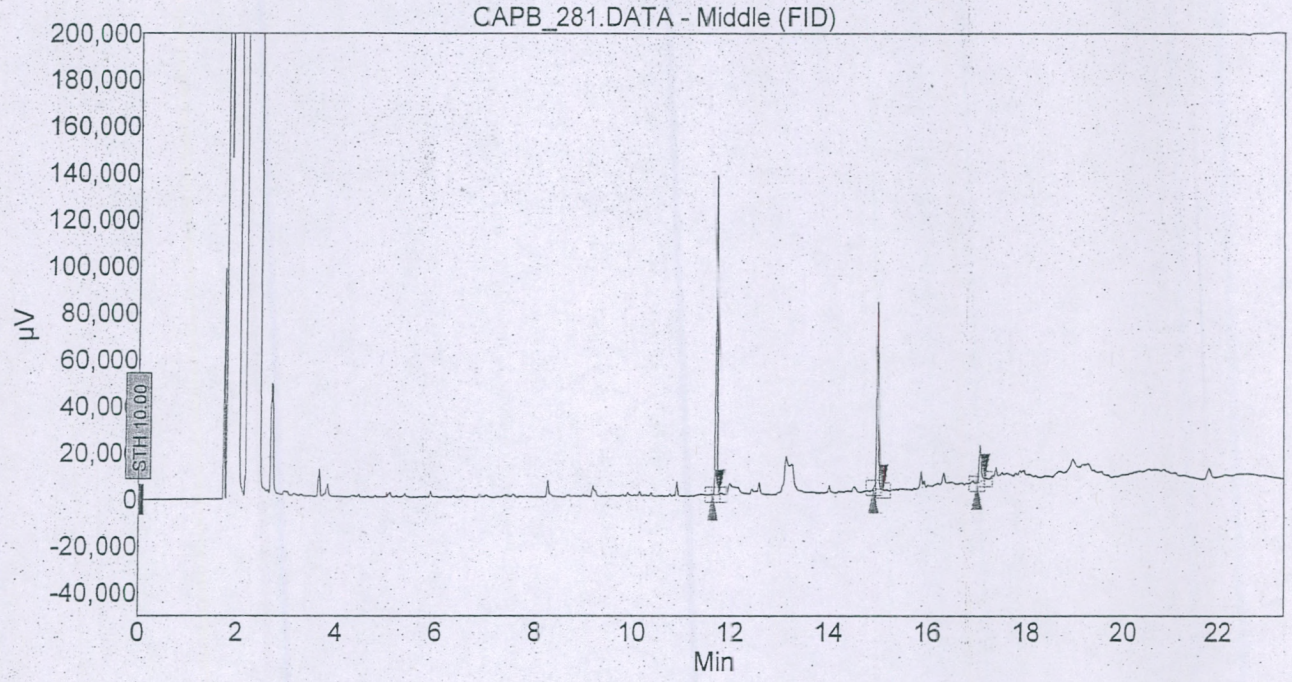
(F) ✓

9 µl injected

Chromatogram : CAPB_281_channel1

System : CP-3800
Method : C8-18 -DB-5
User : Bharat Parab

Acquired : 4/14/2011 7:03:08 PM
Processed : 4/14/2011 7:26:46 PM
Printed : 4/14/2011 7:27:55 PM
Sample Name : N.A.



Peak results :

Index	Name	Time [Min]	Quantity [% Area]	Height [µV]	Area [µV.Min]	Area % [%]
1	UNKNOWN	11.71	54.67	136664.3	4858.7	54.670
2	UNKNOWN	14.98	36.80	80128.0	3270.1	36.796
3	UNKNOWN	17.05	8.53	15749.7	758.4	8.534
Total			100.00	232542.0	8887.2	100.000

$$\frac{35.6\ mg\ /10\ ml}{58.4\ mg\ /3\ ml\ (19.47)} \times \frac{4858}{73576} \times 100 = 1.2\ \%$$

3.56

Page
1/$
PAGECOUNT

PTO

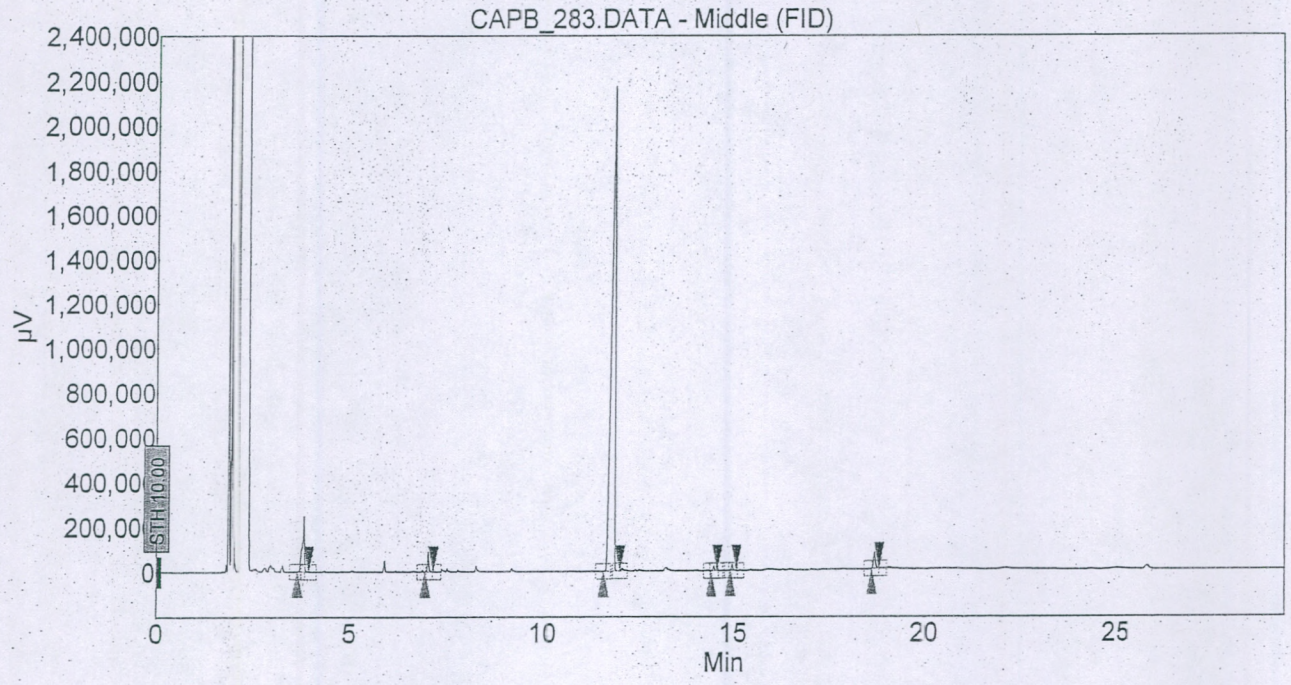
50.8 mg → 3 ml $CHCl_3$ → 9 µl injected

(L)

Chromatogram : CAPB_283_channel1

System : CP-3800
Method : C8-18 -DB-5
User : Bharat Parab

Acquired : 4/14/2011 7:56:54 PM
Processed : 4/14/2011 8:46:36 PM
Printed : 4/14/2011 8:48:30 PM
Sample Name : N.A.



Peak results :

Index	Name	Time [Min]	Quantity [% Area]	Height [µV]	Area [µV.Min]	Area % [%]
1	UNKNOWN	3.83	5.79	246224.9	15800.2	5.794
2	UNKNOWN	7.03	1.28	89885.0	3493.5	1.278
3	UNKNOWN	11.89	90.61	2170209.4	247093.2	90.614
4	UNKNOWN	14.51	0.33	21147.6	910.3	0.334
5	UNKNOWN	14.98	0.40	27326.2	1087.5	0.399
6	UNKNOWN	18.71	1.58	72464.6	4311.5	1.581
Total			100.00	2627257.7	272696.3	100.000

$$\frac{35.6\ mg/10\ ml}{50.8\ mg/3\ ml} \times \frac{247093}{273576} \times 100 = 70.62\%$$

(1693)

Chromatogram : CAPB_284_channel1

System : CP-3800
Method : C8-18 -DB-5
User : Bharat Parab

Acquired : 4/14/2011 8:34:09 PM
Processed : 4/14/2011 8:58:26 PM
Printed : 4/14/2011 8:59:09 PM
Sample Name : N.A.

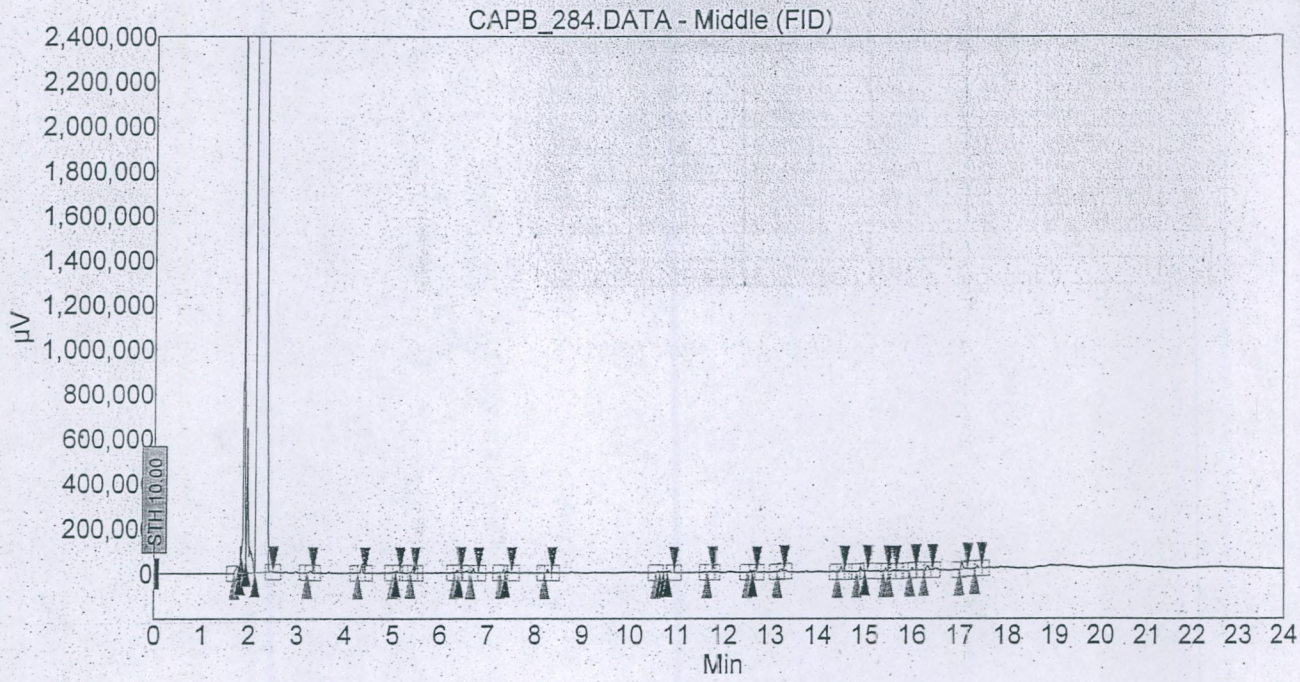
Index	Name	Time [Min]	Quantity [% Area]	Height [µV]	Area [µV.Min]	Area % [%]
27	UNKNOWN	15.00	0.00	2038.3	88.9	0.003
28	UNKNOWN	15.45	0.02	15163.2	605.9	0.021
29	UNKNOWN	15.61	0.02	9397.0	508.3	0.018
30	UNKNOWN	16.00	0.02	11954.3	527.8	0.018
31	UNKNOWN	16.33	0.04	18360.6	1221.2	0.042
32	UNKNOWN	17.05	0.06	33238.1	1614.6	0.056
33	UNKNOWN	17.37	0.02	11663.6	512.9	0.018
Total			100.00	29526464.6	2882291.9	100.000

mg B → 3 ml $CHCl_3$ → 9 µl injected

Chromatogram : CAPB_284_channel1

System : CP-3800
Method : C8-18 -DB-5
User : Bharat Parab

Acquired : 4/14/2011 8:34:09 PM
Processed : 4/14/2011 8:58:26 PM
Printed : 4/14/2011 8:59:09 PM
Sample Name : N.A.



Peak results :

Index	Name	Time [Min]	Quantity [% Area]	Height [µV]	Area [µV.Min]	Area % [%]
1	UNKNOWN	1.75	0.08	36114.4	2295.2	0.080
2	UNKNOWN	1.82	0.11	121395.0	3061.3	0.106
3	UNKNOWN	1.88	3.11	4025014.2	89640.7	3.110
4	UNKNOWN	1.96	0.93	645010.4	26865.0	0.932
5	UNKNOWN	2.14	95.17	24297906.0	2743123.5	95.172
6	UNKNOWN	3.28	0.02	11545.7	576.7	0.020
7	UNKNOWN	4.36	0.05	44614.3	1571.2	0.055
8	UNKNOWN	5.04	0.01	6855.9	240.2	0.008
9	UNKNOWN	5.09	0.01	10678.7	426.3	0.015
10	UNKNOWN	5.43	0.04	30360.2	1018.0	0.035
11	UNKNOWN	5.36	0.02	13020.1	457.9	0.016
12	UNKNOWN	5.41	0.00	2340.4	65.7	0.002
13	UNKNOWN	6.70	0.03	20947.3	882.9	0.031
14	UNKNOWN	7.34	0.02	16245.4	522.3	0.018
15	UNKNOWN	7.43	0.02	13185.0	531.2	0.018
16	UNKNOWN	8.27	0.02	16028.9	610.9	0.021
17	UNKNOWN	10.60	0.01	9018.4	333.5	0.012
18	UNKNOWN	10.67	0.01	6519.0	255.2	0.009
19	UNKNOWN	10.76	0.01	7046.6	308.1	0.011
20	UNKNOWN	10.84	0.01	9883.4	384.6	0.013
21	UNKNOWN	11.69	0.01	7581.0	339.8	0.012
22	UNKNOWN	12.58	0.01	8971.2	380.9	0.013
23	UNKNOWN	12.65	0.01	8542.7	326.5	0.011
24	UNKNOWN	13.21	0.03	11658.2	906.7	0.031
25	UNKNOWN	14.50	0.04	23633.6	1028.1	0.036
26	UNKNOWN	14.91	0.04	20533.3	1060.2	0.037

No coumarin peak seen

Title : Std Coumarin (17.9 mg/~~50~~ 25 ml----2ml/25ml MeOH)
Run File : C:\STAR\MODULE16\AETAC898.RUN (1.432 mg)
Method File : C:\STAR\MODULE16\AETAC898.MTH
Sample ID : Manual Sample

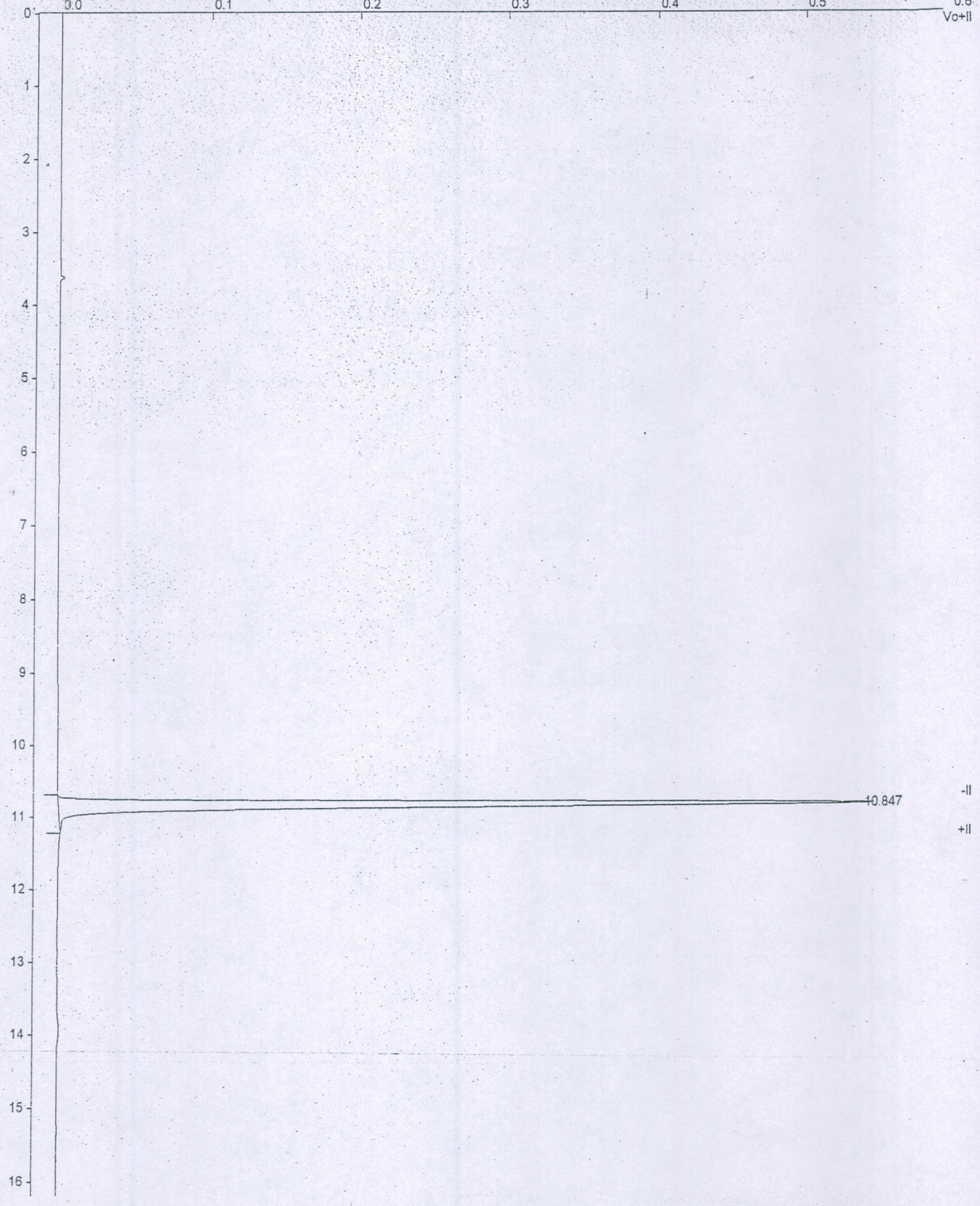
Injection Date: 5-MAY-11 5:13 PM Calculation Date: 5-MAY-11 5:31 PM

Operator : Archana
Workstation:
Instrument : HPLC - UV
Channel : A = A

Detector Type: ADCB (1 Volt)
Bus Address : 16
Sample Rate : 10.00 Hz
Run Time : 16.210 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Chart Speed = 1.22 cm/min Attenuation = 2586 Zero Offset = 2%
Start Time = 0.000 min End Time = 16.210 min Min / Tick = 1.00



Title : Std Coumarin (mg/50ml----2ml/25ml MeOH)
Run File : C:\STAR\MODULE16\AETAC898.RUN
Method File : C:\STAR\MODULE16\AETAC898.MTH
Sample ID : Manual Sample

Injection Date: 5-MAY-11 5:13 PM Calculation Date: 5-MAY-11 5:31 PM

Operator : Archana Detector Type: ADCB (1 Volt)
Workstation: Bus Address : 16
Instrument : HPLC - UV Sample Rate : 10.00 Hz
Channel : A = A Run Time : 16.210 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		100.0000	10.847	0.000	3123071	BB	5.2	
	Totals:	100.0000		0.000	3123071			

Total Unidentified Counts : 3123071 counts

Detected Peaks: 1 Rejected Peaks: 0 Identified Peaks: 0

Multiplier: 1 Divisor: 1

Baseline Offset: -125 microVolts

Noise (used): 33 microVolts - monitored before this run

Manual injection

Revision Log:

5-MAY-11 5:30 PM: Calculated results from channel A using method:
'C:\STAR\STARTUP1.MTH'
5-MAY-11 5:31 PM: Calculated results from channel A using method:
'C:\STAR\MODULE16\AETAC898.MTH'

****

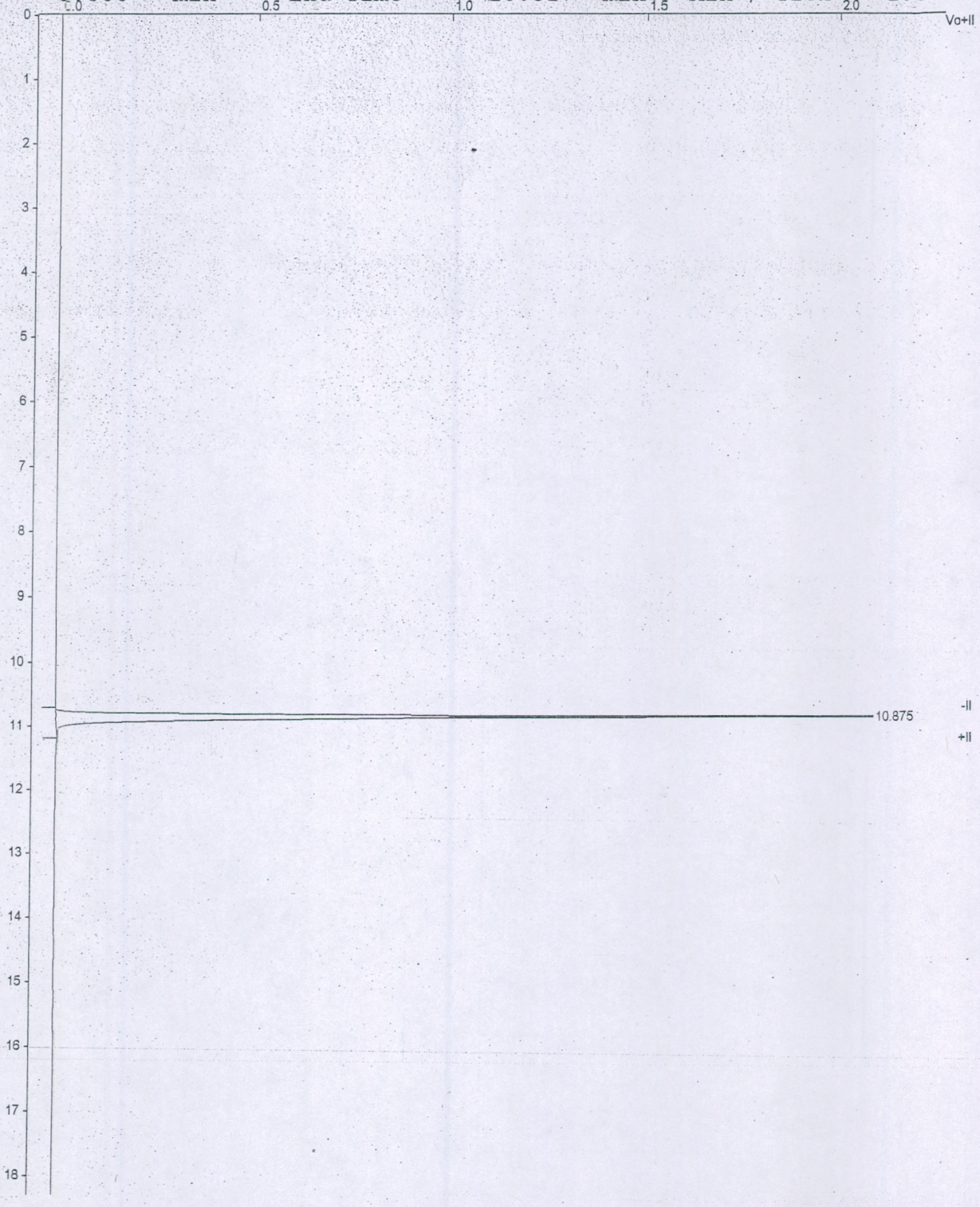
Title : Std Coumarin (17.9mg/50ml---4ml/25ml MeOH)
Run File : C:\STAR\MODULE16\AETAC899.RUN
Method File : C:\STAR\MODULE16\AETAC899.MTH
Sample ID : Manual Sample

Injection Date: 5-MAY-11 5:36 PM Calculation Date: 5-MAY-11 5:55 PM

Operator : Archana Detector Type: ADCB (1 Volt)
Workstation: Bus Address : 16
Instrument : HPLC - UV Sample Rate : 10.00 Hz
Channel : A = A Run Time : 18.317 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Chart Speed = 1.08 cm/min Attenuation = 9916 Zero Offset = 2%
Start Time = 0.000 min End Time = 18.317 min Min / Tick = 1.00



Title : Std Coumarin (mg/50ml---4ml/25ml MeOH)
Run File : C:\STAR\MODULE16\AETAC899.RUN
Method File : C:\STAR\MODULE16\AETAC899.MTH
Sample ID : Manual Sample

Injection Date: 5-MAY-11 5:36 PM Calculation Date: 5-MAY-11 5:55 PM

Operator : Archana Detector Type: ADCB (1 Volt)
Workstation: Bus Address : 16
Instrument : HPLC - UV Sample Rate : 10.00 Hz
Channel : A = A Run Time : 18.317 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		100.0000	10.875	0.000	5990869	BB	5.4	
Totals:		100.0000		0.000	5990869			

Total Unidentified Counts : 5990869 counts

Detected Peaks: 1 Rejected Peaks: 0 Identified Peaks: 0

Multiplier: 1 Divisor: 1

Baseline Offset: 4 microVolts

Noise (used): 48 microVolts - monitored befcre this run

Manual injecticn

Revision Log:

5-MAY-11 5:54 PM: Calculated results from channel A using method:
'C:\STAR\STARTUP1.MTH'
5-MAY-11 5:55 PM: Calculated results from channel A using method:
'C:\STAR\MODULE16\AETAC899.MTH'

****

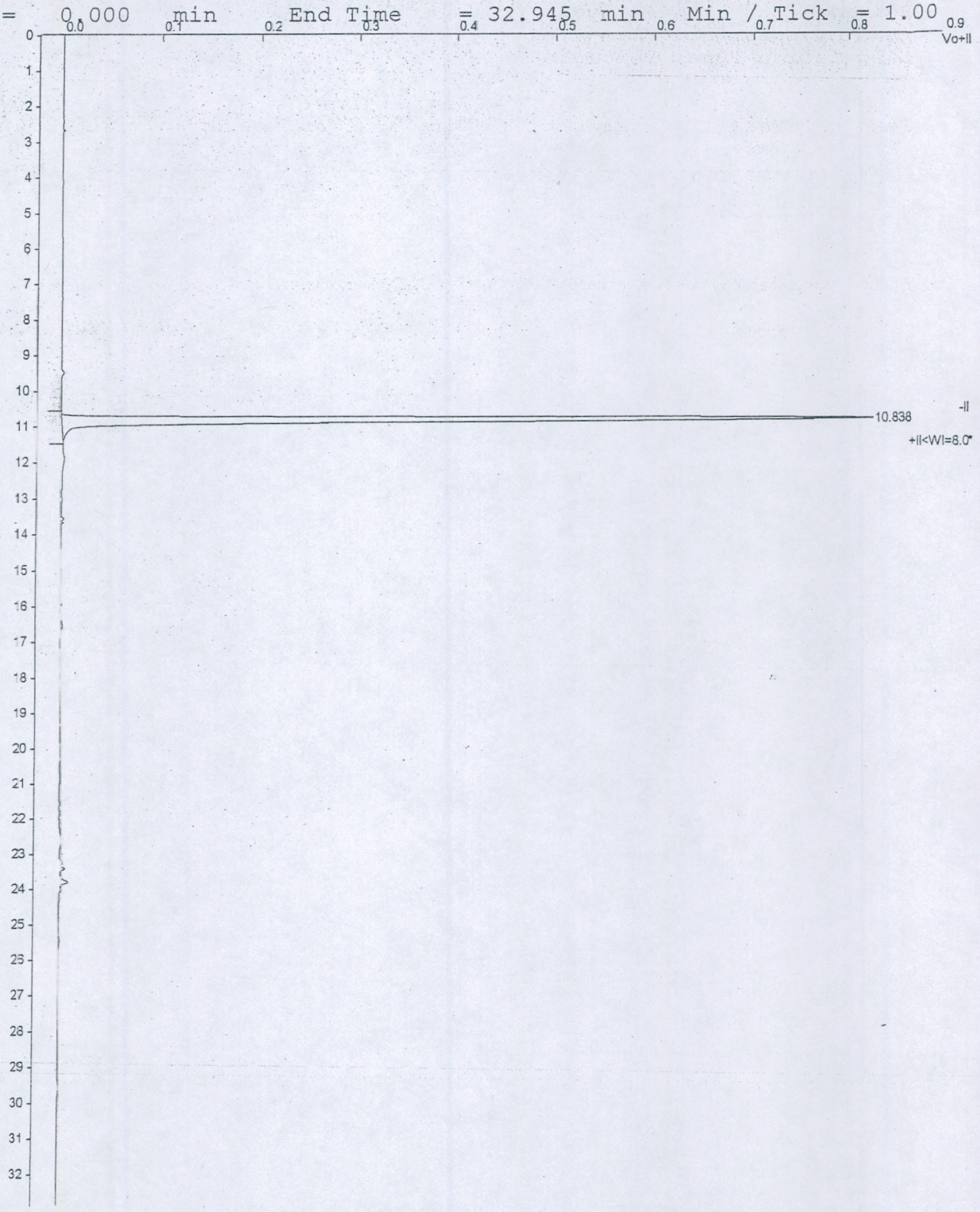
Title : Leaves (42 mg/100ml---5ml/10ml Acetonitrile)
Run File : C:\STAR\MODULE16\AETAC895.RUN
Method File : C:\STAR\MODULE16\AETAC895.MTH
Sample ID : Manual Sample

Injection Date: 5-MAY-11 3:08 PM Calculation Date: 5-MAY-11 3:44 PM

Operator : Archana Detector Type: ADCB (1 Volt)
Workstation: Bus Address : 16
Instrument : HPLC - UV Sample Rate : 10.00 Hz
Channel : A = A Run Time : 32.945 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Chart Speed = 0.60 cm/min Attenuation = 3907 Zero Offset = 2%
Start Time = 0.000 min End Time = 32.945 min Min /Tick = 1.00



Title : Leaves (mg/100ml---5ml/10ml Acetonitrile)
Run File : C:\STAR\MODULE16\AETAC895.RUN
Method File : C:\STAR\MODULE16\AETAC895.MTH
Sample ID : Manual Sample

Injection Date: 5-MAY-11 3:08 PM Calculation Date: 5-MAY-11 3:44 PM

Operator : Archana Detector Type: ADCB (1 Volt)
Workstation: Bus Address : 16
Instrument : HPLC - UV Sample Rate : 10.00 Hz
Channel : A = A Run Time : 32.945 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		100.0000	10.838	0.000	7517208	BB	8.6	
	Totals:	100.0000		0.000	7517208			

Total Unidentified Counts : 7517208 counts

Detected Peaks: 1 Rejected Peaks: 0 Identified Peaks: 0

Multiplier: 1 Divisor: 1

Baseline Offset: 1 microVolts

Noise (used): 14 microVolts - monitored before this run

Manual injection

Revision Log:

5-MAY-11 3:41 PM: Calculated results from channel A using method:
'C:\STAR\STARTUP1.MTH'
5-MAY-11 3:44 PM: Calculated results from channel A using method:
'C:\STAR\MODULE16\AETAC895.MTH'

****

0.114

$$\frac{\text{Std coumarin } 2.864\text{ mg} / 25\text{ ml}}{2.1\text{ mg} / 10\text{ ml}} \times \frac{7517208}{5990869} \times 100 = 68.12\%\ \text{coumarin}$$

Title : Flowers (70 mg/50ml)
Run File : C:\STAR\MODULE16\AETAC900.RUN
Method File : c:\star\module16\aetac900.mth
Sample ID : Manual Sample

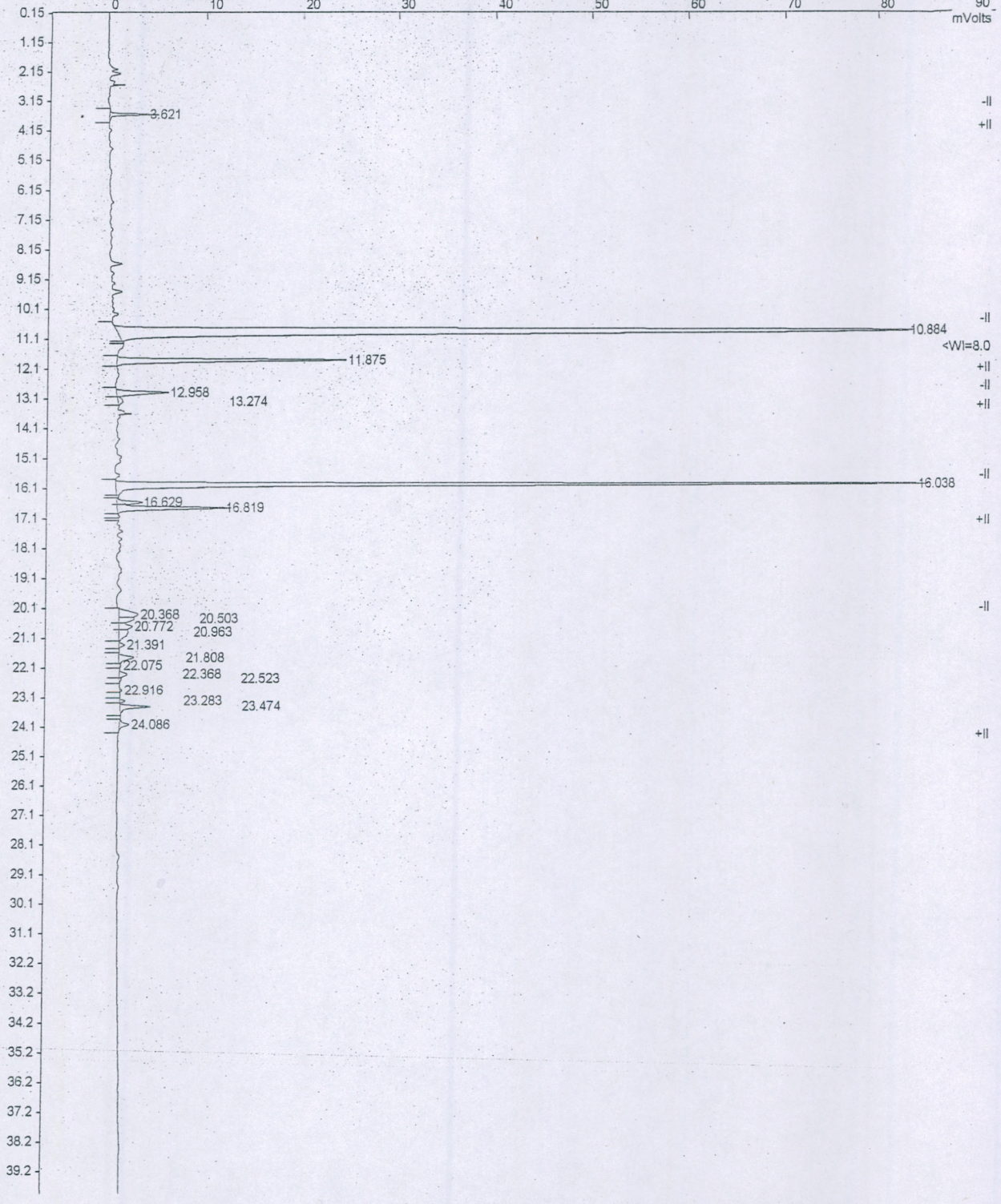
Injection Date: 5-MAY-11 6:03 PM Calculation Date: 5-MAY-11 6:48 PM

Operator : Archana
Workstation:
Instrument : HPLC - UV
Channel : A = A

Detector Type: ADCB (1 Volt)
Bus Address : 16
Sample Rate : 10.00 Hz
Run Time : 40.002 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Chart Speed = 0.49 cm/min Attenuation = 402 Zero Offset = 6%
Start Time = 0.150 min End Time = 39.950 min Min / Tick = 1.00



Title : Flowers (70 mg/50ml)
Run File : C:\STAR\MODULE16\AETAC900.RUN
Method File : c:\star\module16\aetac900.mth
Sample ID : Manual Sample

Injection Date: 5-MAY-11 6:03 PM Calculation Date: 5-MAY-11 6:48 PM

Operator : Archana
Workstation:
Instrument : HPLC - UV
Channel : A = A

Detector Type: ADCB (1 Volt)
Bus Address : 16
Sample Rate : 10.00 Hz
Run Time : 40.002 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		1.0361	3.621	0.000	19351	BB	3.5	
2		50.7787	10.884	0.000	948430	BB	11.2	
3		8.2838	11.875	0.000	154722	BB	6.3	
4		1.8710	12.958	0.000	34947	BV	6.3	
5		0.3059	13.274	0.000	5713	VB	15.8	
6		26.9128	16.038	0.000	502670	BV	5.3	
7		0.5667	16.629	0.000	10584	TS	0.0	
8		3.7858	16.819	0.000	70710	VP	5.6	
9		1.2553	20.368	0.000	23447	BV	13.6	
10		0.7362	20.503	0.000	13750	VV	21.3	
11		0.7069	20.772	0.000	13204	VV	9.7	
12		0.7288	20.963	0.000	13612	VV	24.5	
13		0.2062	21.391	0.000	3851	VV	6.4	
14		0.7015	21.808	0.000	13102	PV	7.9	
15		0.0692	22.075	0.000	1293	VV	11.7	
16		0.3690	22.368	0.000	6891	VV	8.4	
17		0.0745	22.523	0.000	1391	VV	7.5	
18		0.0726	22.916	0.000	1356	VV	7.1	
19		0.1425	23.283	0.000	2662	PV	5.1	
20		1.0181	23.474	0.000	19015	VB	5.5	
21		0.3785	24.086	0.000	7069	BB	6.0	
	Totals:	100.0001		0.000	1867770			

Total Unidentified Counts : 1867771 counts

Detected Peaks: 27 Rejected Peaks: 6 Identified Peaks: 0

Multiplier: 1 Divisor: 1

Baseline Offset: -86 microVolts

Noise (used): 17 microVolts - monitored before this run

Manual injection

Revision Log:

5-MAY-11 6:43 PM: Calculated results from channel A using method:
'C:\STAR\STARTUP1.MTH'
5-MAY-11 6:46 PM: Calculated results from channel A using method:
'C:\STAR\MODULE16\AETAC900.MTH'
5-MAY-11 6:47 PM: Calculated results from channel A using method:
'c:\star\module16\aetac900.mth'

$$\frac{1.432\ mg/25ml}{70mg/50ml} \times \frac{948430}{3123071} \times 100 = 1.24\ \%$$

Title : Bark (mg/50ml)
Run File : C:\STAR\MODULE16\AETAC901.RUN
Method File : c:\star\module16\aetac901.mth
Sample ID : Manual Sample

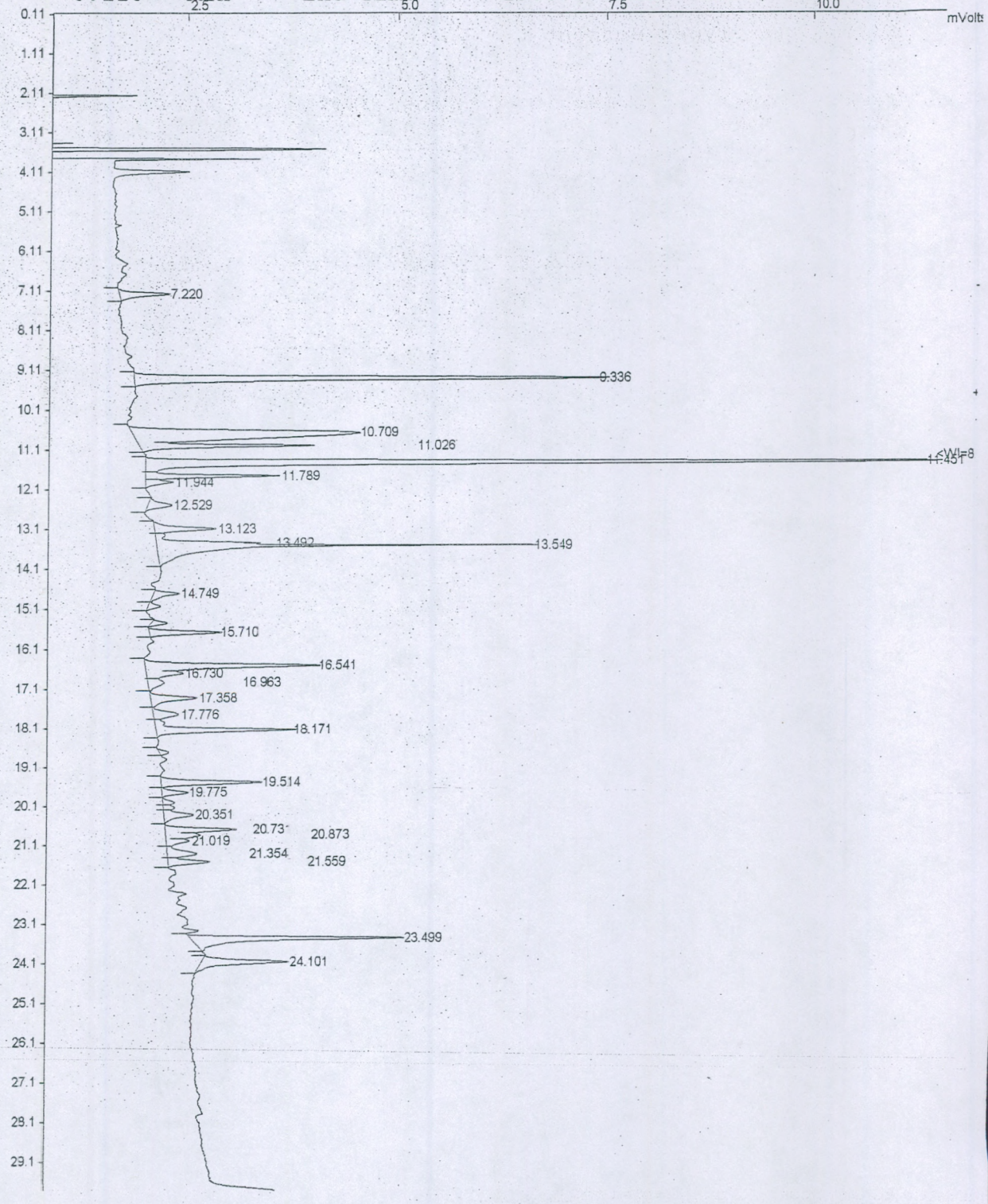
Injection Date: 5-MAY-11 7:05 PM Calculation Date: 5-MAY-11 7:36 PM

Operator : Archana
Workstation:
Instrument : HPLC - UV
Channel : A = A

Detector Type: ADCB (1 Volt)
Bus Address : 16
Sample Rate : 10.00 Hz
Run Time : 29.917 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Chart Speed = 0.66 cm/min Attenuation = 46 Zero Offset = -8%
Start Time = 0.110 min End Time = 29.870 min Min / Tick = 1.00



Title : Bark (mg/50ml)
Run File : C:\STAR\MODULE16\AETAC901.RUN
Method File : c:\star\module16\aetac901.mth
Sample ID : Manual Sample

Injection Date: 5-MAY-11 7:05 PM Calculation Date: 5-MAY-11 7:36 PM

Operator : Archana
Workstation:
Instrument : HPLC - UV
Channel : A = A

Detector Type: ADCB (1 Volt)
Bus Address : 16
Sample Rate : 10.00 Hz
Run Time : 29.917 min

********** Star Chromatography Workstation ******* Version 4.51 **********

Run Mode : Analysis
Peak Measurement: Peak Area
Calculation Type: Percent

Peak No.	Peak Name	Result ()	Ret. Time (min)	Time Offset (min)	Area (counts)	Sep. Code	Width 1/2 (sec)	Status Codes
1		1.3872	7.220	0.000	4066	BB	6.0	
2		12.8313	9.336	0.000	37612	BB	6.1	
3		12.4715	10.709	0.000	36558	BV	13.7	
4		3.6027	11.026	0.000	10561	VB	4.7	
5		19.8963	11.451	0.000	58322	BV	5.6	
6		2.7072	11.789	0.000	7936	VV	4.4	
7		0.6595	11.944	0.000	1933	VB	6.7	
8		0.8264	12.529	0.000	2423	BB	7.5	
9		1.7268	13.123	0.000	5062	BV	6.2	
10		1.8817	13.492	0.000	5516	VV	6.2	
11		7.0893	13.549	0.000	20781	VB	2.0	
12		0.7219	14.749	0.000	2116	BV	5.2	
13		1.6599	15.710	0.000	4866	PB	5.2	
14		5.2192	16.541	0.000	15299	BV	6.7	
15		1.1352	16.730	0.000	3328	VV	12.3	
16		0.7251	16.963	0.000	2126	VV	17.1	
17		1.5458	17.358	0.000	4531	VV	8.0	
18		1.0090	17.776	0.000	2958	VV	13.3	
19		3.9911	18.171	0.000	11699	VB	6.4	
20		2.6418	19.514	0.000	7744	BV	6.1	
21		0.6755	19.775	0.000	1980	VV	6.5	
22		1.0679	20.351	0.000	3130	VV	8.7	
23		1.9357	20.731	0.000	5674	VV	7.2	
24		1.0114	20.873	0.000	2965	VV	0.0	
25		0.6878	21.019	0.000	2016	VV	13.4	
26		1.1798	21.354	0.000	3458	VV	11.4	
27		1.1769	21.559	0.000	3450	VB	7.6	
28		5.7960	23.499	0.000	16990	BB	5.5	
29		2.7404	24.101	0.000	8033	BB	6.3	
	Totals:	100.0003		0.000	293133			

Total Unidentified Counts : 293131 counts

Detected Peaks: 34 Rejected Peaks: 5 Identified Peaks: 0

Multiplier: 1 Divisor: 1

Baseline Offset: -74 microVolts

Noise (used): 21 microVolts - monitored before this run

Manual injection

The bait prepared by fermenting 3%fresh leaves &fresh bark with maize/corn satisfies all these criterias.

The bait was prepared by crushing 100g of fresh corn with 3g of fresh leaves & 3g of fresh bark & molasses of 1g were fed to each animal /day.

The used animals were sent for rehabilitation to Animal Testing Facility, ITA Laboratories, Govandi, Mumbai.

SSRatnaparkhi

Dr. Seema Ratnaparkhi

Principal Investigator