Faculty of Pharmacy, Nursing and Health Professions
Master program in clinical laboratory sciences

Antimicrobial Activity of Essential Oils from thyme and rosemary, and their Potential Uses as Natural Preservatives in Pediatric Pharmaceutical Dosage Forms

By

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Supervised by

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This Thesis was submitted in partial fulfillment of the requirements for the Master's Degree in Clinical Laboratory Science from the Faculty of Graduate Studies at Birzeit University, Palestine.

I dedicate this work to my father and mother, wife and daughters.

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Declaration

I certify that the work provided in this thesis, unless otherwise referenced, is my own work, and to the best of my knowledge and belief has not been submitted elsewhere for any other degree or qualification.

I understand the nature of plagiarism, and I am aware of the University's policy on this.

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Muhannad Khalil Abu Daqa

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List of Abbreviations

CLSI	**Clinical Laboratory Standard Institute**
ATCC	**American Type Culture Collection**
RH	**Relative humidity**
HPLC	**High Performance Liquid Chromatography**
MLT	**Microbial Limit Test**
TAMC	**Total Aerobic Microbial Count**
TYMC	**Total Yeast Microbial Count**
MST	**Microbial Suitability Test**
USP	**United States Pharmacopeia**
TSA	**Trypticase Soy Agar**
SDA	**Sabouraud Dextrose Agar**
CFU	**Colony-forming unit**
TSB	**Trypticase Soy Broth**
MIC	**Minimum Inhibitory Concentrations**
MBC	**Minimum Bactericidal Concentration**
MFC	**Minimum Fungicidal Concentration**

Abstract

Antimicrobial preservatives are substances added to food and pharmaceutical products to inhibit the growth of microorganisms introduced during the manufacturing process or during usage and storage. Antimicrobial agents may contain toxic substances. Therefore, it is important to add the effective concentration of the preservatives to packaged products taking in consideration that it is below a level that may be toxic to humans.

Avoidance of pathogenic and spoilage microorganisms in pharmaceutical products, is usually achieved by using chemical preservatives. However, these chemical preservatives are toxic. The aim of the present study will assess the antimicrobial activities of thymol ($C_{10}H_{14}O$) is the main monoterpene phenol found in thyme essential oil, rosmarinic acid ($C_{18}H_{16}O_8$) which is one of the most abundant individual active antioxidant components in rosemary, *Coridothymus capitatus* L. and *Rosmarinus officinalis* L. essential oils, and to compare their effectiveness against reference ATCC microorganisms.

The aim of the present study will assess the antimicrobial activities of thyme oil, rosemary oil, thymol, and rosmarinic acid, and to study their preservative effectiveness test against reference ATCC microorganisms, to compare their effectiveness with well-known chemical preservative and to evaluate their use as antimicrobial agents (preservatives) for different pharmaceutical preparations especially for pediatric preparations.

ملخص الدراسة

المواد الحافظة المضادة للميكروبات هي مواد تضاف إلى المنتجات الغذائية والصيدلانية لمنع نمو الكائنات الحية الدقيقة التي يتم إدخالها أثناء عملية التصنيع أو أثناء الاستخدام والتخزين. قد تحتوي المواد المضادة للميكروبات على مواد سامة. لذا، فمن المهم إضافة التركيز المناسب من المواد الحافظة إلى المنتجات مع الأخذ في الاعتبار أن تركيزها أقل من المستوى الذي قد يكون ضارا للإنسان. يتم تجنب الكائنات الدقيقة المسببة للأمراض وتلف المنتجات الصيدلانية باستخدام المواد الحافظة الكيميائية. مع العلم بان هذه المواد الحافظة الكيميائية ضارة.

تهدف هذه الدراسة إلى تقييم قدرة الثيمول (C10H14O) كمضاد للميكروبات بواسطة وجود مادة فينول المونوتربين الرئيسية ، وحمض روزمارينيك (C18H16O8) الذي هو أحد أكثر مكونات مضادات الأكسدة الفردية النشطة الموجودة في إكليل الجبل، Coridothymus capitatus L والزيوت العطرية .Rosmarinus officinalis L، ولمقارنة فعاليتها على الكائنات الدقيقة المرجعية لـ ATCC .

تهدف هذه الدراسة إلى تقييم فعالية مضادة الميكروبات لزيت الزعتر وزيت إكليل الجبل والثيمول وحمض روزمارينيك، ودراسة اختبار فعاليتها كمواد حافظة ضد الكائنات الدقيقة المرجعية في ATCC، ومقارنة فعاليتها مع المواد الحافظة الكيميائية المعروفة وتقييمها، والتي تستخدم كمواد مضادة للميكروبات (مواد حافظة) في المستحضرات الصيدلانية المختلفة وخاصة مستحضرات الأطفال.

I. Introduction

Preservatives are substances added to pharmaceutical products to inhibit the growth of microorganisms introduced during the manufacturing process or during usage and storage [British Pharmacopoeia, 2007]. Antimicrobial agents may contain toxic substances. Therefore, it is important to add the effective concentration of the preservatives to packaged products taking in consideration that it is below a level that may be toxic to humans [Breitkreutz and Boos, 2007].

Adding antimicrobial preservatives to sterile and non-sterile pharmaceutical products packaged in multiple dose containers can inhibit the growth of microorganisms that could be accidentally introduced during repeated withdrawal of individual doses. These preservatives in finished dosage forms meet the requirements for added substances under limitations [Sutton and Porter, 2002].

Preservatives commonly found in most oral pharmaceutical products [Mari et al., 2003] (such as tablets, capsules, suspensions and syrups), dental products [Obagwu and Korsten, 2003] (such as toothpaste, mouthwash and gargles), dermal products [Thangavelu et al., 2004] (mostly cosmetic personal care products, such as cream, lotion, ointment, soap, bath gel, hair spray, shampoo and conditioner), nasal products [Suppakul el al., 2003] (such as nasal drops, sprays and aerosols), parenteral products [Nakatani et al., 1994] including vaccines, rectal products (such as suppositories and enema) and ophthalmic products [Dold and Knapp, 1980; Ueda et al., 1982] (such as eye drops, ointments and contact lens solutions) are listed in Table 1.

Pharmaceutical Product	Preservatives
Oral	Methyl, ethyl, propyl parabens and their combinations, sodium benzoate, benzoic acid, calcium lactate, sorbates of calcium, sodium and potassium, sorbic acid
Dermal	Benzalkonium chloride, cetrimide, EDTA, benzoic acid, thiomersal, imidurea, chlorhexidine, chlorocresol, phenyl salicylate
Dental	Sodium benzoate, benzoic acid, potassium sorbate, sodium phosphate, triclosan, cetylpyridinium chloride, methyl and ethyl parabens
Ophthalmic	Benzalkonium chloride, EDTA, benzoic acid, thiomersal, imidurea, chlorhexidine, polyamino propylbiguanide, sodium perborate, boric acid
Nasal	Benzalkonium chloride, phenylcarbinol, potassium sorbate, chlorobutanol, chlorocresol, EDTA
Rectal	Benzyl alcohol, benzoic acid, sodium benzoate, methyl hydroxybenzoate, chlorhexidine gluconate
Parenteral	Methyl, ethyl, propyl, butyl parabens and their combinations, benzyl alcohol, chlorbutanol chlorhexidine, thiomersal, formaldehyde

Table 1: Preservatives used in various pharmaceutical formulations.

Artificial preservatives have negative and potentially life threatening side effects. Examples of artificial preservatives are nitrites, methyl, propyl parabens, see Table 1. Nitrates, upon ingestion, are converted to nitrites that can react with hemoglobin to produce methemoglobin, a substance that can cause loss of consciousness and death, especially in infants. Proteins in the stomach react with nitrites and produce nitrosamines, substances that are carcinogenic. Researchers claim that there is a substantial link between increased levels of nitrates in food and increased deaths from Alzheimer's, Parkinson's and Type 2 diabetes. Headache, sweating, redness of skin, nausea and weakness can occur following consumption of food containing monosodium glutamate (MSG). Sulfite containing food preservatives may cause severe allergic reactions and exacerbation of asthma. The toxic paraben chemicals are often used along with methylchloroisothiazolinone and methylisothiazolinone. These are reported to possibly cause neurological damage in rats and are potent irritants and allergens. The use of these toxic chemicals by pregnant women may adversely affect fetal brain development. Formaldehyde DMDM hydantoin, diazolidinyl urea and imidazolidinyl urea are all potent skin, eye and lung irritants. High levels of exposure to toxins like these can cause DNA damage to sperm. Research has shown that the food additives used in hundreds of children's foods and drinks can cause temper tantrums and disruptive behavior.

Major problems with excipients in pediatric drugs, especially in infants and neonates, have been reported [Breitkreutz and Boos, 2007; Shehab, 2009], e.g. for benzyl alcohol, coloring agents, propylene glycol, ethanol and propyl paraben. Listed in Table 2 are some commonly used preservatives along with health hazards namely hypersensitivity, asthma and cancer, which they can cause.

Preservative	Hypersensitivity (H)	Asthma (A)	Cancer (C)
Potassium & Calcium Sorbates, Sorbic Acid	H	A	-
Benzoic Acid	H	A	-
Sodium Benzoate	H	A	C
Propylparaben	-	A	-
Sulphur Dioxide	H	A	-
Sodium Metabisulphite	-	A	-
Potassium Bisulfite	H	A	-
Hexamethylenetetramine	-	-	C
Sodium Nitrite	H	A	C
Sodium or Potassium Nitrate	H	-	C
Calcium or Potassium or Sodium Propionates, Propionic Acid	H	A	-
Propyl Gallate	-	A	C
tert-butylhydroquinone (TBHQ)	H	A	-
Butylated Hydroxyanisole (BHA)	H	A	C
Butylated Hydroxytoluene (BHT)	H	A	C

Table 2: Health hazards of some commonly used preservatives.

Special considerations must be taken with pediatric drug products in excipient selection to meet regulatory requirements. Major problems with excipients in pediatric drugs, especially in infants and neonates, have been reported [Breitkreutz and Boos, 2007; Shehab, 2009], e.g. for benzyl alcohol, coloring agents, propylene glycol, ethanol and propyl paraben.

The number of excipients and their quantity in the pediatric drug formulation should be the minimum required to ensure an appropriate product stability, microbial control, dose uniformity and other considerations to support product quality, efficacy and safety. Potential alternatives to excipients posing a significant risk to a child should always be considered [Inactive Ingredients in Pharmaceutical Products, 1985].

Recently, natural antimicrobial agents have been extensively tested and used instead of chemical agents in different applications e.g. food, and pharmaceutical industries. Chemical preservatives are notorious for their carcinogenic attributes and residual toxicity as compared to the safer natural antimicrobial agents [Shehab, 2007]. Chemical preservatives are dangerous to use in pediatric formulations for their toxicity and many side effects on children. Therefore, recent research has been focused on natural antimicrobial agents to investigate their preservation abilities and identify the specific effective substance to expand the spectrum of antimicrobial activity over that of the regulatory approved substances.

It is obvious to observe the worldwide concern to search for novel antimicrobial compounds from natural sources. Naturally, derived compounds and other natural products may have applications in controlling bacteria in multiple dose topical and oral dosage forms and for other dosage forms [Sutton and Porter, 2002].

One group of naturally derived antimicrobial compounds is medicinal plants and their essential oils. These compounds are safe, have a certain degree of antimicrobial activity, and could inhibit the growth of pathogens and spoilage microorganisms thereby improving the shelf life of pharmaceutical products. Numerous studies have been reported that medicinal plants produce a large number of secondary metabolites, which have antimicrobial effects on pathogens [Mari et al., 2003; Obagwu and Korsten, 2003]. The ability of medicinal plant extracts to control the growth of pathogens and spoilage bacteria and their use as alternatives to

conventional natural preservatives is emerging because they are generally safe to humans, and friendly to the environment [Thangavelu et al., 2004].

Herbs contain various complexes of organic chemicals that may have different health related effects depending on several factors related to plant growth, effects of crude extracts, and processing of pure active ingredients in the herbal product [Suppakul et al., 2003].

In Palestine, there are numerous medicinal plants described for treatment of many diseases and possess immunomodulatory and antioxidant properties, leading to antibacterial activities, Table 3. Herbal medicine is considered an integral part of the Palestinian culture and plays a pivotal and indispensable role in the current public healthcare. The hills and mountains of Palestine are covered with more than 2600 plant species of which more than 700 are noted for their uses as medicinal herbs or as botanical pesticides [Jaradat, 2005; Alali et at., 2007].

The effectiveness of natural plant extracts need to be evaluated to specify their antimicrobial activity and potential side effects in package pharmaceutical products. The objectives of this study are therefore to investigate antimicrobial activities of essential oils from *Coridothymus capitatus* (thyme) and *Rosemainus offienalis* (rosemary). In addition to thyme and rosemary oils, antimicrobial activities of two active ingredients from thyme and rosemary (Thymol, Rosmarinic acid) will be also studied. Specifically, the preservative effectiveness test of the oils on the following standard reference organisms will be tested: *Candida albicans* (ATCC No. 10231), *Aspergillus niger* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538).

Scientific name	Arabic name	Medicinal use	Parts Used*
Trigonella berythea	Hilbeh	Diabetes, sexual weakness, stomach and intestinal pain	SD
Rosmarinus officinalis L.	Hassalban	Kidney diseases, liver diseases, arteriosclerosis and anemia	ST,FL, LE, SD
Olea europaea L.	Zaitoun	Coughing, diabetes, high blood pressure and stones in kidney and muscle contractions	LE, FR
Teucrium capitatum L.	Jedeh Subian	Kidney, liver diseases, diabetes, stomach, intestine pain and inflammation	LE, AP
Nigella ciliaris	Qezha	Jaundice, blood pressure, heart diseases, sexual weakness and skin diseases	SD
Lupinus albus L.	Turmos	Diabetes and kidney stones	SD
Salvia fruticosa	Mariamieh	Stomach ache, intestinal gas, inflammation, diabetes and sexual weakness	LE, ST
Crataegus aronia L.	Za'roor	Cardiovascular diseases, sexual weakness, cancer and diabetes	LE, FL, ST
Allium sativum L.	Thoum	Poisoning, asthma, blood circulation and muscle relaxation	BL, AP
Matricaria aurea L.	Babounej	Fever, coughing and heart diseases	ST, FL, LE
Allium cepa L.	Basal	Diabetes, loss of appetite, liver diseases, prostate cancer and Coughing	RT, LE, BL
Anisum vulgare L.	Yansoon	Stomach,intestine pain, headache and fertility	SD, FL, LE
Arum palaestinum	Lufe	Internal bacterial infection, cancer, poisoning and circulatory system	LE, SD
Majorana syriaca L.	Za'tar Barri	Intestinal pain, inflammation and high blood pressure	LE
Origanum majorana L.	Mardaqoush	Menstruation regulator, migraine and nerve system	LE, SE
Triticum aestivum L.	Qamh	Cancer	SD
Brassica oleracea L.	Malfof	Respiratory system, asthma, cancer, joint inflammation and bacterial infection	LE, RT
Coridothymus capitatus L.	Za'tar Farsi	Heart diseases, paralysis, diabetes, tract pain, inflammation and respiratory system	LE, FL
Laurus nobilis L.	Ghar	Skin diseases and cancer	LE
Foeniculum vulgare	Shomar	Digestive system, obesity and headache	SD, RT
Amygdalus korschinskii	Louz Barri	Local paralysis and hair loss	FR, SD
Portulaca oleracea L.	Baqleh	Kidney stones	LE
Coriandrum sativum L.	Kozbareh	Intestinal inflammation, weight loss and intestinal gas	LE, SD
Petroselinum sativum	Baqdoones	Urinary system and stones in kidney, period regulator and immune system	LE
Amygdalus communis L.	Louz Hilo	Local paralysis and hair loss	FR
Citrullus colocynthis L.	Hanthal	Liver diseases and diabetes	SD
Ficus carica L.	Teen	Warts	LE
Ziziphus spina-christi L.	Doom Seder	Cholesterol reduction, cancer, eye inflammation and hair loss	LE

Table 3: Local medicinal plant species used within the Palestinian population in traditional Arabic medicine.

* AP: aerial parts, BA: bark, LE: leaves, FL: flowers, FR: fruits, SD: seeds, ST: stem, RT: roots.

The preservative effectiveness test then will be compared with routinely used chemical preservatives like methyl and propyl paraben.

II. Objectives

The objectives of this study are:

II.1 To determine the antimicrobial activities of essential oils from *Coridothymus capitatus* (thyme) and *Rosemainus offienalis* (rosemary) on bacterial and fungal strains and compare that with routinely used preservatives.

II.2 To perform the Antimicrobial Effectiveness Test or efficacy test of the oils on standard reference organisms. The following organisms are included:

- *Candida albicans* (ATCC No. 10231)
- *Aspergillus niger* (ATCC No. 16404)
- *Escherichia coli* (ATCC No. 8739)
- *Pseudomonas aeruginosa* (ATCC No. 9027)
- *Staphylococcus aureus* (ATCC No. 6538).

II.3 Incorporate the oils into pediatric pharmaceutical formulation such as paracetamol syrup and subsequently evaluate the antimicrobial effectiveness of these essential oils and determine the stability of the finished product and the shelf life.

III. Literature Review

III.1 Medicinal Plants (Herbs)

The word “herbs” has a meaning *herba* in Latin where it means a medicinal plant. An herb is a plant or plant part used for its smell, flavor or therapeutic properties. Herbs contain various complexes of organic chemicals that may have different health related effects depending on several factors related to plant growth, effects of crude extracts, and processing of pure active ingredients in the herbal product [Suppakul et al., 2003].

Natural substances of herbs are usually obtained from seeds, berries, buds, leaves, bark and roots of plants growing mainly in the tropical, the subtropical and the temperate zones. Essential oils of herbs have been used extensively for many years in food products, perfumery, dental and oral products due to their therapeutic medicinal properties [Suppakul et al., 2003].

At the end of the last century, antimicrobial activities of medicinal plants and spices had already been examined. Ground mustard, clove and cinnamon and their essential oils were known to retard microbial spoilage in food [Nakatani, 1994].

Dold and Knapp [Dold and Knapp, 1980] examined the antimicrobial activity of 27 plant extracts against 8 bacterial isolates such as *Escherichia coli*, *Salmonella typhosa*, *Shigella paradysenteria* and others. The outcome of this work have showed garlic to be active against all organisms tested. Onion, nutmeg and clove inhibited the growth of all tested organisms, except *Bacillus subtilis*. From the viewpoint of plant taxonomy, the Liliaceae family showed the highest activity, followed by the Myrtaceae, Cruciferae and Labiatae family.

Ueda et al. [Ueda et al., 1982], have tested the ethanolic extracts of medicinal plants and spices for their role in the inhibition of bacteria and fungi in culture media at different pH levels. Clove exhibited remarkable antibacterial activity against all organisms tested

including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella Typhimurium*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris* and *Proteus morganii*. Oregano and cinnamon extracts showed a wide range of inhibitory effects against Gram-positive bacteria with less potent effects against Gram-negative bacteria. Higher activity was exhibited at a lower pH level.

Mustard oil manifested excellent activity against both Gram positive and negative bacteria while cinnamon, marjoram, oregano and thyme have showed good activity as reported by Galli et al. [Galli et al., 1985].

Shelef et al. [Shelef et al., 1984], studied the sensitivity of common food borne bacteria (24 Gram positive and 22 Gram negative bacteria) to sage, rosemary and allspice. Gram-positive bacteria were more sensitive than Gram-negative bacteria to these herbs. Sage or rosemary have induced bacteriostatic effects at a concentration of 0.3%, and bactericidal effects at 0.5%. However, combination of sage and rosemary extracts displayed enhanced antimicrobial activity.

According to Aureli et al. [Aureli et al., 1992], essential oils of pimento, clove, origano and thyme showed effective inhibition the growth of *Listeria monocytogenes* proportional to the concentrations used. This pathogen was completely killed within 2-4 hours by the essential oils at concentration of 5µl/ml in a saline solution system.

Garlic has also antimicrobial activity against several pathogenic bacteria. Arora and Kaur [Arora and Kaur, 1999] reported that garlic has bactericidal effect against *Staphylococcus aureus* and *Salmonella typhi* with a 1-2 Log_{10} decrease in 2 hours. However, the inhibitory effects of garlic have diminished after 3 hours, which may indicate according to the author that resistant bacteria continued growth.

III.2 *Coridothymus capitatus* L.

III.2.1 General information

Coridothymus capitatus L., shown in Figure 1 is a well-known plant with many names, *Satureja capitata* (L)., *Thymbra capitata* (L.), *Thymus capitatus* (L.), *Thymus cephalatos* (L.) and Zaâter [A Guide to Medicinal Plants in North Africa, 2005].

"This is a sweet smelling bush with erect spreading branches, between 20 and 40 cm high. The leaves are simple, small, linear and glandulous spotted; in spring those on the flower bearing branches are very caducous; in the dry season those on non-flowering branches are tightly packed and seem to overlap at the tips. The floral leaves are lengthily ciliated. The inflorescence is a compact, ovoid, terminal capitulum. The pink corolla is twice the calyx, which is compressed and laterally utricular. Flowering occurs from April to July" [A Guide to Medicinal Plants in North Africa, 2005].

Coridothymus capitatus is very widespread in the Mediterranean, except France. The plant is greatly sought after, particularly for its aromatic quality. It is frequently picked, often without supervision. Sometimes it is grown in gardens. The flowering branches and the leaves are sold fresh or dried by herbalists and by people who sell medicinal and sweet smelling plants in the souks of the region [A Guide to Medicinal Plants in North Africa, 2005].

III.2.2 The uses of *Coridothymus capitatus*

The Parts use of *Coridothymus capitatus* are branches, leaves and inflorescences. The essential oil of this thyme is rich in carvacrol, thymol and contains a small quantity of tannin. The pharmacological action of essential oil of this thyme is an antiseptic for the respiratory. *Coridothymus capitatus* use in herbal medicine as genitourinary tracts, bactericide and fungicide. The pharmaceutical industry mainly uses its essential oil [A Guide to Medicinal Plants in North Africa, 2005].

III.3 *Rosemainus offienalis* L.

III.3.1 General information

Rosemainus offienalis (Fig.2) is a well-known plant with many names, *Rosmarinus laxiflorus, Rosmarinus lavandulaceus,* Klil, Romarin officinal and Rosemary [Abu Shanab et al., 2004].

"Rosemary is a perennial plant forming a stiff shrub, much branched and densely bushy, with a characteristic aromatic smell. The leaves are simple, tough, linear with revolute margins, greenish and crinkled on top and tomentose underneath, 2-4 mm wide. The flowers are grouped in little axillary and terminal clusters with bracts. The calyx is bell shaped and bi-labiate and has a pale to bright blue corolla, the upper lip is entire and lower lip tri-lobate. Two prominent stamens with a simple filament bearing a fertile, a long very exert style. The nutlets are smooth" [Abu Shanab et al., 2004].

The geographical distribution of *Rosemainus offienalis* in the northern Mediterranean. It extends from Portugal to Turkey, in the southern Mediterranean, it extends from eastern Morocco to Cyrenaica. It is also present in the Near East [Abu Shanab et al., 2004].

III.3.2 The uses of *Rosemainus offienalis*

The leaves and some parts of the flower contain volatile oil are used in aromatherapy. Rosemary oil was also found to possess antioxidant properties, a few research studies also indicate that it has antibacterial and antifungal qualities. Its essential oil made up especially of cineol, camphor and a pinene, tricyclic phenolic diterpenes including carnosolic acid and carnosol, tannins, methylated flavons, triterpenes, steroids, lipids, especially in the young shoots, polysaccharides and traces of salicylate. The pharmacological action of Rosemary has many diverse actions like antibacterial and antiseptic, limited antiparasitic, antioxidant action of rosmarinic acid and anti-inflammatory. The essential oil of rosemary use in herbal medicine is part of many antiseptic and antibacterial medicines for the respiratory passages [Abu Shanab et al., 2004].

Figure 1: *Coridothymus capitatus* L.

Figure 2: *Rosemainus offienalis* L.

IV. Hypothesis and specific Aims

IV.1 Hypothesis

We hypothesize that natural preservation using essential oils can be used as natural preservatives in pharmaceutical preparations.

IV.2 Specific Aims

IV.2.1 To test the antimicrobial effects of oils from the following natural preservatives; Thymol, Rosmarinic acid, Rosemary and thyme essential oils by testing these substances on reference ATCC microorganisms including: *E. coli, Staphylococcus aureus, Bacillus subtilis, Micrococcus luteus, Pseudomonas aeruginosa, and Saccharomyces cerevisiae*

IV.2.2 To test the preservative efficacy in pharmaceutical dosage by the microbial limit test (MLT) and preservative effectiveness test

IV.2.3 To determine the shelf life of the above mentioned natural preservatives and their oils. This will include the following:

a. pH of the final product

b. Viscosity

c. Density

d. Paracetamol active using the HPLC

IV.3 Novelty Aspects

This study will look in depth at the development of natural preservative system in multi-dose formulation pharmaceutical products using natural oils to replace it against the chemical preservatives that may be considered toxic substances. The pediatric pharmaceutical product such as paracetamol (Acetaminophen) syrup were chosen because this medication is widely used in the form of the syrup, cover a wide age range from newborn to adolescents and it is one of the most commonly used nonprescription pharmaceutical drugs (OTC).

V. Materials and Methods

Various formulations were stored for 6 months of accelerated storage conditions (at 40 ±2 °C and 75 ±5% relative humidity (RH)). Another set of various formulations will be stored for one year at room temperature.

Samples of the paracetamol syrup formulations were monitored weekly regarding organoleptic features (potential changes in color, smell, transparency, crystallization, etc.), whilst in monthly intervals (at Preparation, one month, 2 months, 3 months, 4 months and 5 months) pH and the content of the paracetamol and the essential oil were analyzed, with the spectrophotometric method (HPLC). Preservative effectiveness testing also known as preservative challenge testing based on the USP test method will be also conducted for these samples. The above mentioned information has been obtained from Guidance for Industry [ANDAS, 2013].

V.1 Formulation development

In this part of the study, various formulations of paracetamol syrup of 0.25% (12.5 mg/5mL) were prepared with different concentrations of the essential oil and / or a combination of two essential oils were used as a preservation system of the syrup.

The Tables specifying the different formulas preparation are shown in the Tables below (Tables 4 to 11).

Item #	Item	Function	Theoretical Quantity	
			(mg/ml)	(mg/5ml)
1	Paracetamol	Active ingredient	24	120
2	PVP 25	Solubilizing agent	150	750
3	Glycerin	solvent; sweetening agent	80	400
4	Sorbitol (70%)	sweetening agent	100	500
5	Sodium Cyclamate	sweetening agent	5	25
6	Sodium Saccharine	sweetening agent	3	15
7	Red Color 40	Coloring agent	0.03	0.15
8	Methylparaben	Preservative	1	5
9	Sodium Disulfate	Antioxidant	0.001	0.25
10	Disodium Edetate	chelating agent	0.2	1
11	Magnasweet “Ammonium Glycyrrhizinate”	After taste agent	2	10
12	Acesulfame Potassium	sweetening agent	0.2	1
13	Cherry	Flavoring agent	9	45
14	R.O Water "up to"	Solvent	1ml	5ml
15	Sodium Hydroxide*			

Table 4: Positive control formula.

* sodium hydroxide was used to adjust the pH of the syrup as needed.

Item #	Item	Theoretical Quantity	
		(mg/ml)	(mg/5ml)
1	Paracetamol	24	120
2	PVP 25	150	750
3	Glycerin	80	400
4	Sorbitol (70%)	100	500
5	Sodium Cyclamate	5	25
6	Sodium Saccharine	3	15
7	Red Color 40	0.03	0.15
8	Disodium Edetate	0.2	1
9	Magnasweet “Ammonium Glycyrrhizinate”	2	10
10	Acesulfame Potassium	0.2	1
11	Cherry	9	45
12	R.O Water "up to"	1ml	5ml
13	Sodium Hydroxide*		

Table 5: Negative control formula.

* sodium hydroxide was used to adjust the pH of the syrup as needed.

Item #	Item	Theoretical Quantity	
		(mg/ml)	(mg/5ml)
1	Paracetamol	24	120
2	PVP 25	150	750
3	Glycerin	80	400
4	Sorbitol (70%)	100	500
5	Sodium Cyclamate	5	25
6	Sodium Saccharine	3	15
7	Red Color 40	0.03	0.15
8	Thymol	2.5	12.5
10	Disodium Edetate	0.2	1
11	Magnasweet “Ammonium Glycyrrhizinate”	2	10
12	Acesulfame Potassium	0.2	1
13	Cherry	9	45
14	R.O Water "up to"	1ml	5ml
15	Sodium Hydroxide*		

Table 6: Formula with thymol as natural preservative.

* sodium hydroxide was used to adjust the pH of the syrup as needed.

Item #	Item	Theoretical Quantity	
		(mg/ml)	(mg/5ml)
1	Paracetamol	24	120
2	PVP 25	150	750
3	Glycerin	80	400
4	Sorbitol (70%)	100	500
5	Sodium Cyclamate	5	25
6	Sodium Saccharine	3	15
7	Red Color 40	0.03	0.15
8	Rosmarinic Acid	2.5	12.5
10	Disodium Edetate	0.2	1
11	Magnasweet “Ammonium Glycyrrhizinate”	2	10
12	Acesulfame Potassium	0.2	1
13	Cherry	9	45
14	R.O Water "up to"	1ml	5ml
15	Sodium Hydroxide*		

Table 7: Formula with rosmarinic Acid as natural preservative.

* sodium hydroxide was used to adjust the pH of the syrup as needed.

Item #	Item	Theoretical Quantity	
		(mg/ml)	(mg/5ml)
1	Paracetamol	24	120
2	PVP 25	150	750
3	Glycerin	80	400
4	Sorbitol (70%)	100	500
5	Sodium Cyclamate	5	25
6	Sodium Saccharine	3	15
7	Red Color 40	0.03	0.15
8	Thymol	2.5	12.5
9	Rosmarinic Acid	2.5	12.5
10	Disodium Edetate	0.2	1
11	Magnasweet “Ammonium Glycyrrhizinate”	2	10
12	Acesulfame Potassium	0.2	1
13	Cherry	9	45
14	R.O Water "up to"	1ml	5ml
15	Sodium Hydroxide*		

Table 8: Formula with thymol & rosmarinic Acid as natural preservative.

* sodium hydroxide was used to adjust the pH of the syrup as needed.

Item #	Item	Theoretical Quantity	
		(mg/ml)	(mg/5ml)
1	Paracetamol	24	120
2	PVP 25	150	750
3	Glycerin	80	400
4	Sorbitol (70%)	100	500
5	Sodium Cyclamate	5	25
6	Sodium Saccharine	3	15
7	Red Color 40	0.03	0.15
8	Thyme Oil	2.5	12.5
10	Disodium Edetate	0.2	1
11	Magnasweet “Ammonium Glycyrrhizinate”	2	10
12	Acesulfame Potassium	0.2	1
13	Cherry	9	45
14	R.O Water "up to"	1ml	5ml
15	Sodium Hydroxide*		

Table 9: Formula with thyme oil as natural preservative.

* sodium hydroxide was used to adjust the pH of the syrup as needed.

Item #	Item	Theoretical Quantity	
		(mg/ml)	(mg/5ml)
1	Paracetamol	24	120
2	PVP 25	150	750
3	Glycerin	80	400
4	Sorbitol (70%)	100	500
5	Sodium Cyclamate	5	25
6	Sodium Saccharine	3	15
7	Red Color 40	0.03	0.15
8	Rosemary Oil	2.5	12.5
10	Disodium Edetate	0.2	1
11	Magnasweet “Ammonium Glycyrrhizinate”	2	10
12	Acesulfame Potassium	0.2	1
13	Cherry	9	45
14	R.O Water "up to"	1ml	5ml
15	Sodium Hydroxide*		

Table 10: Formula with rosemary oil as natural preservative.

* sodium hydroxide was used to adjust the pH of the syrup as needed.

Item #	Item	Theoretical Quantity	
		(mg/ml)	(mg/5ml)
1	Paracetamol	24	120
2	PVP 25	150	750
3	Glycerin	80	400
4	Sorbitol (70%)	100	500
5	Sodium Cyclamate	5	25
6	Sodium Saccharine	3	15
7	Red Color 40	0.03	0.15
8	Thyme Oil	2.5	12.5
9	Rosemary Oil	2.5	12.5
10	Disodium Edetate	0.2	1
11	Magnasweet “Ammonium Glycyrrhizinate”	2	10
12	Acesulfame Potassium	0.2	1
13	Cherry	9	45
14	R.O Water "up to"	1ml	5ml
15	Sodium Hydroxide*		

Table 11: Formula with thyme oil & rosemary oil as natural preservative.

* sodium hydroxide was used to adjust the pH of the syrup as needed.

V.2 Research Methodology

V.2.1 Microbial Limit Test

V.2.1.1 General overview

Microbial limit or enumeration test (MLT) is a quantitative procedure which determines the Total Aerobic Microbial (TAMC) and Total Yeast and Mold Counts (TYMC) present in the test product. A non-sterile pharmaceutical products are not completely free of microorganisms. MLT test was carried out to ensure that these organisms are present in low or high numbers. MLT was performed to check degree of safety of pharmaceutical products before release to the market.

V.2.1.2 Agar Media for Microbial Growth

- Soybean-Casein Digest Agar- has been used as a suitable growth medium for the determination of the Total Aerobic Microbial Count (TAMC).
- Sabouraud Dextrose Agar - has been used as a suitable growth medium for the determination of the Total Yeast Microbial Count (TYMC).

V.2.1.3 Methods for Microbial Enumeration

- Pour-Plate Method.
- Surface-Spread Method.
- Membrane Filtration Test Method. This technique is usually used for microbial enumeration of the products, which can be filtered through a membrane.

V.2.1.4 Interpretation of Microbial Enumeration Test results

TAMC - the total number of microorganisms was determined. Yeasts and molds must be included in the counts.

TYMC - the total number of yeasts and molds was determined. Visible bacterial colonies were included in this count.

V.2.1.5 Microbial Suitability Test (MST)

MST has been conducted following the guidelines of United States Pharmacopeia [USP 35-NF30, 2012]. The essence of this test as described below is to inoculate the final product with ≤ 100 cfu of several specific test organisms including Gram positive, Gram negative, yeast and mold.

MST is carried out to assure that any antimicrobial activity of the test agent does not affect the reliability of recovering the microorganisms that may be present in the product. The test method was carried out by using neutralization and dilution techniques which induce inhibiting activity or quenching substances that may interfere (if present) with the recovery of the microorganisms inoculated in the product. The average microbial counts obtained for the total aerobic count and the total yeast and mold count, must be ≥ 50% and ≤ 200% of those obtained from the positive control plates [The United States Pharmacopeia, 2015].

V.2.1.6 Procedure for MST

The final product was diluted 1:10 (10 ml in 100 ml) in sterile Fluid number 3. The diluted suspension was then serially diluted to 1:100 and 1:1000. Each of these three dilutions was plated in duplicate on TSA and SDA plates. Specifically, one ml from each dilution was placed in melted TSA and SDA at 45^{o} C, poured into 90 mm Petri dishes, and incubated after solidification in an inverted position. TSA plates were incubated at 30 -35° C for 3 days while SDA plates at 20-25° C for 5 days.

V.2.1.7 Fluid number 3 Preparation

Fluid number 3 (buffered sodium chloride peptone powder, Himedia) has been prepared according to manufacturer's instructions; 14.6 g of the dehydrated powder was dissolved in one liter of distilled water. This was followed by the addition of 1.0 g polysorbate 80 and heated until completely dissolved to form a homogenous fluid. This fluid was then sterilized by autoclaving for 15 minutes at 121° C and the final pH adjusted to 7.0.

V.3 Test for efficacy of antimicrobial preservation

V.3.1 General overview

This test was performed according to USP guidelines. In brief, the final product was inoculated with $1x10^8$ cfu (1 McFarland standard) with each of the specified microorganisms. At the time of inoculation (Day zero), 100µl (1:100 dilution) of microorganism suspension was transferred to two tubes, one containing 10 ml of the final product and the other containing normal saline. However, for *A. niger*, the saline tube contained 0.05% polysorbate 80. The tubes containing the final product that has been inoculated with the specific microorganisms were incubated at 20 -25^o C for time intervals of 2 to 4 weeks. In order to establish a base line for later comparison, we diluted the saline tubes by transferring one ml serially to a final dilution of 1 x 10^{-8}. One ml of the last 4 dilutions ($1x10^{-5}$ to $1x10^{-8}$) were removed, mixed with 19 ml melted TSA and SDA (in duplicate) and incubated at 30 -35^o C for 3 days and 20 -25^o C 3 to 5 days respectively. For the final product, this step was repeated after 2 and 4 weeks of incubation. Dilutions of $1x10^{-3}$ to $1x10^{-6}$ were plated and scored [British Pharmacopoeia, 2007].

V.3.2 Enumeration

At the end of incubation, the plates containing microbial growth were enumerated. To determine the amounts of organisms for Day zero, we counted the plates containing 30 to 300 CFUs. For these dilutions, the number of microorganisms was determined as follows:

$$\text{CFU/ml} = \text{Average number of colonies} \times \text{dilution factor}$$

These steps were repeated for all 5-test microorganisms at 2 weeks and 4 weeks. The counts were then tabulated and analyzed according to USP acceptance criteria.

V.3.3 Test microorganisms

The following microorganisms were recommended by the USP and used throughout this project:

- *Candida albicans* ATCC 10231
- *Aspergillus niger* ATCC 16404
- *Escherichia coli* ATCC 8739
- *Pseudomonas aeruginosa* ATCC 9027
- *Staphylococcus aureus* ATCC 6538

V.3.4 Preparation of inoculum

Preparatory to the test, inoculate the surface of tryptic soy agar for bacteria and sabouraud dextrose agar for fungi, with the recently grown stock culture of each of the specified microorganisms. Incubate the bacterial cultures at 30-35 °C for 18-24 h, the culture of *C. albicans* at 20-25°C for 48 h, and the culture of *A. niger* at 20-25°C for 1 week or until good conidia formation is obtained. Subcultures may be needed after revival before the microorganism is in its optimal state, but it is recommended that their number be kept to a minimum.

To harvest the bacterial and *C. albicans* cultures, the organisms are suspended in sterile normal saline (9 g/l of sodium chloride) to produce inoculums of $1X10^8$ cfu/ml. For *A. niger,* sterile normal saline containing 0.5 g/l polysorbate 80 and the conidia count is adjusted to $1X10^8$ conidia/ml. The inoculums are adjusted using a 1.0 McFarland standard to get an inoculum of $1x10^8$ cfu/ml.

V.3.5 Criteria of acceptance

The criteria for evaluation of antimicrobial activity are given in terms of the log reduction in the number of viable microorganisms against the value obtained for the initial inoculum.

The acceptance criteria indicate that no less than 1.0 log reduction from the initial count should be observed on day 14. In addition, there should be no increase in the counts at day 28 as compared to counts obtained at day 14. The criteria for testing yeasts and molds varies from that applied to bacteria. There should be no increase in the counts on days 14 and 28 as compared to the initial counts at day zero.

V.4 Determination of the Minimum Inhibitory Concentrations (MIC)

The antimicrobial susceptibility testing was performed following the recommendations of the Clinical and Laboratory Standards Institute [M100, 27th edition, 2015]

V.4.1 Test Microorganisms

Staphylococcus aureus ATCC 29737, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027, *Micrococcus luteus* ATCC 10240, *Bacillus subtilis* ATCC 6633 and *Saccharomyces cerevisiae* ATCC 2601 were cultured on soybean-casein digest agar and incubated at 35°C for 24 hours. For *S. cerevisiae*, the yeast was cultured onto sabouraud dextrose agar and incubated at 25°C for 48 hours. Subsequently, the inoculums for all bacterial and yeast isolates were prepared in sterile saline at 0.5 McFarland standard, which is equivalent to 1 x 10^4 CFU/ml.

V.4.2 Medium Preparation

Soybean-casein digest broth (TSB) has been prepared according to manufacturer instructions and autoclaved at 121° C for 15 minutes.

V.4.3 Natural preservatives preparation for antibacterial testing

1.0 g of thymol and rosmarinic acid have been carefully weighed using an analytical balance and dissolved in 100 ml of the previously prepared TSB. Samples were serially diluted 1:2 with TSB. The final concentrations of the oils tested were 5.00, 2.50, 1.25, 0.625 and 0.312 mg/ml. As for the rosemary and thyme oils, the stock was prepared in TSB by dissolving one

ml of the oil in 100 ml of the TSB. Doubling dilution was done to give concentrations as previously indicated.

V.4.4 Minimal Inhibitory Concentration of the Natural Preservatives

The MIC was conducted using 12 chamber tissue culture plates. In brief, 2 ml of the preservative solution (10 mg/ml) was placed in the first chamber. One ml of the TSB was placed in chambers 2 through 6, and the serial doubling dilution was made. The inoculum of the bacterial and yeast strains was prepared at 0.5 McFarland standard in the designated broth medium, and one ml was added to each chamber. In addition, one well contained only sterile medium as negative control and one well contained one ml of the medium and one ml of the inoculum as positive control. The plates were incubated at 37^{o} C for 24 hours for the bacterial strains, and 48 hours at 25^{o} C for the yeast. The plates were read and interpreted at the end of the incubation period.

V.5 Determination of the Minimum Bactericidal/Fungicidal Concentrations (MBC)

Referring to the results of the MIC assay, all wells showing complete absence of growth were considered the MIC. One ml from the MIC chamber and from the two higher dilutions was removed and suspended in 9 ml TSB broth and a serial dilution of 1:10 was made. One ml of each dilution was added to 16 ml of TSA (for bacteria) and SDA (for yeast), mixed well and poured into 90 mm Petri dishes, incubated for 24 to 48 hours at the proper temperature and counted. The plate that contained 99.9% killing (10 colonies or less according to the reference Table) is considered as MBC/MFC.

VI. Results

VI.1 Microbial Limit Test (MLT)

The rosmarinic acid, thymol, thyme oil and rosemary oil has been tested for MLT. The compounds tested were free of microbial contamination. Our results indicated the absence of bacterial and fungal growth after the incubation period.

VI.2 Minimum Inhibitory Concentration (MIC)

The antimicrobial effects of the tested natural preservative compounds have been evaluated by their MIC levels. The results obtained are shown in Table12.

		Minimum inhibitory concentration - MIC			
Microorganism	ATCC	Thymol ($mg.ml^{-1}$, w/v)	Rosmarinic acid ($mg.ml^{-1}$, w/v)	Thyme oil ($\mu l.ml^{-1}$, v/v)	Rosemary oil ($\mu l.ml^{-1}$, v/v)
S. aureus	29737	0.625	1.25	1.25	2.5
M. luteus	10240	1.25	1.25	1.25	2.5
E. coli	8739	1.25	2.5	1.25	2.5
P. aeruginosa	9027	2.5	2.5	2.5	2.5
B. subtilis	6633	1.25	1.25	1.25	2.5
S. cerevisiae	2601	2.5	2.5	2.5	2.5

Table 12: MIC of the natural preservative compounds used in the study to test their effectiveness against different strains of microorganisms.

VI.3 Minimum bactericidal/fungicidal concentration (MBC, MFC)

The minimum bactericidal and fungicidal concentrations for the tested organisms were determined by plating aliquots taken from the MIC well and three wells with higher concentration than the MIC. The results obtained are shown in the Table below (Table 13)

Microorganism	ATCC	Initial Count cfu/ml	Concentration	Minimum bactericidal/fungicidal concentration - MBC/MFC cfu/ml×10^{-1}			
				Thymol (mg.ml-1), (w/v)	Rosmarinic acid (mg.ml^{-1}), (w/v)	Thyme oil (µl.ml^{-1}), (v/v)	Rosemary oil (µl.ml^{-1}), (v/v)
S. aureus	29737	4.0×10^4	0.625	37	44	62	113
			1.250	11	15	18	75
			2.500	NG	NG	3	18
			5.000	NG	NG	NG	6
M. luteus	10240	2.0×10^4	0.625	31	147	103	122
			1.250	8	85	52	84
			2.500	NG	7	10	8
			5.000	NG	NG	NG	NG
E. coli	8739	7.0×10^4	0.625	124	169	153	148
			1.250	67	67	61	81
			2.500	21	34	28	31
			5.000	4	7	NG	14
P. aureginosa	9027	2.0×10^4	0.625	111	135	127	133
			1.250	54	73	66	68
			2.500	4	7	7	6
			5.000	NG	NG	NG	NG
B. subtilis	6633	3.0×10^4	0.625	68	49	89	124
			1.250	35	16	34	64
			2.500	9	15	11	13
			5.000	NG	NG	NG	3
S. cerevisiae	2601	4.0×10^4	0.625	96	147	114	132
			1.250	43	88	61	76
			2.500	9	19	13	16
			5.000	NG	7	2	4

Table 13: Minimum bactericidal and fungicidal concentrations for the ATCC tested microorganisms against the natural substances and their oils.

VI.4 Minimum bactericidal/fungicidal concentration (MBC, MFC)

A summary of the results obtained in the previous Table (Table 13) is shown in the Table below (Table 14)

		Minimum bactericidal/fungicidal concentration - MBC/MFC			
Microorganism	ATCC	Thymol ($mg.ml^{-1}$, w/v)	Rosmarinic acid ($mg.ml^{-1}$, w/v)	Thyme oil ($\mu l.ml^{-1}$, v/v)	Rosemary oil ($\mu l.ml^{-1}$, v/v)
S. aureus	29737	1.25	1.25	1.25	2.5
M. luteus	10240	1.25	2.5	2.5	2.5
E. coli	8739	2.5	2.5	2.5	2.5
P. aureginosa	9027	2.5	2.5	2.5	2.5
B. subtilis	6633	2.5	1.25	2.5	2.5
S. cerevisiae	2601	2.5	2.5	2.5	2.5

Table 14: MBC/MFC results for the ATCC tested microorganisms against the natural substances and their oils.

Microorganism	Preparation of test Strain	Growth Promotion		Suitability of Counting Method in the Presence of Product															
		TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC	TAMC	TYMC
				Positive Control		Negative Control		With Thymol		With Rosmarinic acid		With Thymol & Rosmarinic acid		With Thyme oil		With Rosemary oil		With Thyme & Rosemary oils	
S. aureus ATCC 6538	TSA 30°–35° C 18–24 hours	73		65		42		52		60		47		47		71		63	
P. aeruginosa ATCC 9027	TSA 30°–35° C 18–24 hours	87		77		55		35		51		61		39		59		57	
B. subtilis ATCC 6633	TSA 30°–35° C 18–24 hours	52		57		65		41		38		47		44		38		39	
C. albicans ATCC 10231	TSA/SDA 20°–25° C 2–3 days	46	37	37	39	47	28	32	28	26	27	18	25	29	28	16	47	24	28
A. niger ATCC 16404	TSA/SDA 20°–25° C 5–7 days	37	34	32	35	31	24	17	21	14	24	27	14	22	18	21	31	22	19

Table 15: Bacterial counts in the presence and absence of medicinal oils and compounds tested for their potential preservation abilities.

VI.5 Physical properties

The physical properties of all the formulas prepared were determined according to the recommendations of USP [The United States Pharmacopeia, 2016].

For the color, there was no obvious changes at all except with rosmarinic acid, it became slightly deeper red color. The natural smell of the thyme oil and rosemary oil was present in the preparations containing these preservatives.

Formula	At preparation (%)	Temperature (°C)	After three months (%)	After six months (%)
Positive control	100.8	RT*	99.8	103.8
		30	101.4	102.6
		40	101.2	101.7
Negative control (Not preserved)	100.3	RT	99.9	103.3
		30	99.6	103.4
		40	99.6	103.3
Thymol	101.3	RT	98.3	103.5
		30	99.8	103.7
		40	101.8	102.5
Rosmarinic acid	101.2	RT	100.0	103.4
		30	100.0	101.7
		40	100.1	101.5
Thymol and Rosmarinic acid	101.8	RT	100.1	103.3
		30	99.7	101.6
		40	101.6	101.5
Thyme oil	100.3	RT	98.6	104.8
		30	100.6	103.4
		40	102.3	104.0
Rosemary oil	102.4	RT	99.0	103.9
		30	101.6	103.3
		40	101.1	102.9
Thyme oil and Rosemary oil	102.4	RT	99.7	103.6
		30	100.0	103.6
		40	101.2	103.3

Table 16: Paracetamol active results.

The Table above shows the results obtained for the stability of the active ingredient in the formula (Paracetamol) after three months and six months of preparation. Its noteworthy to see that the active ingredient maintained its full activity in all the formulas prepared and tested.

Formula	At preparation (cPS*)	Temperature (°C)	After three months (cPS)	After six months (cPS)
Positive control	25.0	RT**	18.0	20
		30	18.0	20.5
		40	18.5	19.0
Negative control	24.0	RT	22.0	19.0
		30	21.0	18.5
		40	19.0	17.0
Thymol	26.0	RT	19.0	20.0
		30	19.0	20.5
		40	18.0	17.0
Rosmarinic acid	26.0	RT	21.0	22.0
		30	20.5	22.5
		40	21.0	21.0
Thymol and Rosmarinic acid	26.0	RT	22.5	21.5
		30	22.0	19.0
		40	20.0	18.5
Thyme oil	26.0	RT	19.0	19.5
		30	17.5	18.5
		40	16.5	17.0
Rosemary oil	24.0	RT	18.5	19.0
		30	17.0	16.5
		40	18.0	18.0
Thyme oil and Rosemary oil	27.0	RT	17.5	17.0
		30	17.5	19.0
		40	17.0	19.5

Table 17: Viscosity results.

The formulas prepared were evaluated for their viscosity at different temperatures after three and six months period after preparation. The results obtained (Table 17) showed consistent results with minimal variation.

* cPS definition: centipoise is a non-System International measurement unit of dynamic viscosity per second.
** RT: Room Temperature.

Formula	At preparation	Temperature (°C)	After three months	After six months
Positive control	4.0	RT*	4.1	4.1
		30	4.1	4.2
		40	4.2	4.0
Negative control	4.1	RT	4.2	4.1
		30	4.1	4.1
		40	4.2	4.2
Thymol	4.1	RT	4.2	4.1
		30	4.1	4.0
		40	4.2	4.1
Rosmarinic acid	3.9	RT	4.0	3.8
		30	3.9	3.8
		40	3.9	3.9
Thymol and Rosmarinic acid	3.9	RT	3.9	3.8
		30	3.9	3.9
		40	3.9	3.8
Thyme oil	4.0	RT	4.2	4.2
		30	4.1	4.3
		40	4.2	4.3
Rosemary oil	4.0	RT	4.1	4.2
		30	4.2	4.2
		40	4.1	4.1
Thyme oil and Rosemary oil	4.0	RT	4.3	4.1
		30	4.1	4.1
		40	4.2	4.2

Table 18: pH results.

The formulas prepared were evaluated for their pH at different temperatures after three and six months period after preparation as.

* Room Temperature

Formula	At preparation ($g.ml^{-1}$)	Temperature (° C)	After three months ($g.ml^{-1}$)	After six months ($g.ml^{-1}$)
Positive control	1.086	RT*	1.085	1.084
		30	1.085	1.084
		40	1.084	1.087
Negative control	1.086	RT	1.086	1.085
		30	1.083	1.086
		40	1.084	1.081
Thymol	1.086	RT	1.085	1.084
		30	1.084	1.085
		40	1.084	1.088
Rosmarinic acid	1.085	RT	1.084	1.080
		30	1.085	1.084
		40	1.086	1.085
Thymol and Rosmarinic acid	1.085	RT	1.085	1.085
		30	1.084	1.083
		40	1.086	1.085
Thyme oil	1.087	RT	1.086	1.083
		30	1.084	1.088
		40	1.084	1.084
Rosemary oil	1.086	RT	1.084	1.083
		30	1.084	1.085
		40	1.084	1.086
Thyme oil and Rosemary oil	1.085	RT	1.085	1.086
		30	1.084	1.085
		40	1.084	1.087

Table 19: Density results.

The formulas prepared were evaluated for their density at different temperatures after three and six months period after preparation.

* Room Temperature

VI.6 Antimicrobial preservation test

The preservation abilities of thymol, rosmarinic acid, thyme oil and rosemary oil were comparable with the positive control after 14 and 28 days, emphasizing their preservation properties on all microorganisms tested as shown in the Table below (Table 20). However, the negative control formula without preservation system maintained the growth of the microorganisms introduced in the formula throughout the testing period. The results of *A. niger* showed increased growth in the negative control. However, there was three log reductions in growth with rosmarinic acid preservation system, which is acceptable according to the USP criteria.

		Organism				
	Reference	*S. aureus* ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
		cfu/ml				
Formula		2.2×10^{8}	1.9×10^{8}	1.2×10^{8}	1.1×10^{8}	9.0×10^{7}
Positive control	14 days	NG*	NG	NG	NG	TFTC**
	28 days	NG	NG	NG	NG	NG
Negative control	14 days	1.2×10^{7}	1.9×10^{7}	1.2×10^{8}	7.4×10^{6}	TNTC***
	28 days	6.8×10^{6}	1.7×10^{6}	7.9×10^{7}	5.3×10^{6}	TNTC
Thymol	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Rosmarinic acid	14 days	NG	NG	NG	NG	2.1×10^{5}
	28 days	NG	NG	NG	NG	1.7×10^{4}
Thymol and Rosmarinic acid	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Thyme oil	14 days	NG	NG	NG	NG	8.9×10^{6}
	28 days	NG	NG	NG	NG	TFTC
Rosemary oil	14 days	NG	NG	TFTC	NG	TFTC
	28 days	NG	NG	NG	NG	NG
Thyme oil and Rosemary oil	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG

Table 20: The preservation abilities of thymol, rosmarinic acid, thyme oil and rosemary oil compared with the positive and negative control after 14 and 28 days.

* NG: No growth, ** TFTC: Two few to count, *** TNTC: Two numerous to count

Formula	Reference	Organism				
		S. aureus ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
		cfu/ml				
		1.2×10^{8}	2.1×10^{8}	2.2×10^{8}	9.5×10^{7}	8.6×10^{7}
Positive control	14 days	NG*	NG	NG	NG	TFTC**
	28 days	NG	NG	NG	NG	TFTC
Negative control	14 days	9.7×10^{7}	1.8×10^{8}	1.7×10^{8}	6.9×10^{7}	TNTC***
	28 days	7.7×10^{7}	1.2×10^{8}	1.4×10^{8}	4.1×10^{7}	TNTC
Thymol	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Rosmarinic acid	14 days	NG	NG	TFTC	NG	3.8×10^{4}
	28 days	NG	NG	NG	NG	1.1×10^{3}
Thymol and Rosmarinic acid	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Thyme oil	14 days	NG	NG	NG	NG	7.3×10^{4}
	28 days	NG	NG	NG	NG	TFTC
Rosemary oil	14 days	NG	NG	NG	NG	1.1×10^{5}
	28 days	NG	NG	NG	NG	TFTC
Thyme oil and Rosemary oil	14 days	NG	NG	NG	NG	TFTC
	28 days	NG	NG	NG	NG	NG

Table 21: The preservation abilities of thymol, rosmarinic acid, thyme oil and rosemary oil were comparable with the positive control after 14 and 28 days after 3 months at room temperature.

* No growth, ** Two few to count, *** Tow numerous to count

Formula	Reference	Organism				
		S. aureus ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
		cfu/ml				
		1.2×10^{8}	2.1×10^{8}	2.2×10^{8}	9.5×10^{7}	8.6×10^{7}
Positive control	14 days	NG*	NG	3.3×10^{4}	NG	9.4×10^{5}
	28 days	NG	NG	NG	NG	3.9×10^{4}
Negative control	14 days	7.7×10^{7}	8.3×10^{7}	7.1×10^{7}	6.0×10^{7}	TNTC**
	28 days	5.8×10^{6}	4.5×10^{6}	9.7×10^{6}	7.6×10^{6}	TNTC
Thymol	14 days	NG	NG	NG	NG	TFTC***
	28 days	NG	NG	NG	NG	TFTC
Rosmarinic acid	14 days	NG	NG	TFTC	NG	8.3×10^{4}
	28 days	NG	NG	TFTC	NG	4.6×10^{3}
Thymol and Rosmarinic acid	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Thyme oil	14 days	NG	NG	TFTC	NG	5.7×10^{4}
	28 days	NG	NG	NG	NG	TFTC
Rosemary oil	14 days	NG	NG	TFTC	NG	3.2×10^{5}
	28 days	NG	NG	NG	NG	8.8×10^{4}
Thyme oil and Rosemary oil	14 days	NG	NG	NG	NG	2.2×10^{4}
	28 days	NG	NG	NG	NG	NG

Table 22: The preservation abilities of thymol, rosmarinic acid, thyme oil and rosemary oil were comparable with the positive control after 3 months at 30° C.

* No growth, ** Two few to count, *** Tow numerous to count

Formula	Reference	Organism				
		S. aureus ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
		cfu/ml				
		1.2×10^{8}	2.1×10^{8}	2.2×10^{8}	9.5×10^{7}	8.6×10^{7}
Positive control	14 days	NG*	NG	NG	NG	7.2×10^{5}
	28 days	NG	NG	NG	NG	4.3×10^{4}
Negative control	14 days	6.1×10^{7}	5.7×10^{7}	7.7×10^{7}	7.2×10^{7}	TNTC**
	28 days	9.0×10^{6}	1.1×10^{6}	5.3×10^{6}	6.7×10^{6}	TNTC
Thymol	14 days	NG	NG	NG	NG	TFTC***
	28 days	NG	NG	NG	NG	TFTC
Rosmarinic acid	14 days	NG	NG	TFTC	NG	3.1×10^{4}
	28 days	NG	NG	TFTC	NG	TFTC
Thymol and Rosmarinic acid	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Thyme oil	14 days	NG	NG	TFTC	NG	2.8×10^{4}
	28 days	NG	NG	NG	NG	TFTC
Rosemary oil	14 days	NG	NG	TFTC	NG	6.7×10^{5}
	28 days	NG	NG	NG	NG	1.9×10^{4}
Thyme oil and Rosemary oil	14 days	NG	NG	NG	NG	4.4×10^{4}
	28 days	NG	NG	NG	NG	TFTC

Table 23: The preservation abilities of thymol, rosmarinic acid, thyme oil and rosemary oil were comparable with the positive control after 3 months at 40° C.

* No growth, ** Two few to count, *** Tow numerous to count

Formula	Reference	Organism				
		S. aureus ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
		cfu/ml				
		1.8×10^{8}	1.1×10^{8}	1.9×10^{8}	2.1×10^{8}	1.1×10^{8}
Positive control	14 days	NG*	NG	NG	NG	2.2×10^{4}
	28 days	NG	NG	NG	NG	TFTC**
Negative control	14 days	1.9×10^{7}	5.5×10^{7}	1.2×10^{8}	3.3×10^{6}	7.9×10^{7}
	28 days	6.3×10^{6}	3.8×10^{6}	7.5×10^{7}	6.1×10^{5}	TNTC***
Thymol	14 days	NG	NG	NG	NG	TFTC
	28 days	NG	NG	NG	NG	NG
Rosmarinic acid	14 days	NG	NG	TFTC	NG	4.0×10^{4}
	28 days	NG	NG	TFTC	NG	TFTC
Thymol and Rosmarinic acid	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Thyme oil	14 days	NG	NG	TFTC	NG	TFTC
	28 days	NG	NG	NG	NG	TFTC
Rosemary oil	14 days	NG	NG	TFTC	NG	4.4×10^{4}
	28 days	NG	NG	NG	NG	TFTC
Thyme oil and Rosemary oil	14 days	NG	NG	NG	NG	TFTC
	28 days	NG	NG	NG	NG	TFTC

Table 24: The preservation abilities of thymol, rosmarinic acid, thyme oil and rosemary oil were comparable with the positive control after 6 months at Room temperature.

* No growth, ** Two few to count, *** Tow numerous to count

Formula	Reference	Organism				
		S. aureus ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
		cfu/ml				
		1.8×10^{8}	1.1×10^{8}	1.9×10^{8}	2.1×10^{8}	1.1×10^{8}
Positive control	14 days	NG*	NG	NG	NG	TFTC**
	28 days	NG	NG	NG	NG	TFTC
Negative control	14 days	7.2×10^{6}	1.9×10^{7}	7.0×10^{7}	4.7×10^{6}	TNTC***
	28 days	2.5×10^{5}	2.0×10^{6}	1.1×10^{6}	9.2×10^{5}	TNTC
Thymol	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Rosmarinic acid	14 days	NG	NG	TFTC	NG	TFTC
	28 days	NG	NG	TFTC	NG	TFTC
Thymol and Rosmarinic acid	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Thyme oil	14 days	NG	NG	TFTC	NG	TFTC
	28 days	NG	NG	NG	NG	TFTC
Rosemary oil	14 days	NG	NG	TFTC	NG	TFTC
	28 days	NG	NG	NG	NG	TFTC
Thyme oil and Rosemary oil	14 days	NG	NG	NG	NG	TFTC
	28 days	NG	NG	NG	NG	TFTC

Table 25: The preservation abilities of thymol, rosmarinic acid, thyme oil and rosemary oil were comparable with the positive control after 6 months at 30° C.

* No growth, ** Two few to count, *** Tow numerous to count

Formula	Reference	Organism				
		S. aureus ATCC 6538	*E. coli* ATCC 8739	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 10231	*A. niger* ATCC 16404
		cfu/ml				
		1.8×10^{8}	1.1×10^{8}	1.9×10^{8}	2.1×10^{8}	1.1×10^{8}
Positive control	14 days	NG*	NG	NG	NG	TFTC**
	28 days	NG	NG	NG	NG	TFTC
Negative control	14 days	3.8×10^{6}	3.3×10^{7}	2.8×10^{7}	1.8×10^{6}	1.6×10^{8}
	28 days	8.1×10^{4}	6.0×10^{6}	9.1×10^{5}	9.8×10^{4}	TNTC***
Thymol	14 days	NG	NG	NG	NG	NG
	28 days	NG	NG	NG	NG	NG
Rosmarinic acid	14 days	NG	NG	TFTC	NG	TFTC
	28 days	NG	NG	NG	NG	TFTC
Thymol Rosmarinic acid	14 days	NG	NG	NG	NG	TFTC
	28 days	NG	NG	NG	NG	NG
Thyme oil	14 days	NG	NG	NG	NG	TFTC
	28 days	NG	NG	NG	NG	TFTC
Rosemary oil	14 days	NG	NG	TFTC	NG	3.8×10^{4}
	28 days	NG	NG	TFTC	NG	TFTC
Thyme Rosemary oils	14 days	NG	NG	TFTC	NG	3.1×10^{4}
	28 days	NG	NG	NG	NG	TFTC

Table 26: The preservation abilities of thymol, rosmarinic acid, thyme oil and rosemary oil were comparable with the positive control after 6 months at 40° C.

* No growth, ** Two few to count, *** Tow numerous to count

VII. Discussion

VII.1 Minimal inhibitory concentration and Bactericidal/Fungicidal Concentration for the Natural Preservatives

MIC and MBC for the bacterial strains and yeast were determined using microbroth dilution methods. The results obtained for the bacterial strains ranged from 0.625 to 2.5 mg/ml. The results for MBC/MFC ranged from 1.25 to 2.5 mg/ml. Considering the concentrations of the chemical preservatives, the concentrations obtained for the natural ones are encouraging. The natural preservatives have several desirable effects in terms of the flavor, taste, antioxidant activity and the long lasting effects as compared to the chemical ones. It is known that chemical preservatives have many undesirable side effects and frequently their concentrations may be harmful particularly for children.

It is worth mentioning that our stability study for these natural preservatives is continuing. The one-year follow up data will be obtained during June 2016, and thereafter on June 2017. Additional information will be obtained two years post expiration date on June 2018 according to USP guidelines.

VII.2 Microbial Limit Test (MLT)

The MLT was performed according to the USP recommendations to ensure that our natural substances are free of contaminating microorganisms. This essential step must be done before proceeding to use the natural substances and the oils as natural preservatives in pharmaceutical preparations. This step has provided us with the green light to proceed and conduct the MIC/MBC experiments to determine the specific antimicrobial effects of these natural preservatives and oils in-vitro. Furthermore, MLT was also repeated after adding the

natural substances and the oils alone and in combination separately to pharmaceutical preparations. These two steps have provided us with the confidence to proceed in further testing the antimicrobial activities of these substances in the microbial challenge test. The outcome of these tests as shown in the results provided us with valuable information regarding the stability and preservation power of our natural substances and oils.

Minimum Inhibitory Concentration (MIC)

The MIC was performed according to the CLSI recommendations. Our results showed that the MIC for the natural substances and oils was 2-3 milligrams/ml. These amounts are encouraging to use to preserve pharmaceutical preparations. The advantages of these substances as preservatives instead of chemical ones such as Methylparaben is due to the similar concentrations that has to be added to these preparations. In addition, it is worth mentioning the importance of avoiding consumption of chemicals that may have adverse effects on humans in general and pediatrics and elderly in particular. Acetaminophen for example, is a drug that is commonly used in infants over one month old, children, adolescents and adults. It is the most frequently obtained without a physician prescription - ('over-the-counter' (OTC). Acetaminophen has been used for relief of pain and reduce of fever. The dose of Acetaminophen for a patient is weight dependent, usually 15 mg per kilogram. This dose can be taken once every 4 to 6 hours, up to 4 times in 24 hours if needed. All the available products in the market in the form of Syrup contains chemical preservatives such as Benzoic acid, Potassium Sorbate, Methylparaben, Propylparaben and others.

VII.3 Preservative Efficacy Test Results

We followed the USP criteria to evaluate the effects of the natural and chemical preservatives and compared to a negative control. For the test to be acceptable, 2 log reduction in growth must be observed for all bacterial strains and no increase in the growth of the yeast and mold strains at day 14 as compared to the initial counts. In addition, there should be no increase for all the microbial strains tested on day 28 as compared with day 14. It was observed that our results conform to the USP criteria as shown in Tables (Table 20 to 26).

VII.4 Evaluation of the Paracetamol activity

In order to confirm the stability of the active ingredient in the syrup in the presence of the different combinations of our natural preservatives, we evaluated the concentration of the paracetamol alone (in the negative control) and with the preservatives. Our results are shown in Table 16. The results showed that the activity (or concentration) of all the preparations were almost identical. This has been determined by the HPLC according to the USP requirements. The results of the HPLC for the tests and controls are shown in annex 1. Table instead with a concentration of 100 units for the active ingredients.

VII.5 Physical properties: PH, Viscosity, Density, precipitation, color and taste

It is important for this work to have the least amount of variations in the physical properties of the preparations. All the physical properties were tested regularly on preparation, 3 months and six months. They will be further tested after one year, 2 years and one year after the expiration date. The pH for all the preparations was within acceptable range of 3.8 to 6.9 according to guidelines.

Precipitation, color and taste has been macroscopically evaluated by 3 people. The outcome of these tests have minimal changes, which confirmed their conformity with guidelines.

The experiments conducted in this research projected tried to answer the main objectives raised in terms of natural versus chemical preservation. It was apparent that the chemical preservative methyl and ethyl parabens and others mentioned earlier proved to be effective. The question here is its safety for pediatric use? A wealth of literature has indicated the undesirable qualities of chemical preservation in general and for pediatric use in particular. As mentioned earlier, the physical properties of the natural preservatives are far better and preferable to use than their chemical counterparts. The side effects of the chemical preservatives are more pronounced in children considering allergies effects on tissues and organs, taste and many others. On the other hand, natural preservatives are more appealing to children for their pleasant smell, good taste and minimal or even absence of side effects. Our experiments proved the effectiveness of the natural substances preservation capabilities. They have been repeatedly tested for prolonged periods of time from preparation to six months. The counts obtained met the criteria of acceptance because of the logarithmic reduction and stability throughout the testing period.

In conclusion, we feel confident to recommend the use of thyme oil, rosemary oil, thymol and rosmarinic acid as preservatives in the pediatric formulas instead of the chemical ones. However, we also recommend the testing of combinations of these oils and their respective compounds hoping to find better effectiveness at lower concentrations and possible longer shelf life. Furthermore, we also wish to recommend expanding the testing of the natural oils and compounds tested here or other if possible against the wide variety of chemical preservatives used in syrup formulations prepared for pediatric use.

VIII. References

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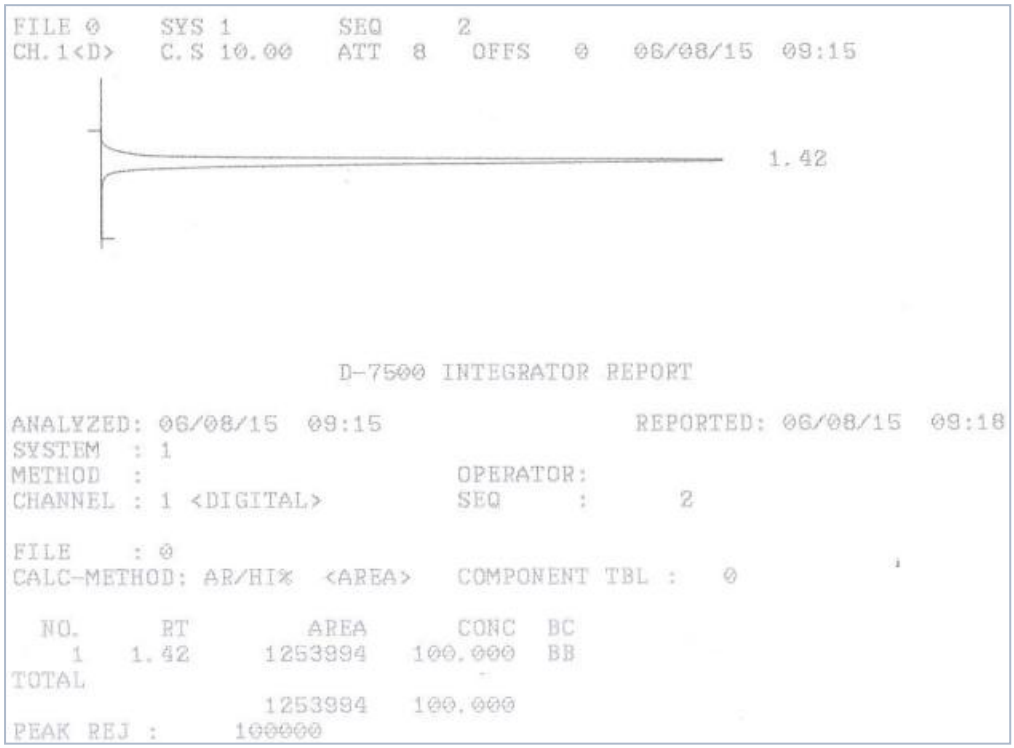
Annex 1

HPLC output of Paracetamol active at preparation:



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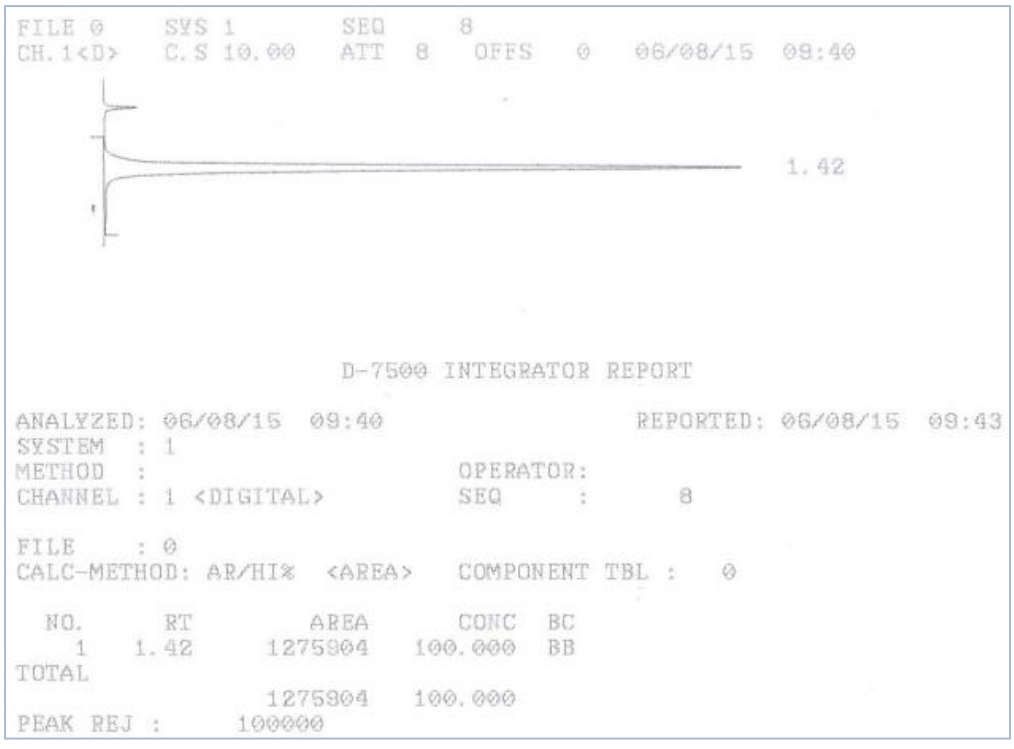

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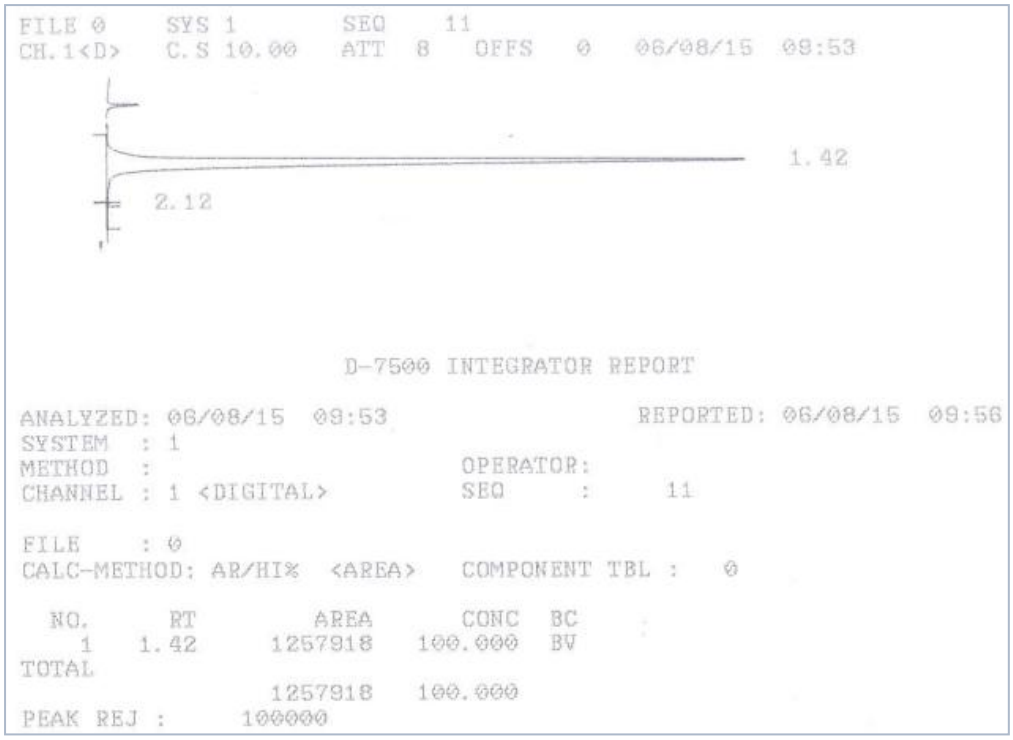

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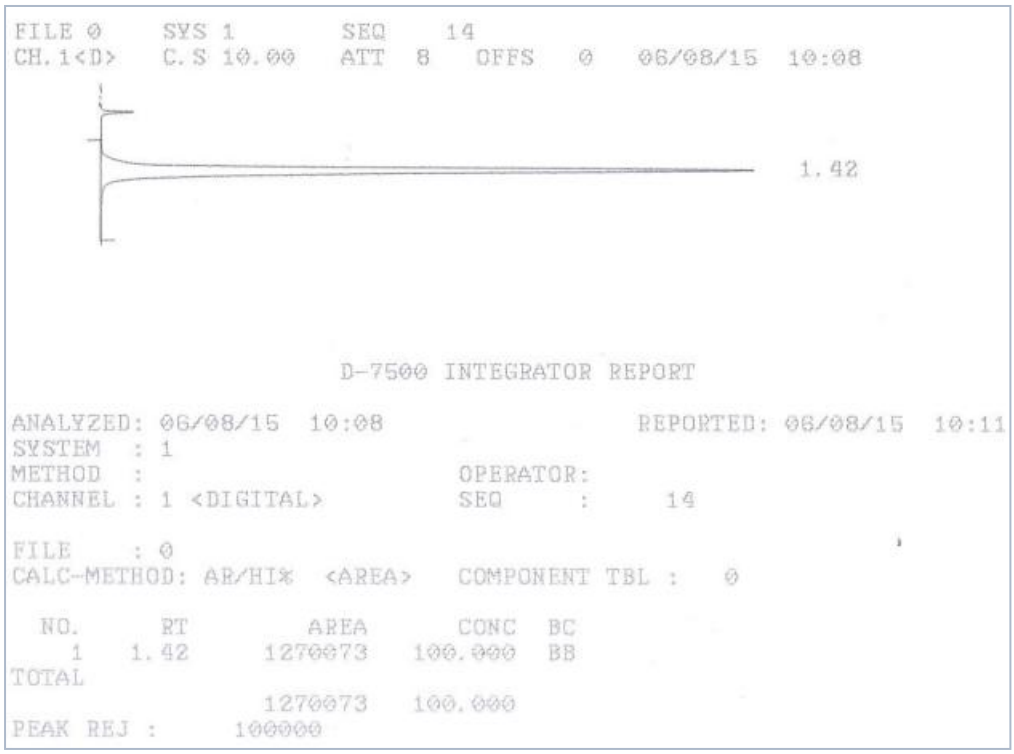

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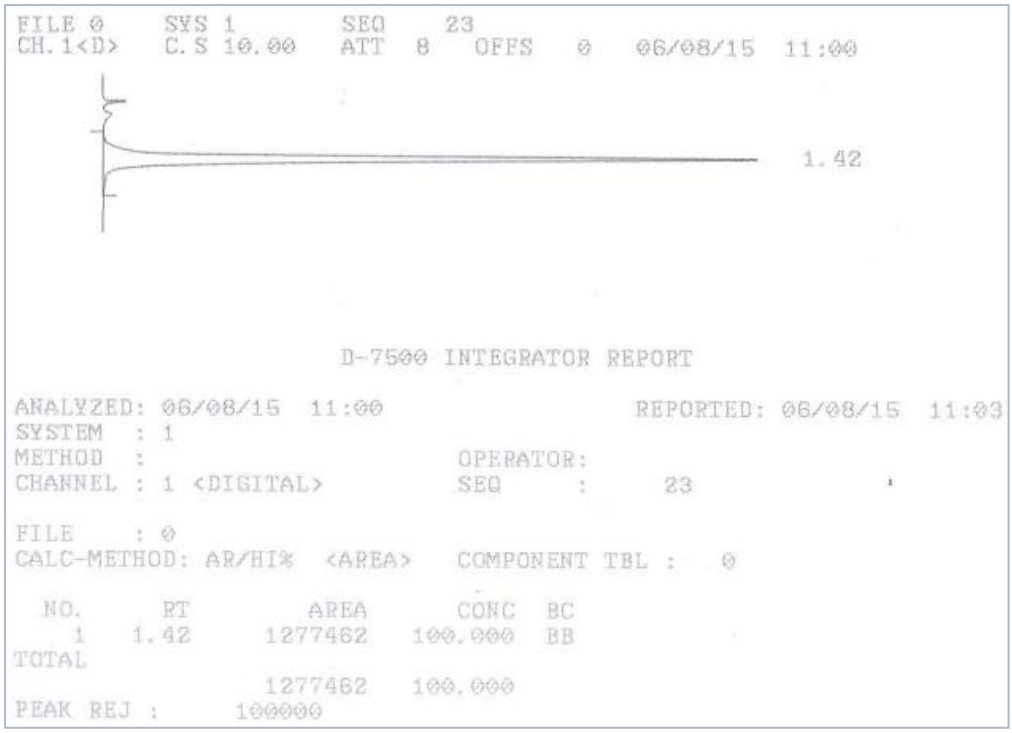

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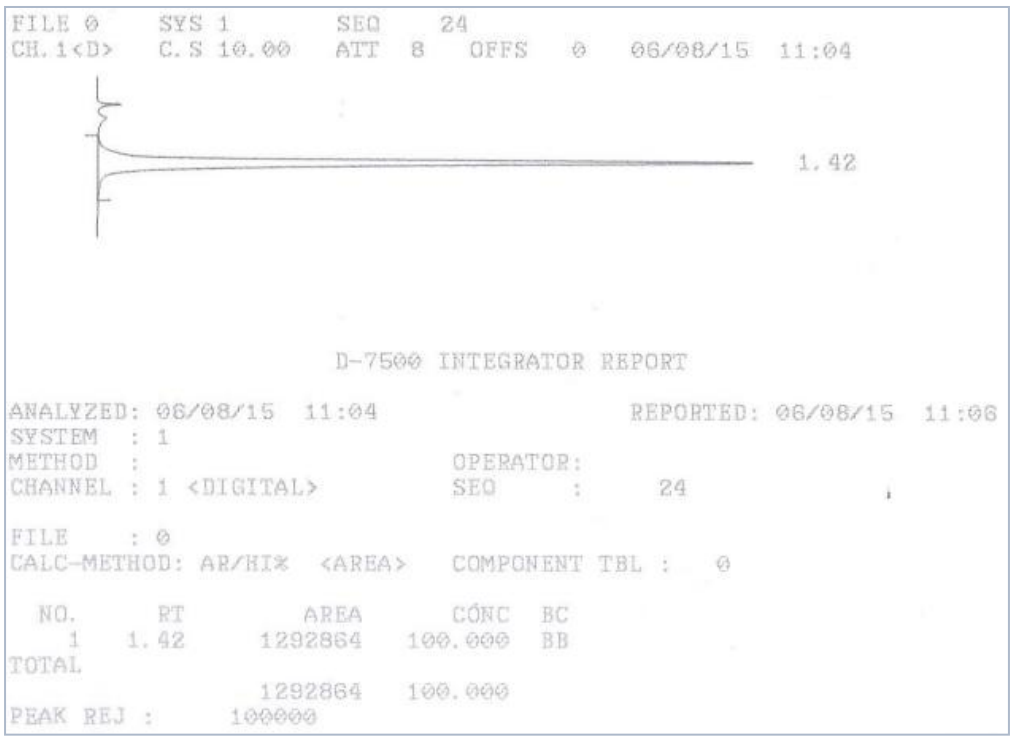

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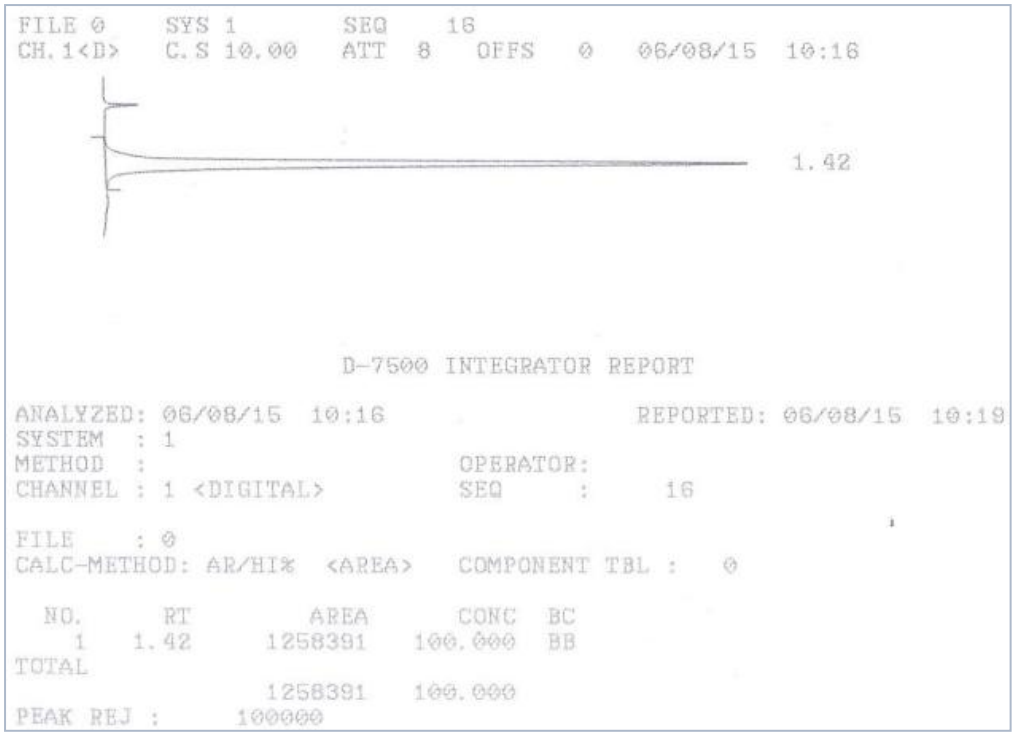

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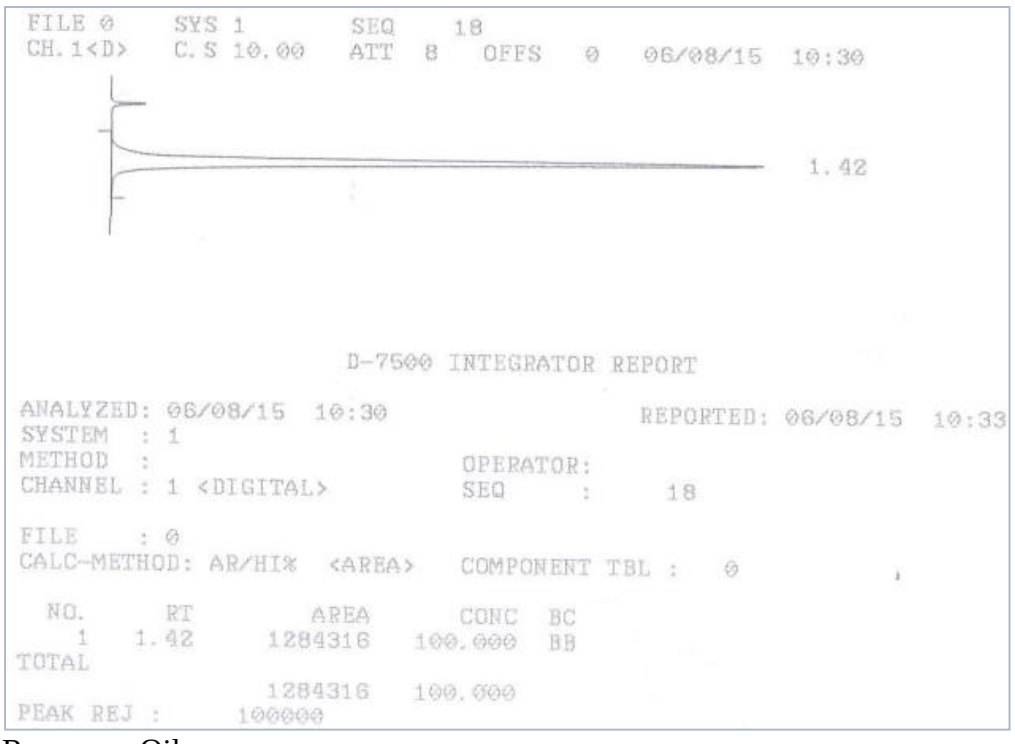

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PEAK REJ :       100000
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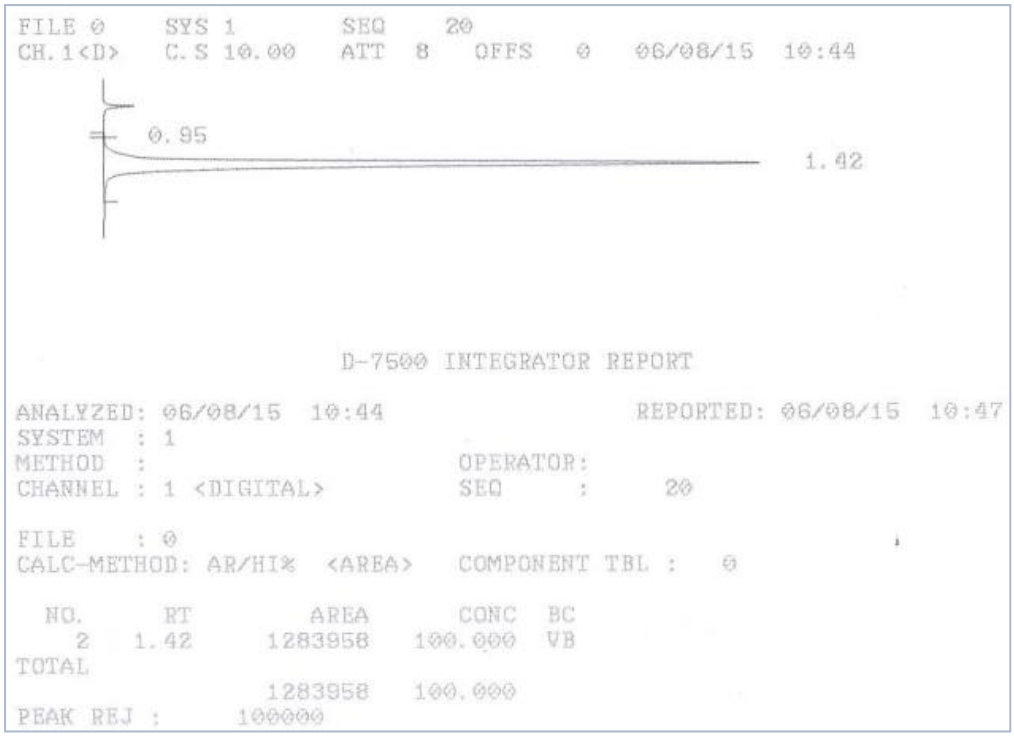
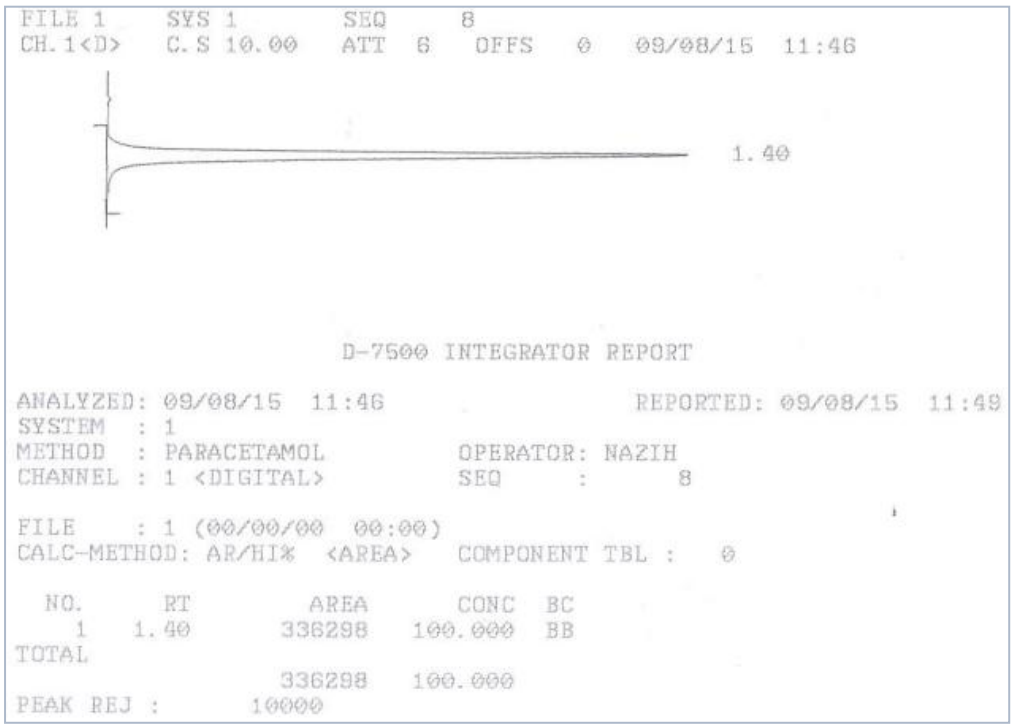
Rosemary Oil

.

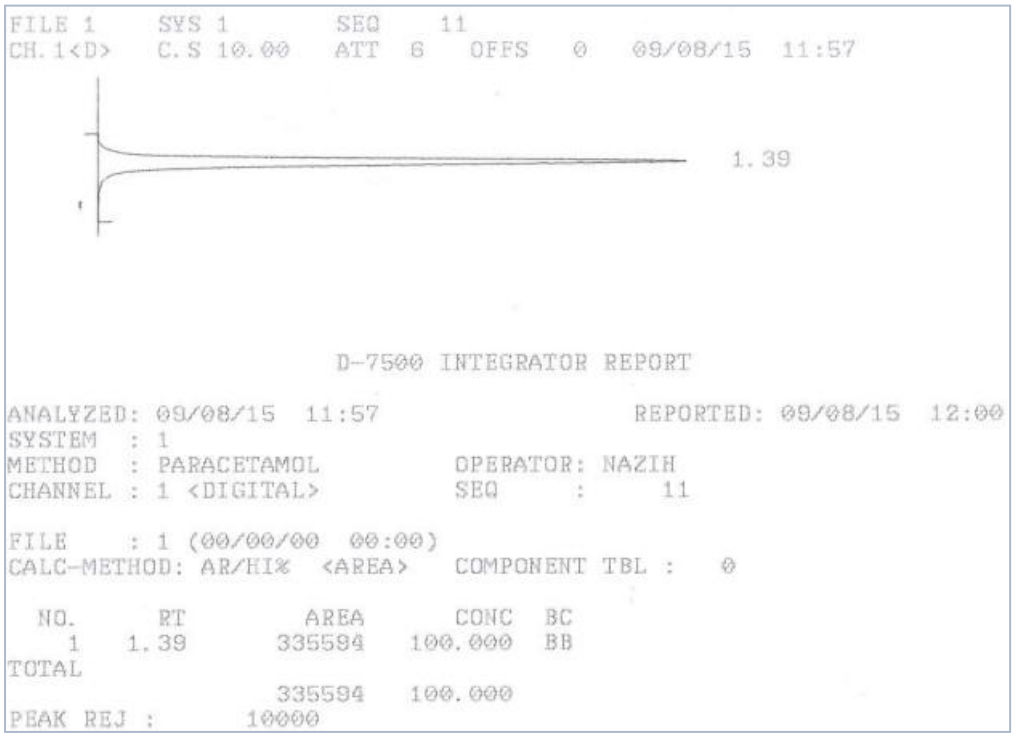


Thyme Oil and Rosemary Oil

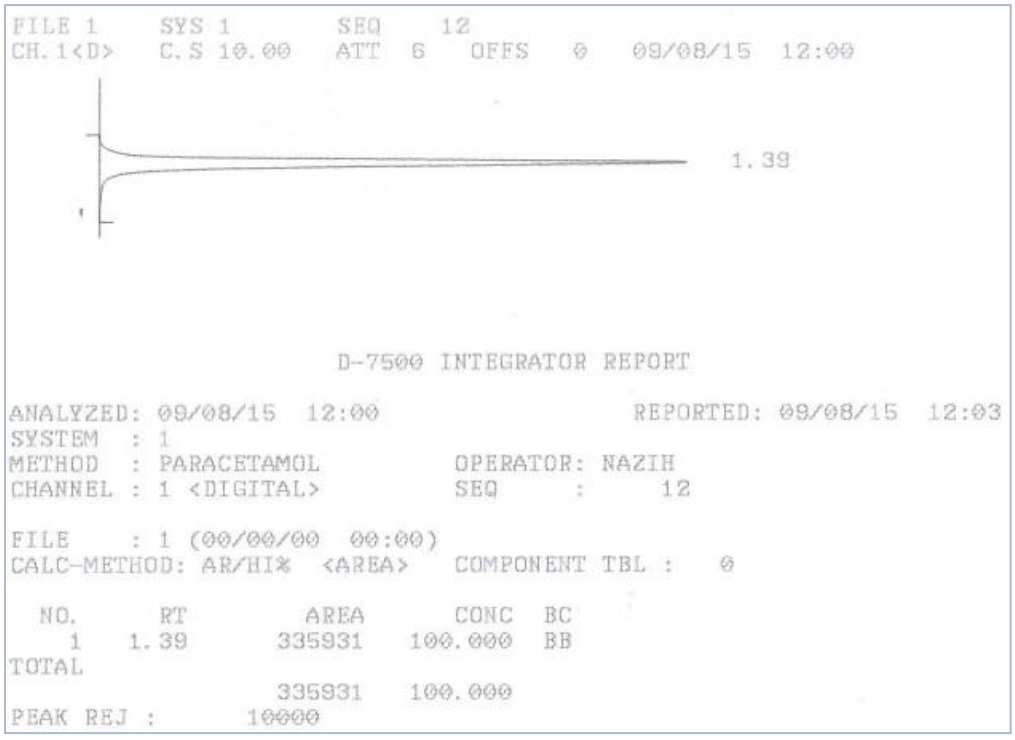
HPLC output of Paracetamol active after three months:



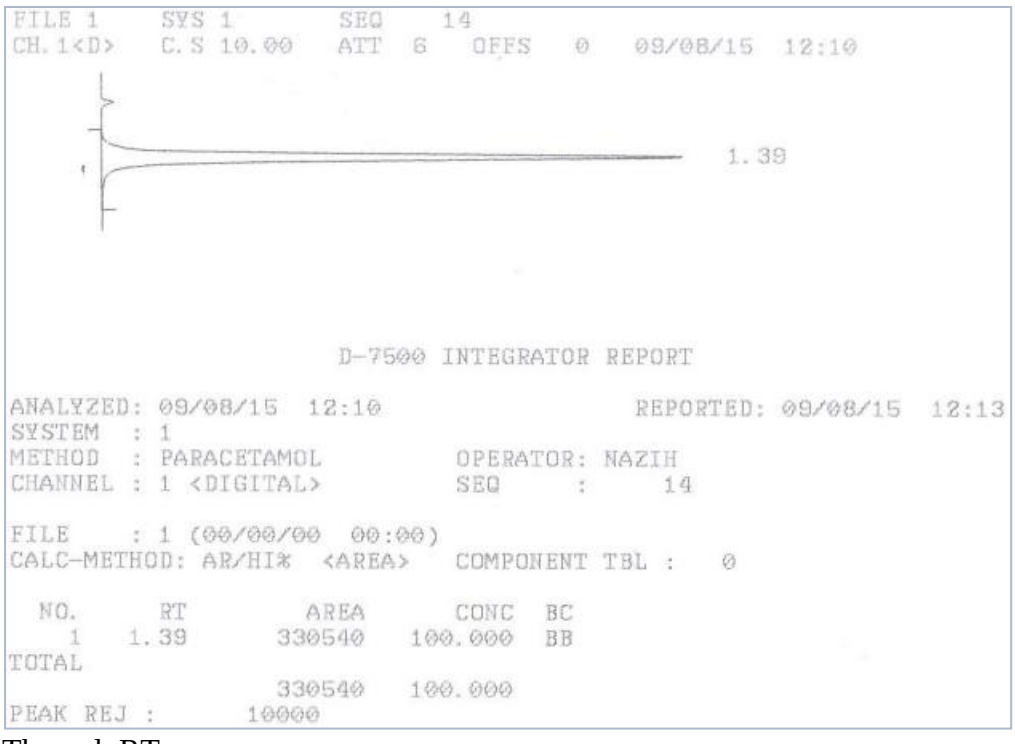
Standard



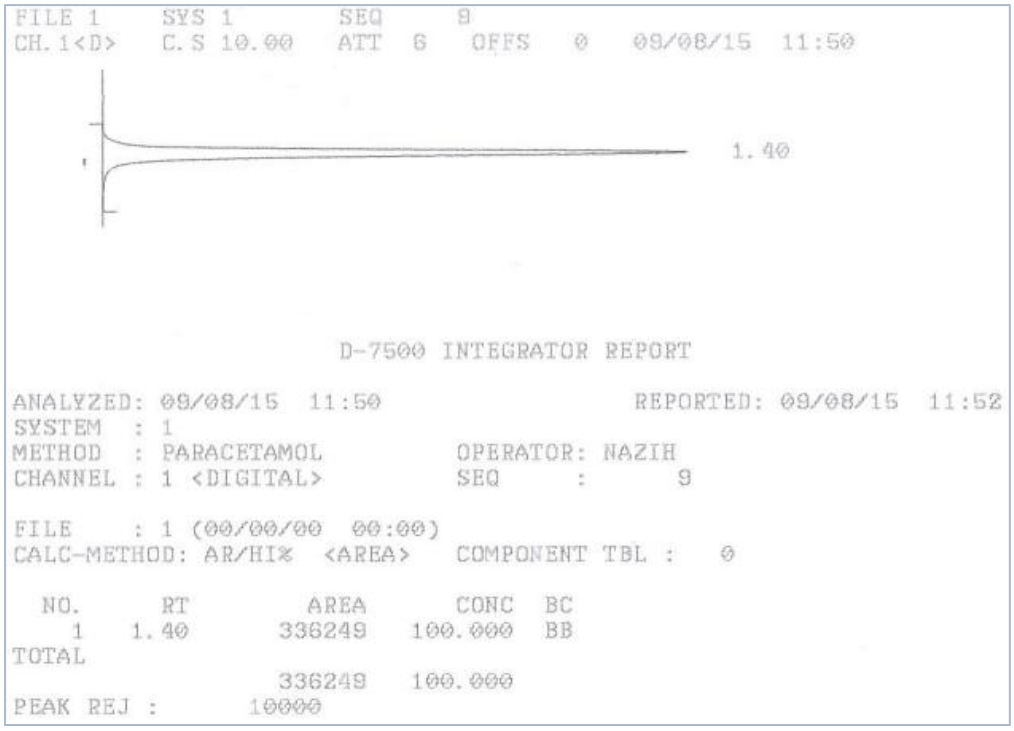
Positive control, RT



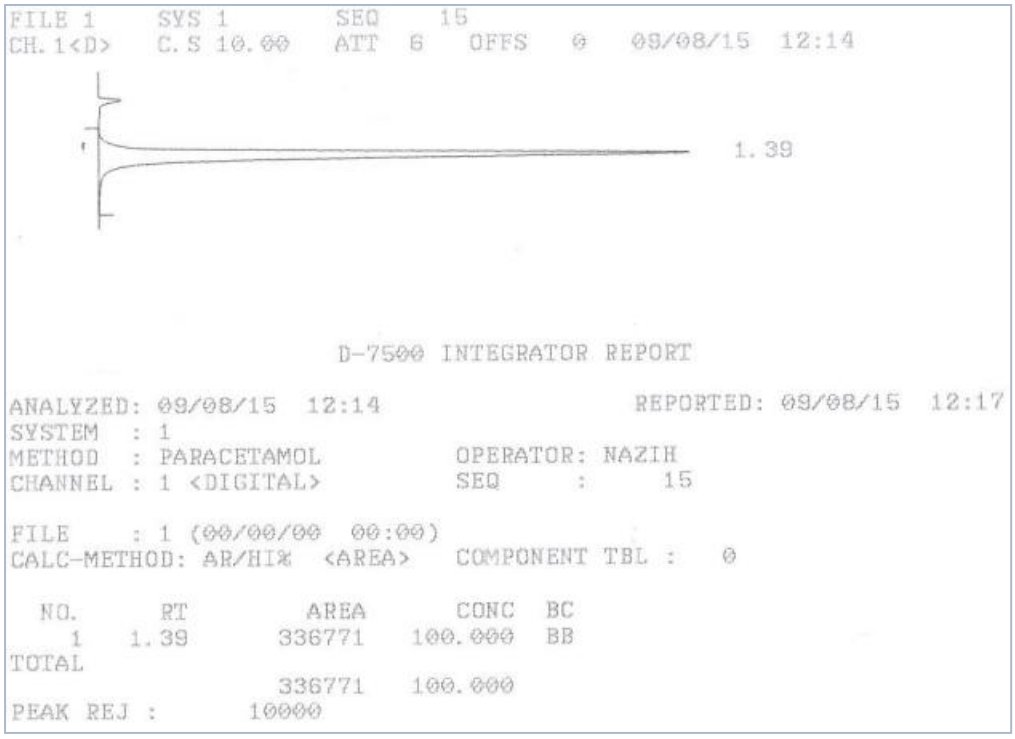
Negative control, RT



Thymol, RT

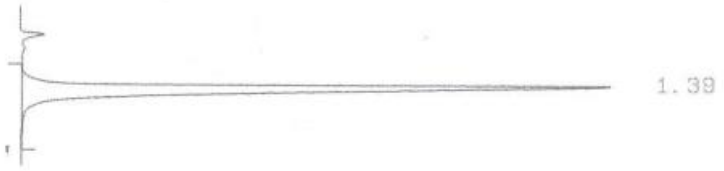


Rosmarinic Acid, RT



Thymol and Rosmarinic Acid, RT

FILE 1 SYS 1 SEQ 16
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 12:19



D-7500 INTEGRATOR REPORT

ANALYZED: 09/08/15 12:19 REPORTED: 09/08/15 12:21
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 16

FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

NO.	RT	AREA	CONC	BC
1	1.39	331459	100.000	BB
TOTAL		331459	100.000	

PEAK REJ : 10000

Thyme Oil, RT

FILE 1 SYS 1 SEQ 17
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 12:23

D-7500 INTEGRATOR REPORT

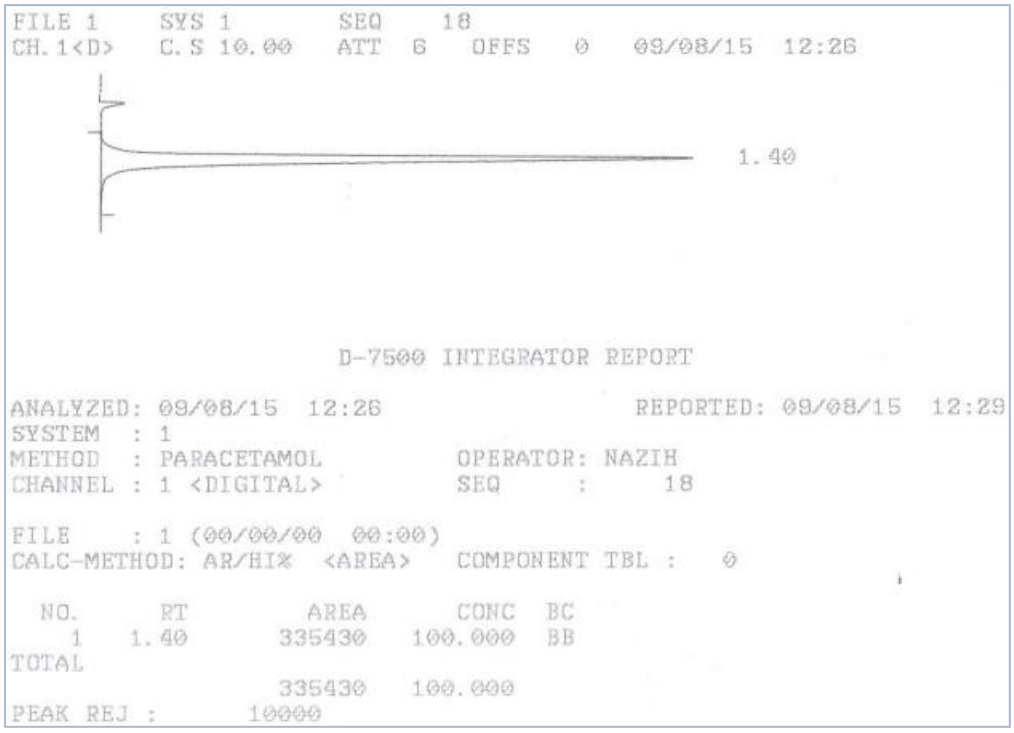
ANALYZED: 09/08/15 12:23 REPORTED: 09/08/15 12:26
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 17

FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

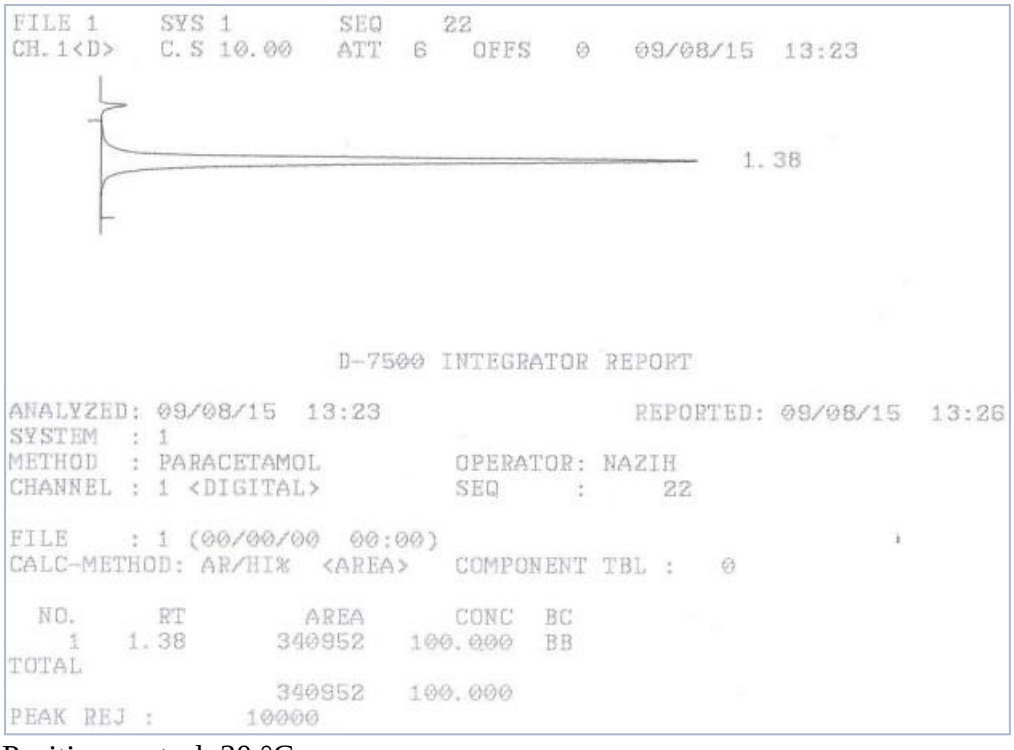
NO.	RT	AREA	CONC	BC
1	1.39	332780	100.000	BB
TOTAL		332780	100.000	

PEAK REJ : 10000

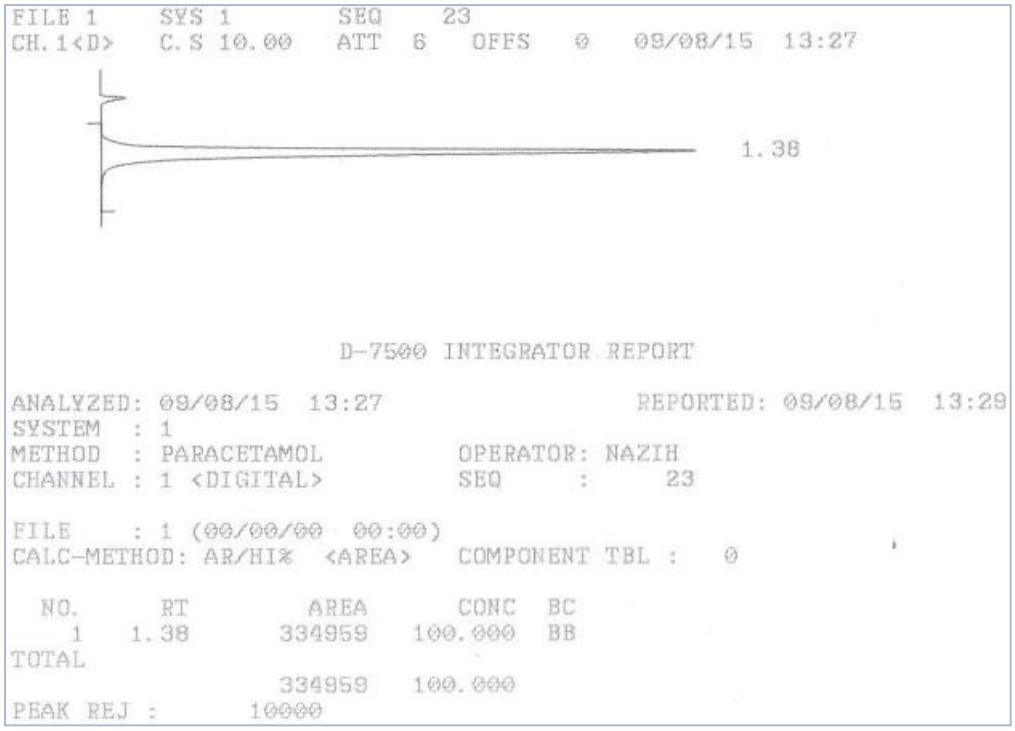
Rosemary Oil, RT



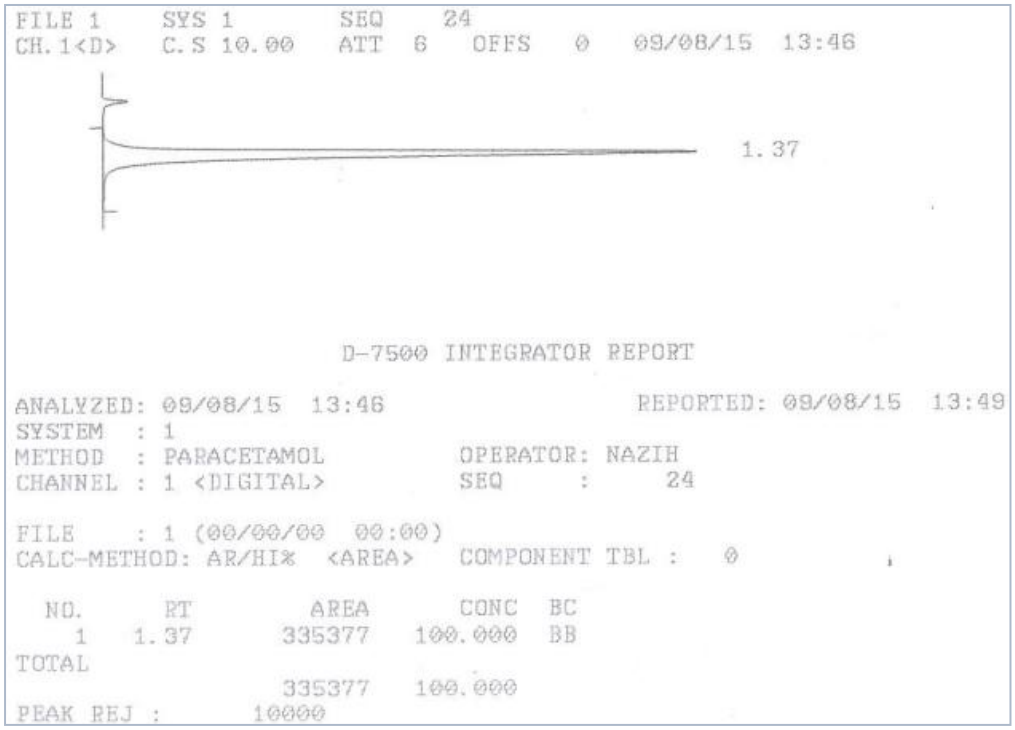
Thyme Oil and Rosemary Oil, RT



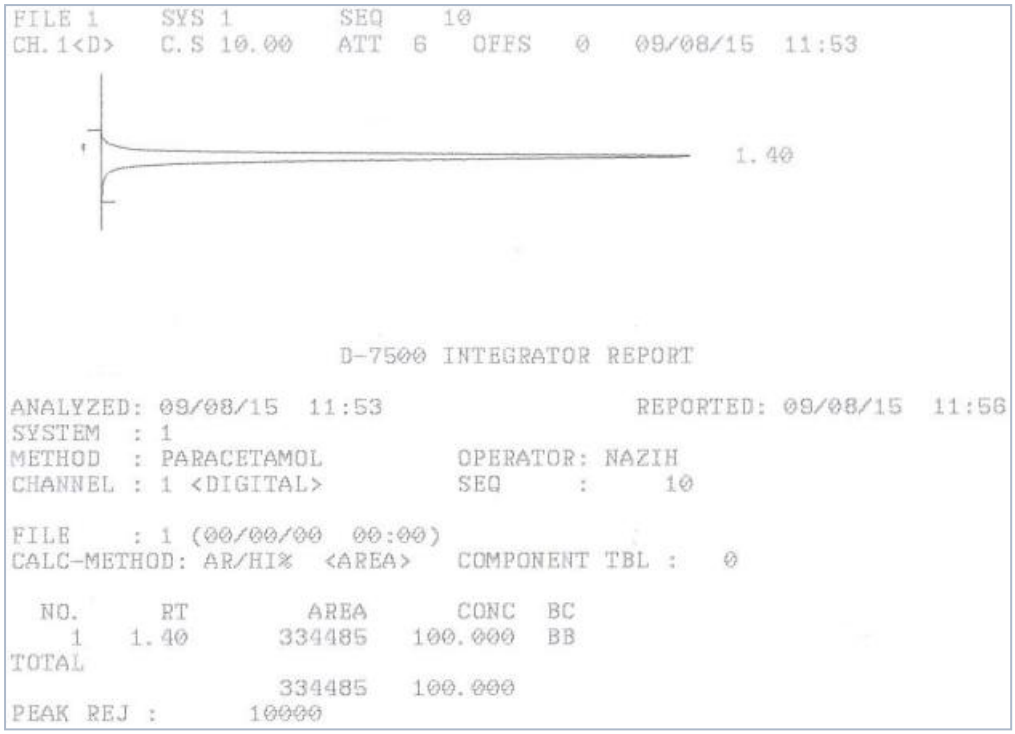
Positive control, 30 °C



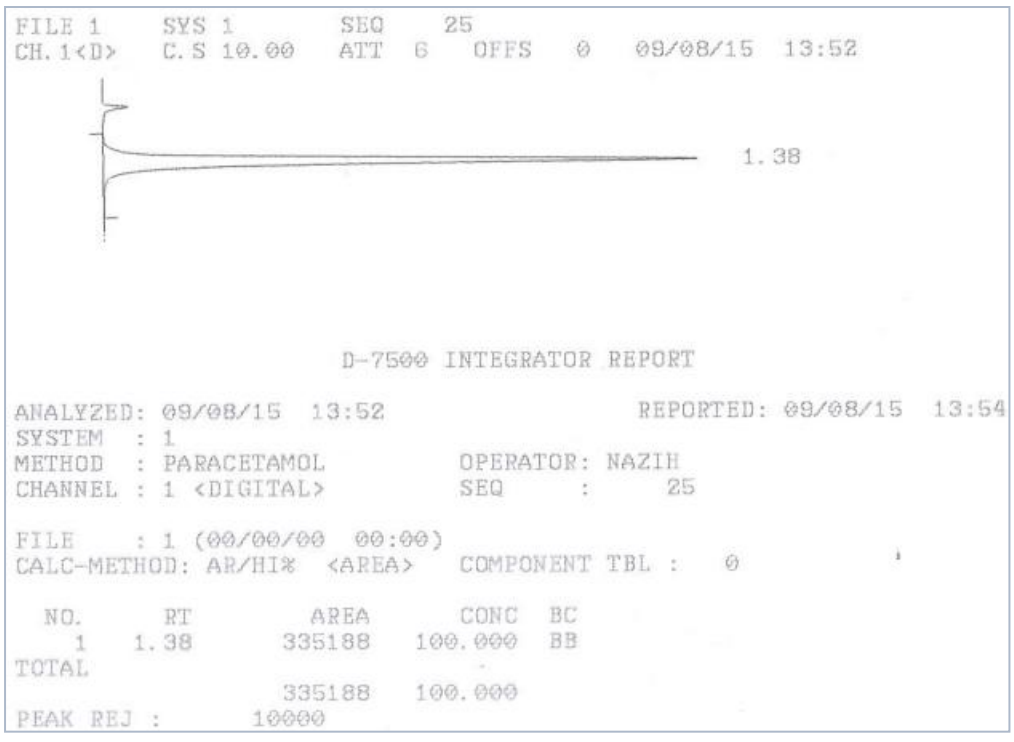
Negative control, 30 °C



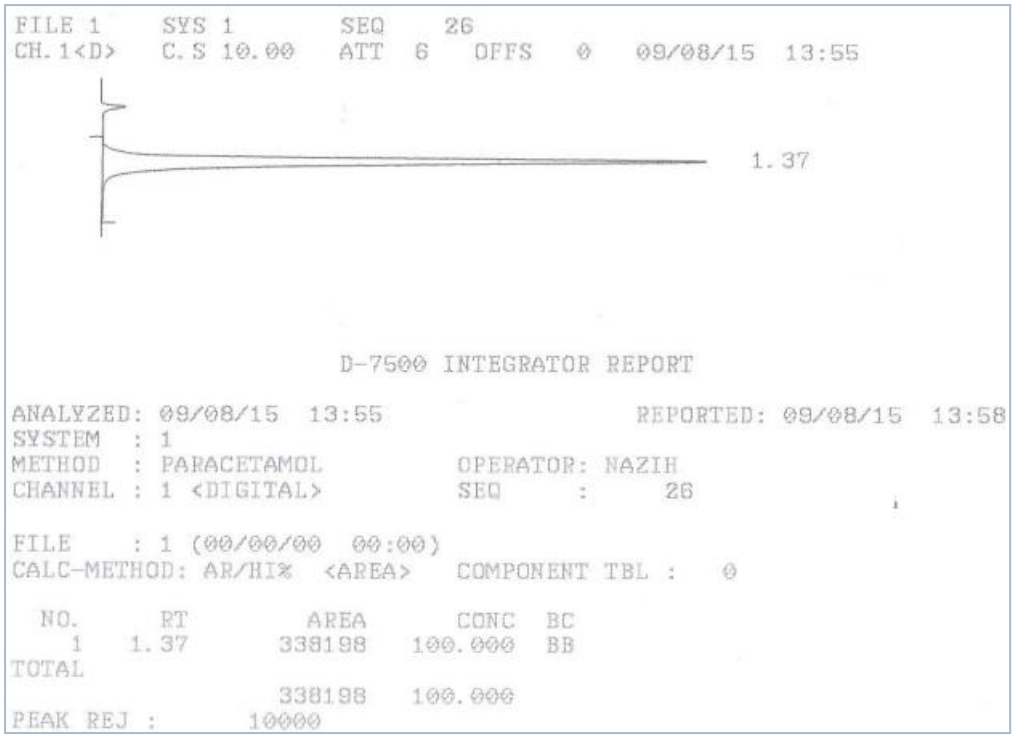
Thymol, 30 °C



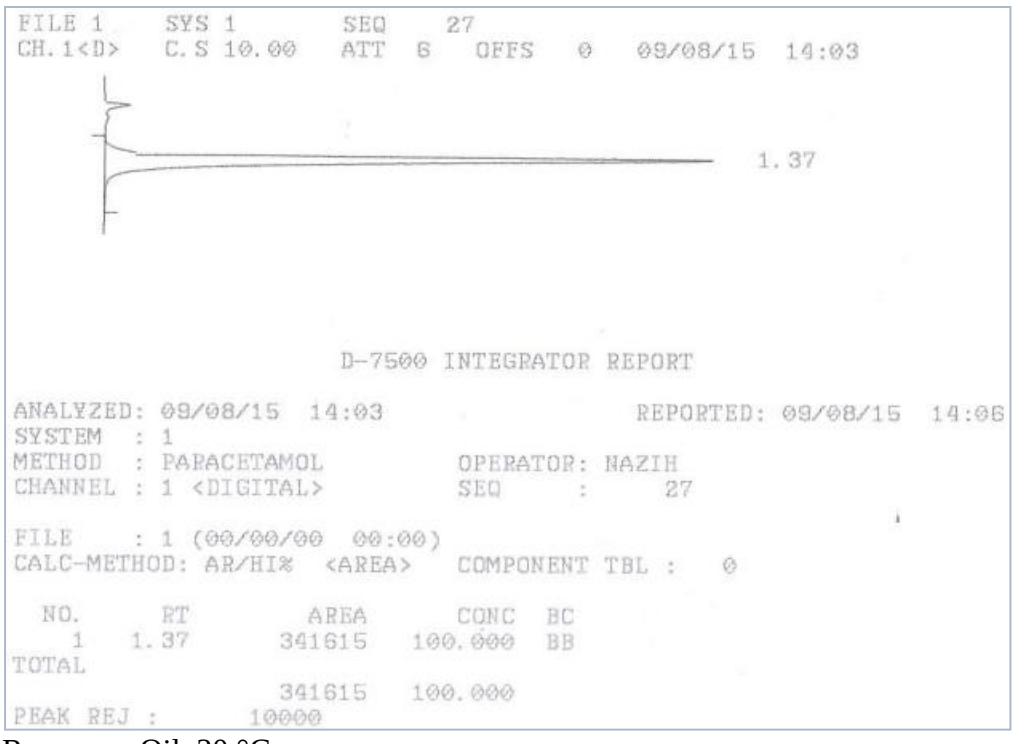
Rosmarinic Acid, 30 °C



Thymol and Rosmarinic Acid, 30 °C

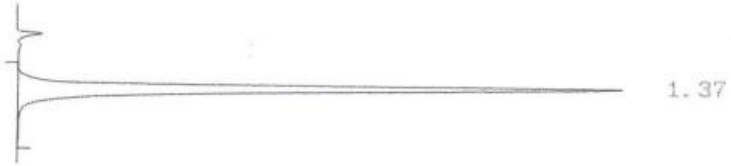


Thyme Oil, 30 °C



Rosemary Oil, 30 °C

FILE 1 SYS 1 SEQ 28
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 14:07



D-7500 INTEGRATOR REPORT

ANALYZED: 09/08/15 14:07 REPORTED: 09/08/15 14:09
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 28

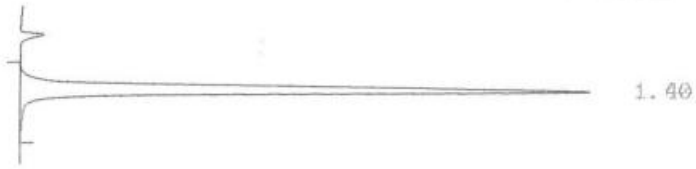
FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

NO.	RT	AREA	CONC	BC
1	1.37	336342	100.000	BB
TOTAL				
		336342	100.000	

PEAK REJ : 10000

Thyme Oil and Rosemary Oil, 30 °C

FILE 1 SYS 1 SEQ 32
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 14:39



D-7500 INTEGRATOR REPORT

ANALYZED: 09/08/15 14:39 REPORTED: 09/08/15 14:42
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 32

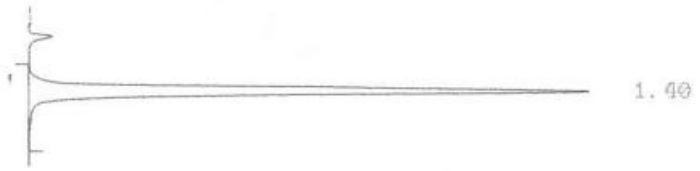
FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

NO.	RT	AREA	CONC	BC
1	1.40	340308	100.000	BB
TOTAL				
		340308	100.000	

PEAK REJ : 10000

Positive control, 40 °C

FILE 1 SYS 1 SEQ 33
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 14:42



D-7500 INTEGRATOR REPORT

ANALYZED: 09/08/15 14:42 REPORTED: 09/08/15 14:45
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 33

FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

NO.	RT	AREA	CONC	BC
1	1.40	334863	100.000	BB
TOTAL				
		334863	100.000	

PEAK REJ : 10000

Negative control, 40 °C

FILE 1 SYS 1 SEQ 34
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 14:46

D-7500 INTEGRATOR REPORT

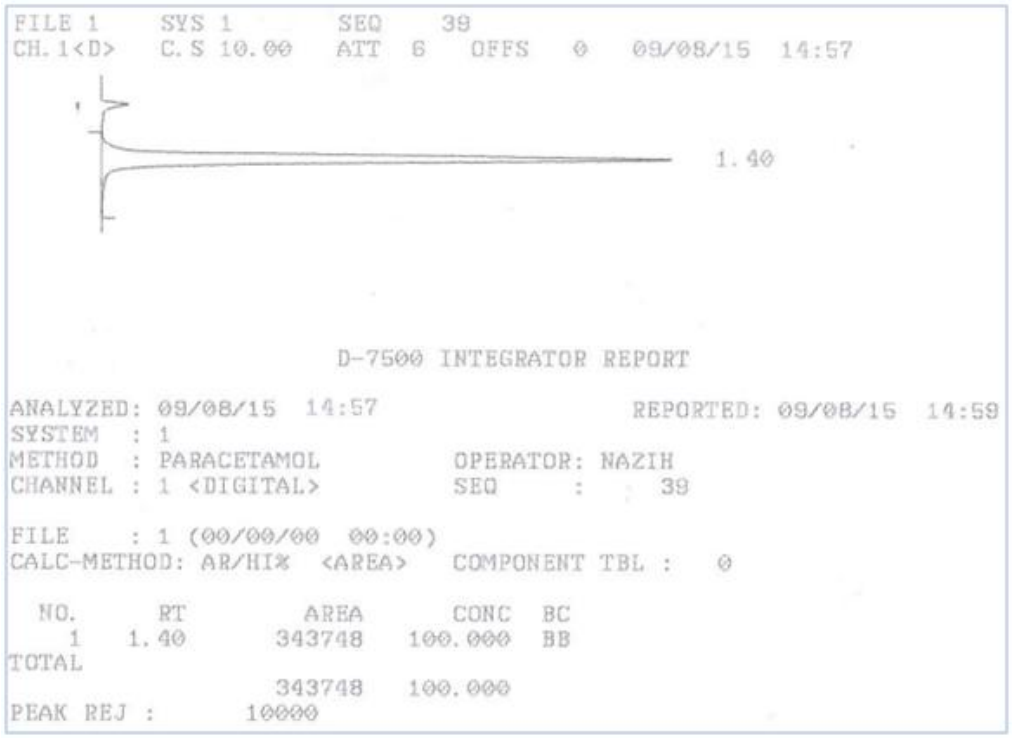
ANALYZED: 09/08/15 14:46 REPORTED: 09/08/15 14:48
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 34

FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

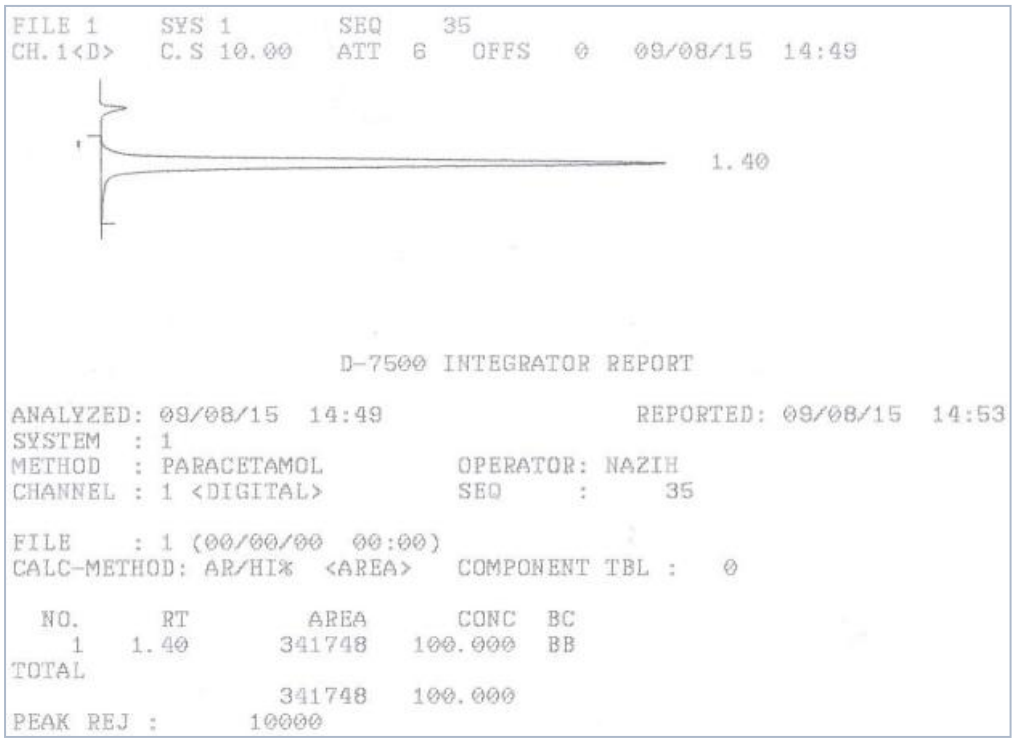
NO.	RT	AREA	CONC	BC
1	1.40	342262	100.000	BB
TOTAL				
		342262	100.000	

PEAK REJ : 10000

Thymol, 40 °C



Rosmarinic Acid, 40 °C



Thymol and Rosmarinic Acid, 40 °C

FILE 1 SYS 1 SEQ 36
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 14:55

D-7500 INTEGRATOR REPORT

ANALYZED: 09/08/15 14:55 REPORTED: 09/08/15 14:58
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 36

FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

NO.	RT	AREA	CONC	BC
1	1.40	343814	100.000	BB
TOTAL				
		343814	100.000	

PEAK REJ : 10000

Thyme Oil, 40 °C

FILE 1 SYS 1 SEQ 38
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 15:02

D-7500 INTEGRATOR REPORT

ANALYZED: 09/08/15 15:02 REPORTED: 09/08/15 15:05
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 38

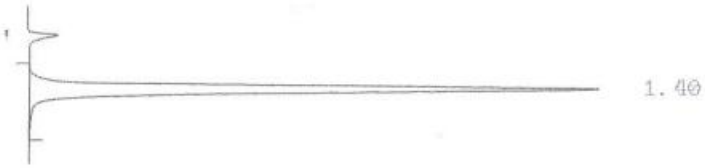
FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

NO.	RT	AREA	CONC	BC
1	1.40	339937	100.000	BB
TOTAL				
		339937	100.000	

PEAK REJ : 10000

Rosemary Oil, 40 °C

FILE 1 SYS 1 SEQ 40
CH.1<D> C.S 10.00 ATT 6 OFFS 0 09/08/15 15:10



D-7500 INTEGRATOR REPORT

ANALYZED: 09/08/15 15:10 REPORTED: 09/08/15 15:13
SYSTEM : 1
METHOD : PARACETAMOL OPERATOR: NAZIH
CHANNEL : 1 <DIGITAL> SEQ : 40

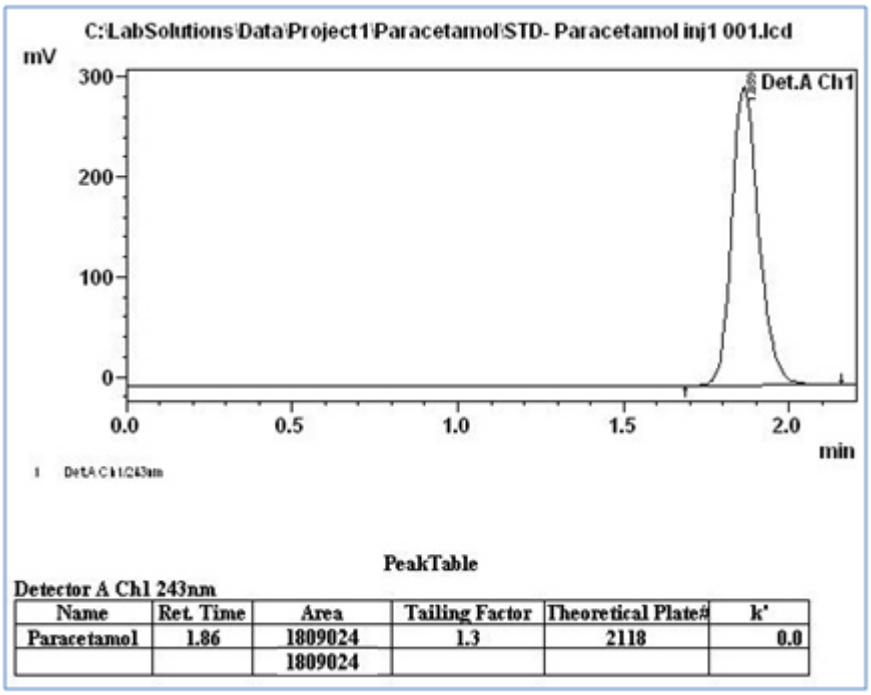
FILE : 1 (00/00/00 00:00)
CALC-METHOD: AR/HI% <AREA> COMPONENT TBL : 0

NO.	RT	AREA	CONC	BC
1	1.40	340262	100.000	BB
TOTAL		340262	100.000	

PEAK REJ : 10000

Thyme Oil and Rosemary Oil, 40 °C

HPLC output of Paracetamol active after six months:

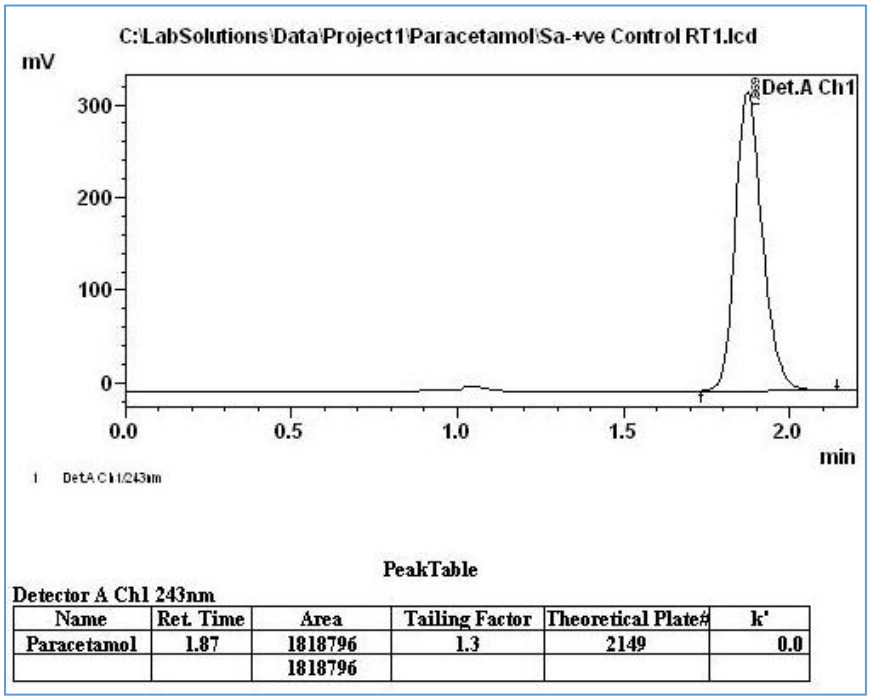


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.86	1809024	1.3	2118	0.0
		1809024			

Standard

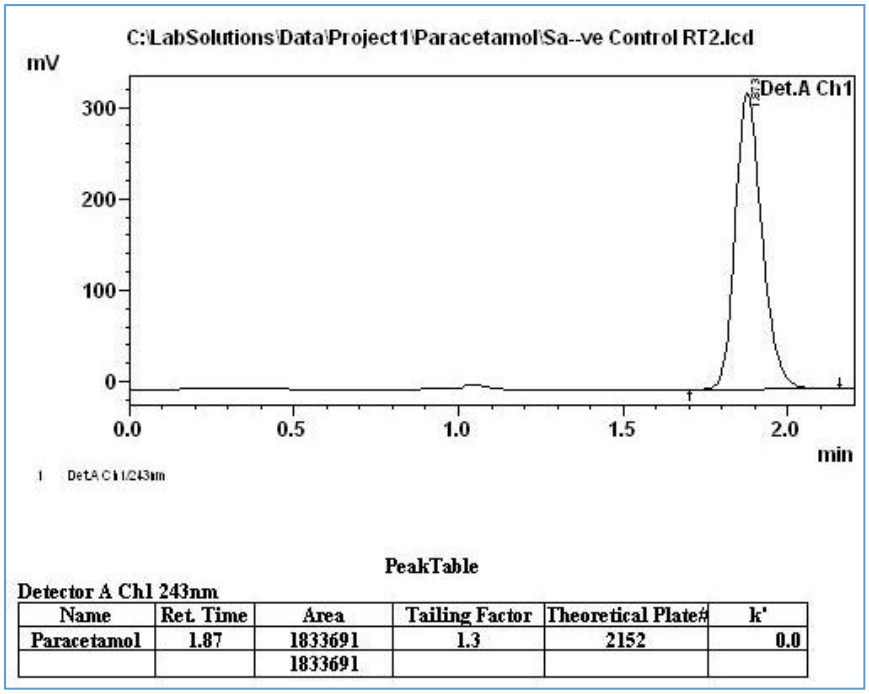


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.87	1818796	1.3	2149	0.0
		1818796			

Positive control, RT

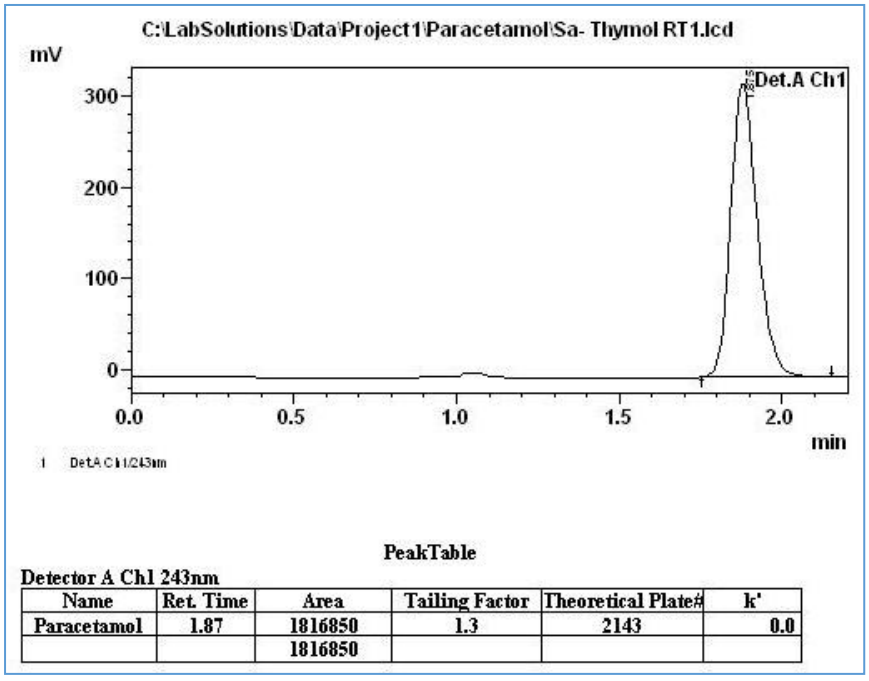


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.87	1833691	1.3	2152	0.0
		1833691			

Negative control, RT

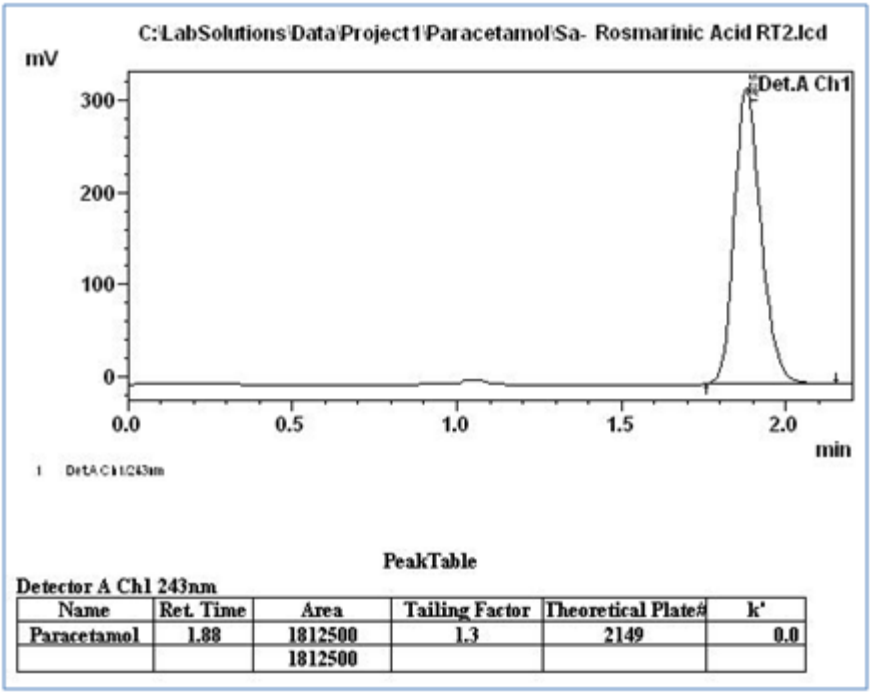


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.87	1816850	1.3	2143	0.0
		1816850			

Thymol, RT

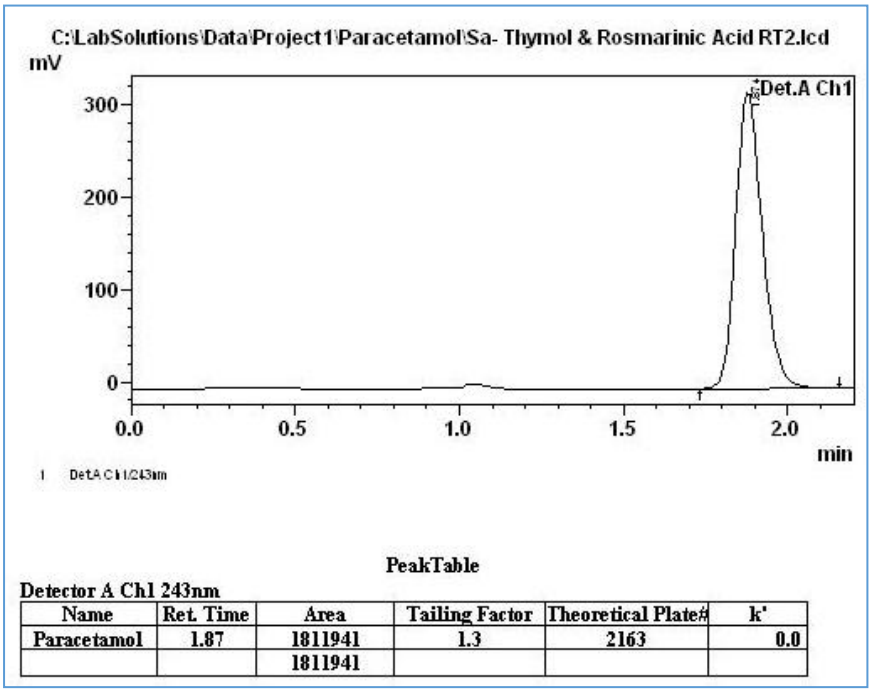


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1812500	1.3	2149	0.0
		1812500			

Rosmarinic Acid, RT

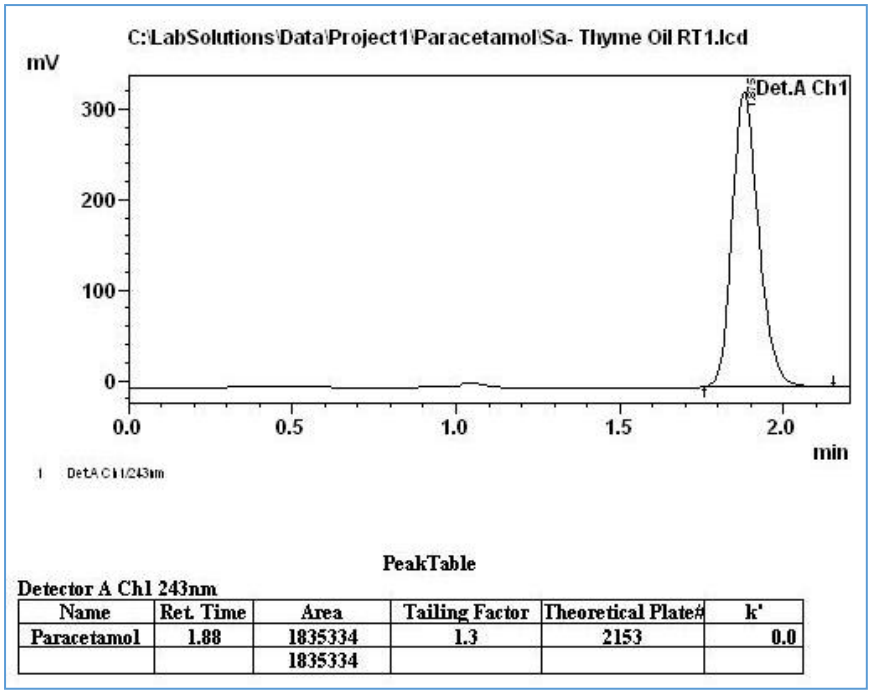


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.87	1811941	1.3	2163	0.0
		1811941			

Thymol and Rosmarinic Acid, RT

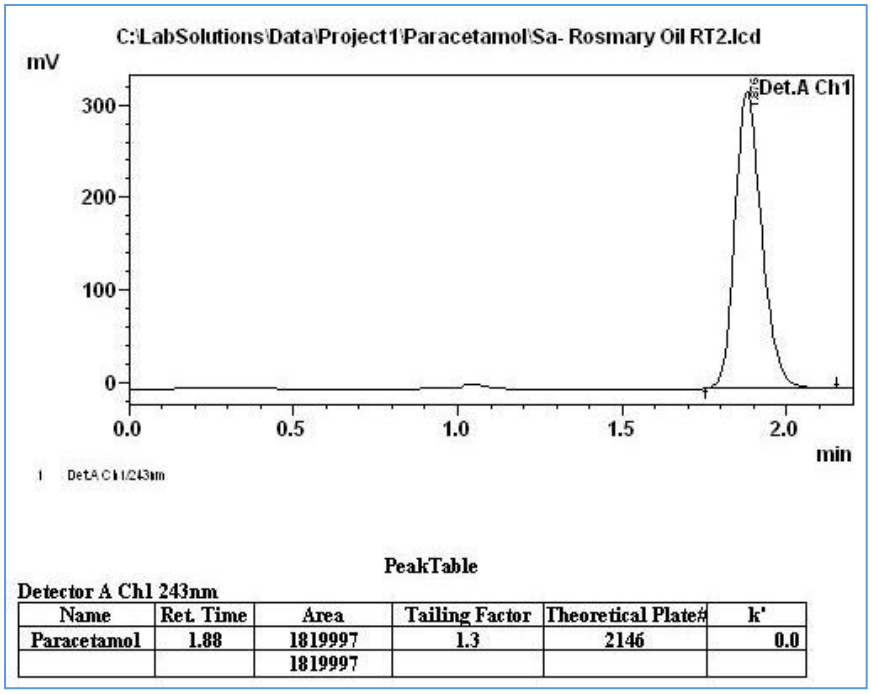


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1835334	1.3	2153	0.0
		1835334			

Thyme Oil, RT

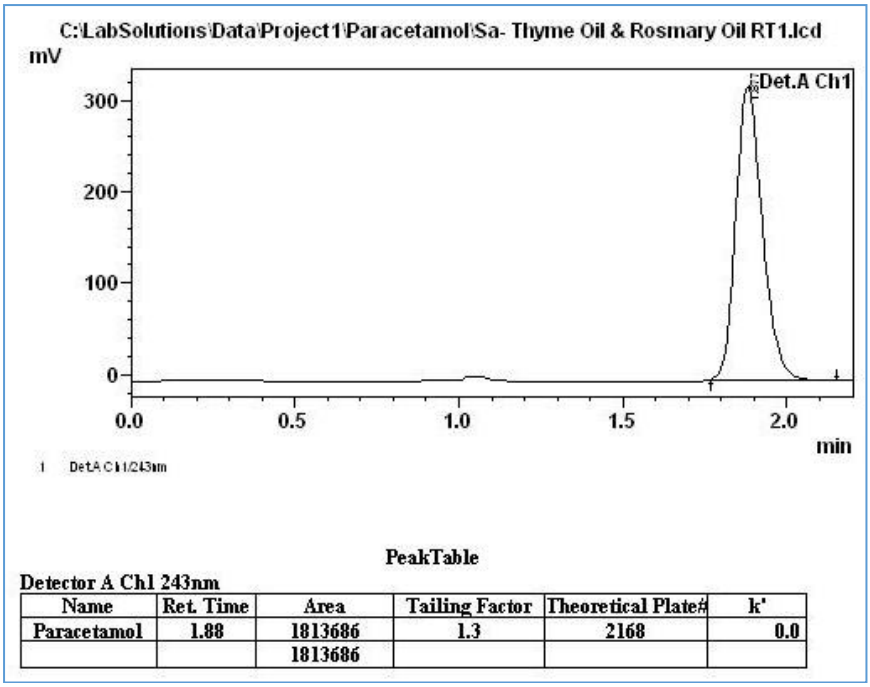


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1819997	1.3	2146	0.0
		1819997			

Rosemary Oil, RT

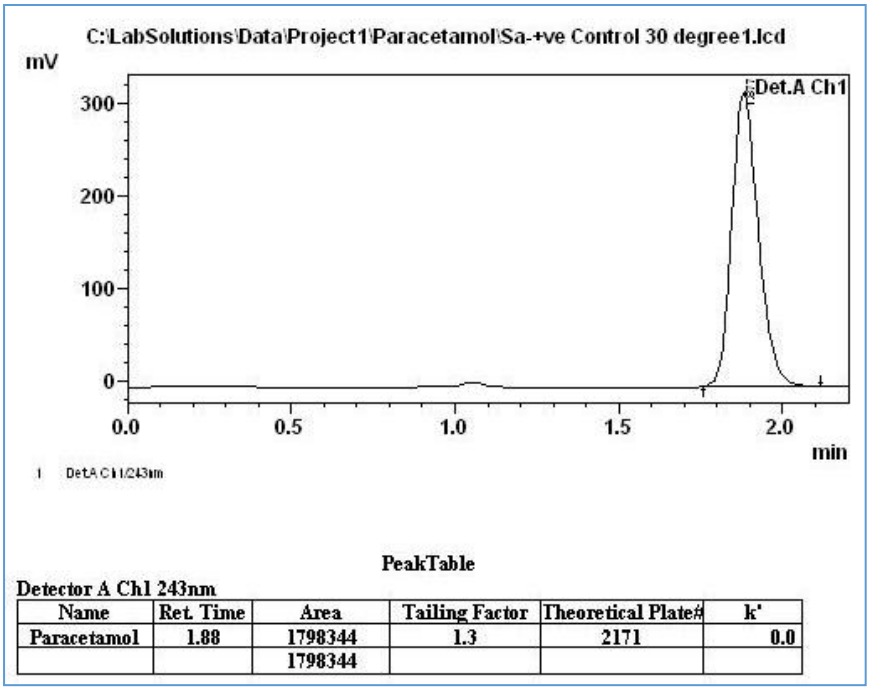


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1813686	1.3	2168	0.0
		1813686			

Thyme Oil and Rosemary Oil, RT

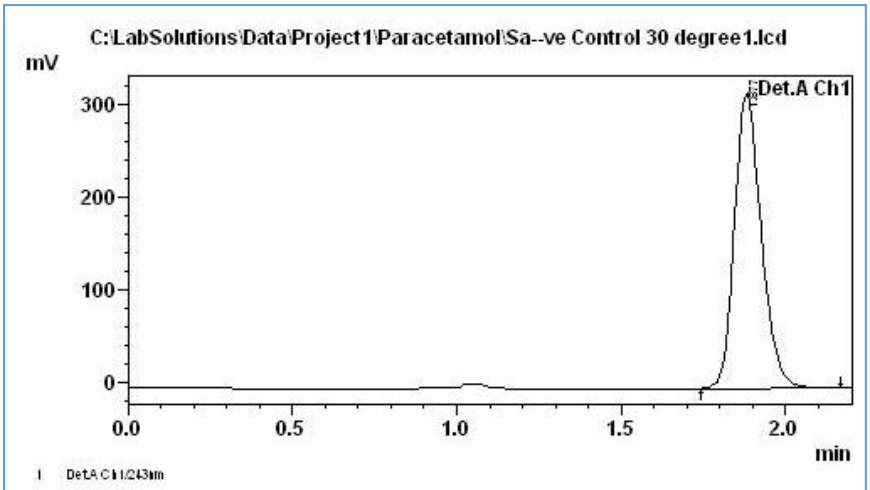


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1798344	1.3	2171	0.0
		1798344			

Positive control, 30 °C

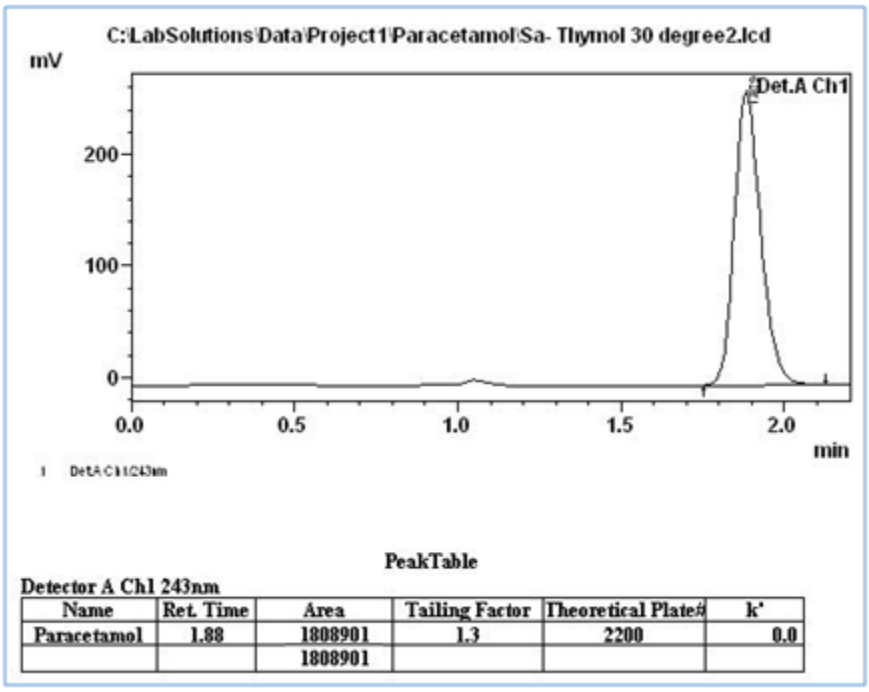


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1810933	1.3	2161	0.0
		1810933			

Negative control, 30 °C

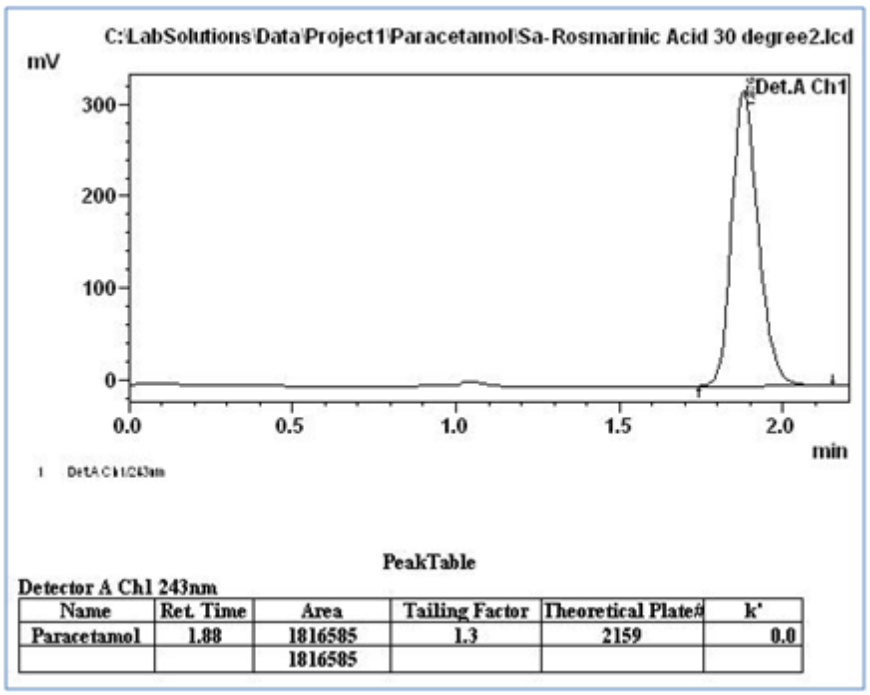


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1808901	1.3	2200	0.0
		1808901			

Thymol, 30 °C

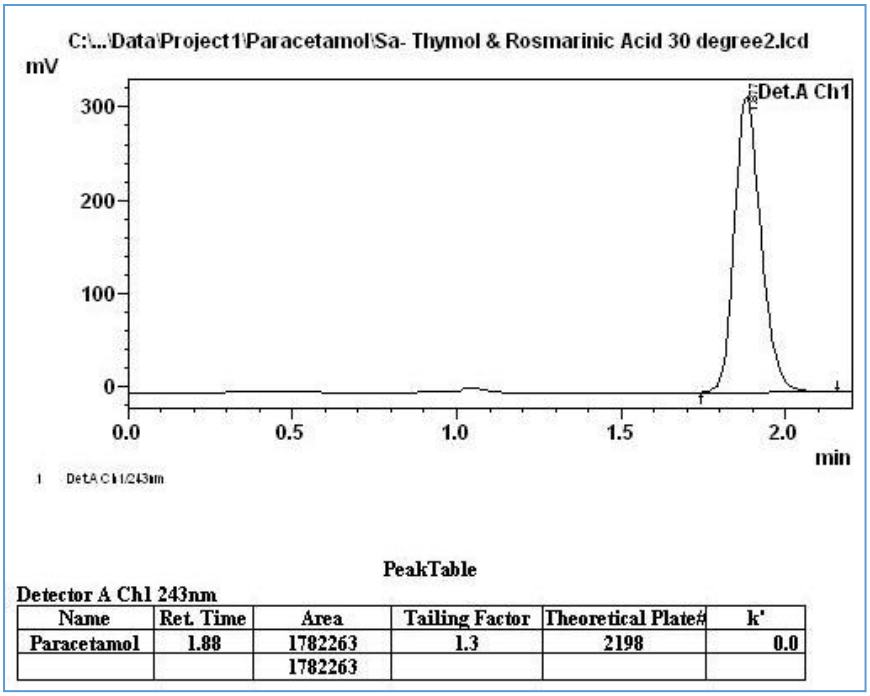


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1816585	1.3	2159	0.0
		1816585			

Rosmarinic Acid, 30 °C

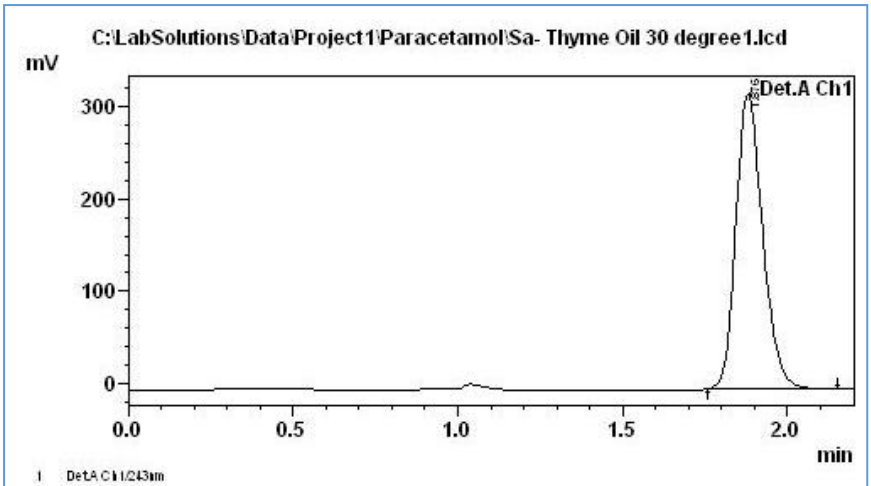


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1782263	1.3	2198	0.0
		1782263			

Thymol and Rosmarinic Acid, 30 °C

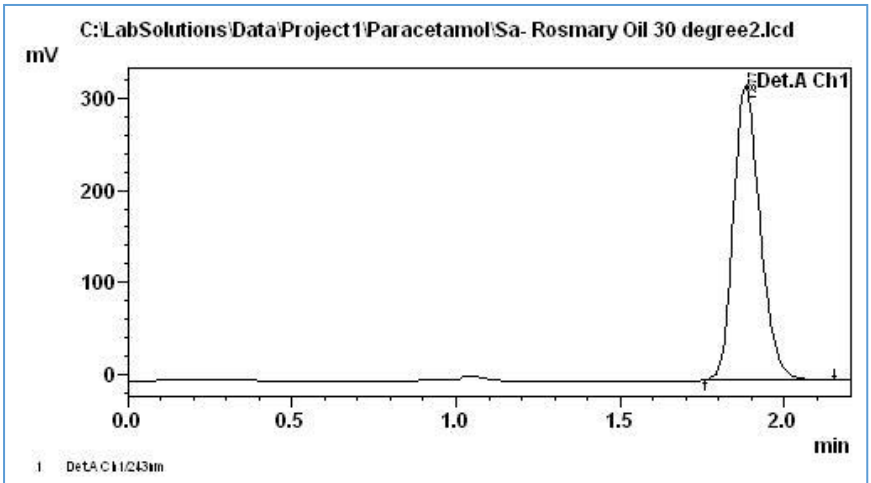


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1814343	1.3	2160	0.0
		1814343			

Thyme Oil, 30 °C

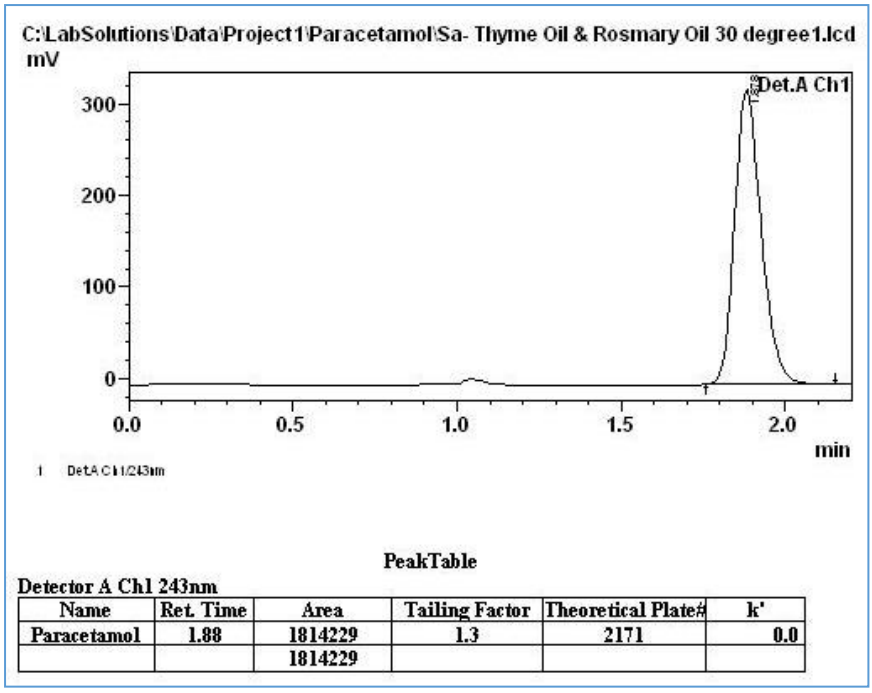


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1809537	1.3	2169	0.0
		1809537			

Rosemary Oil, 30 °C

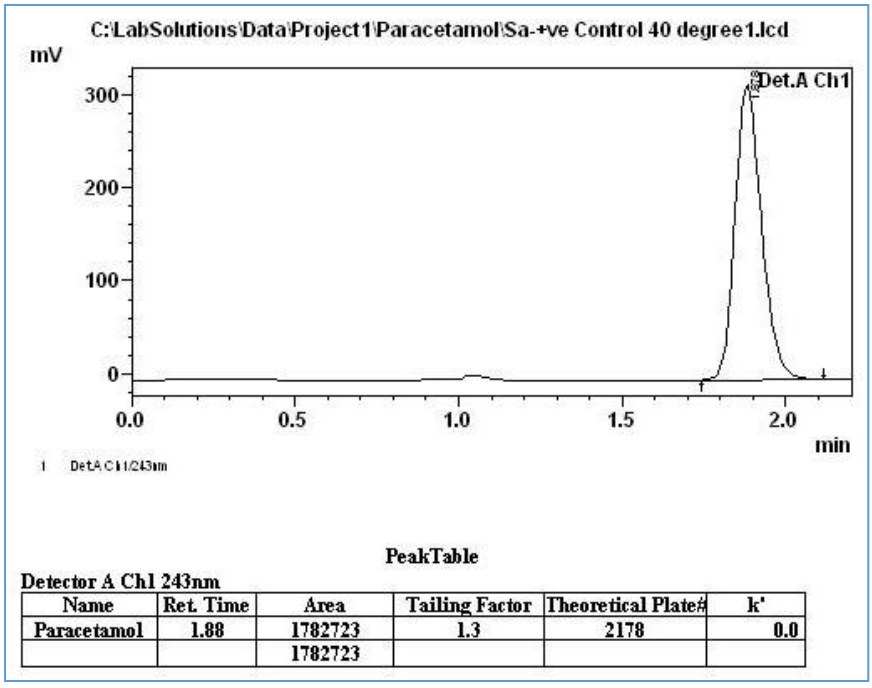


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1814229	1.3	2171	0.0
		1814229			

Thyme Oil and Rosemary Oil, 30 °C

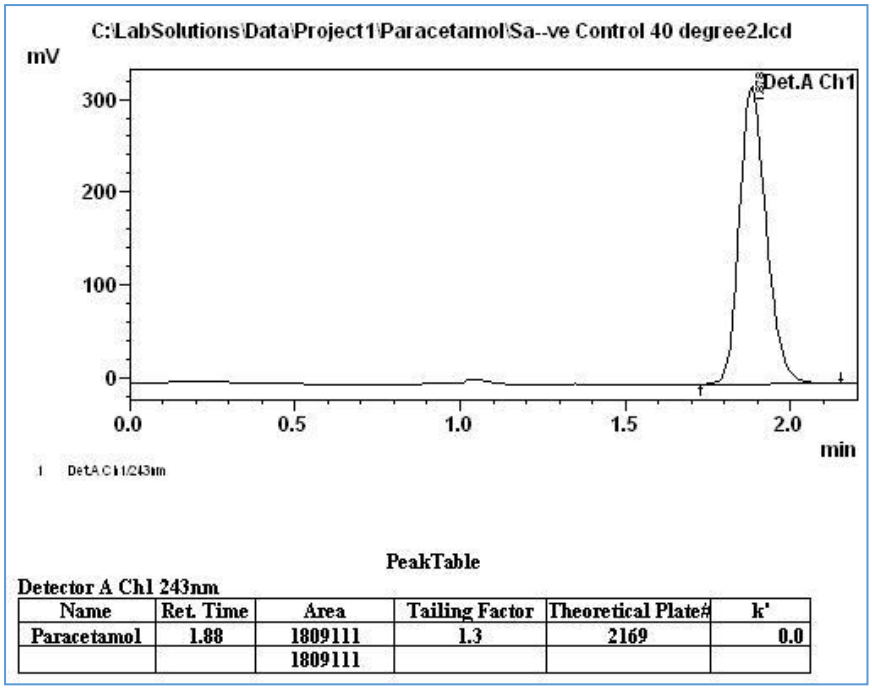


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1782723	1.3	2178	0.0
		1782723			

Positive control, 40 °C

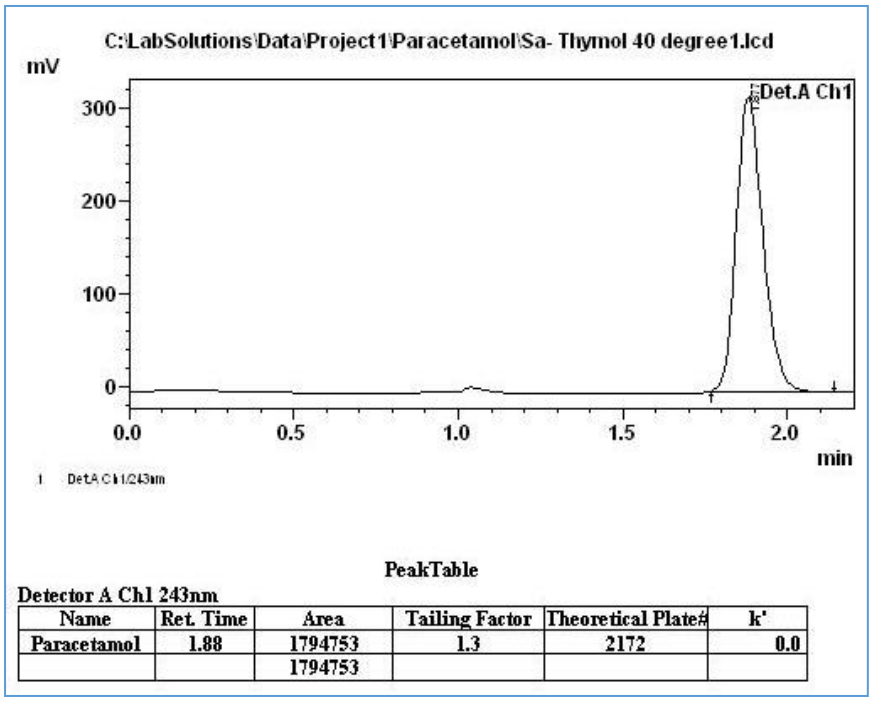


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1809111	1.3	2169	0.0
		1809111			

Negative control, 40 °C

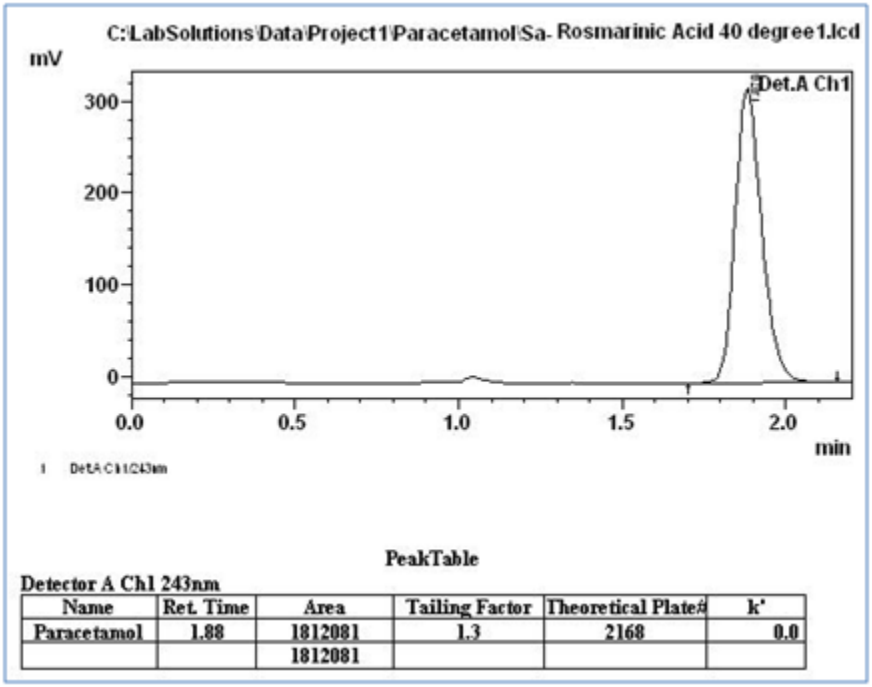


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1794753	1.3	2172	0.0
		1794753			

Thymol, 40 °C

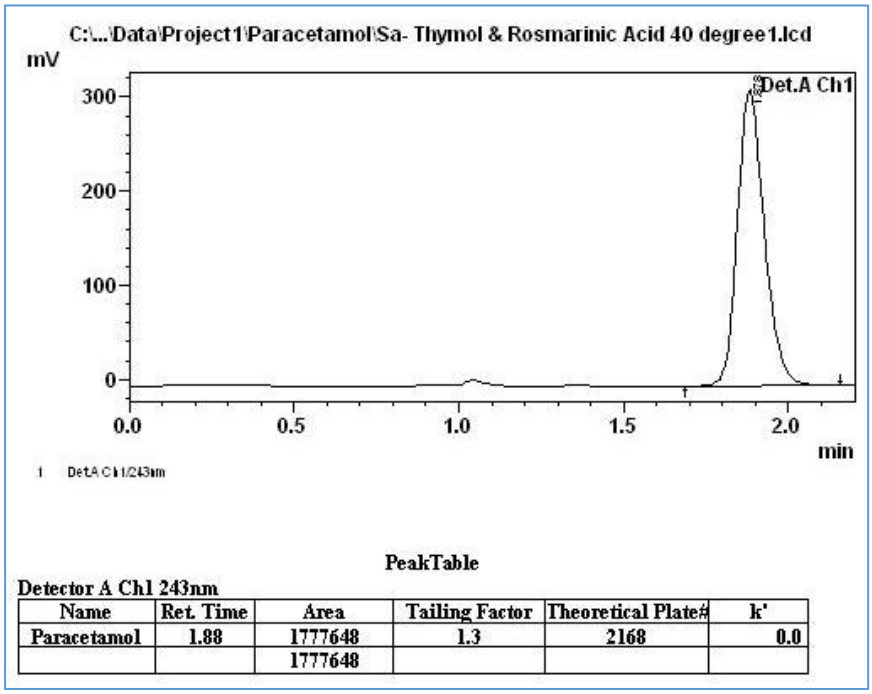


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1812081	1.3	2168	0.0
		1812081			

Rosmarinic Acid, 40 °C

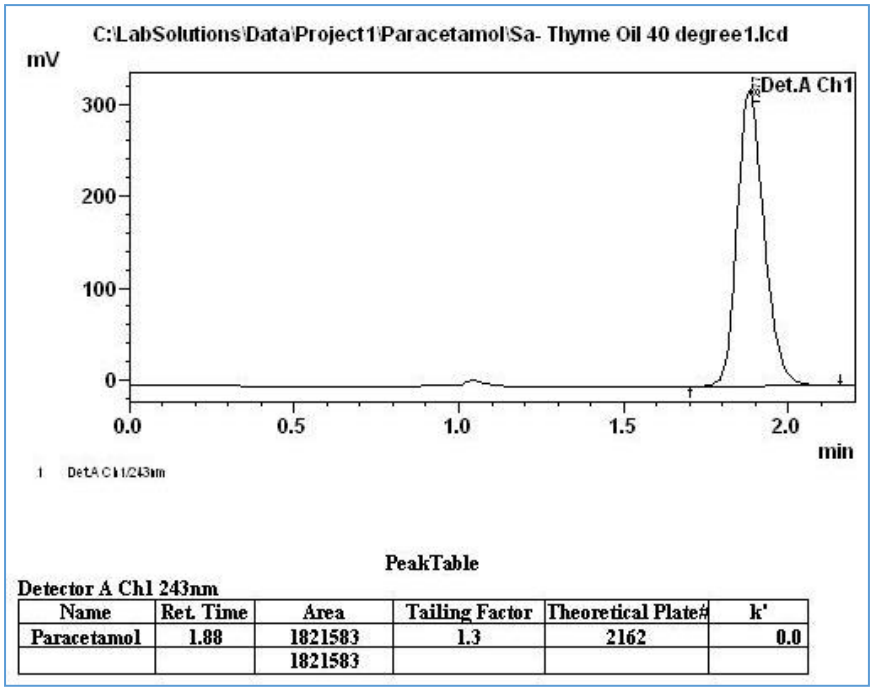


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1777648	1.3	2168	0.0
		1777648			

Thymol and Rosmarinic Acid, 40 °C

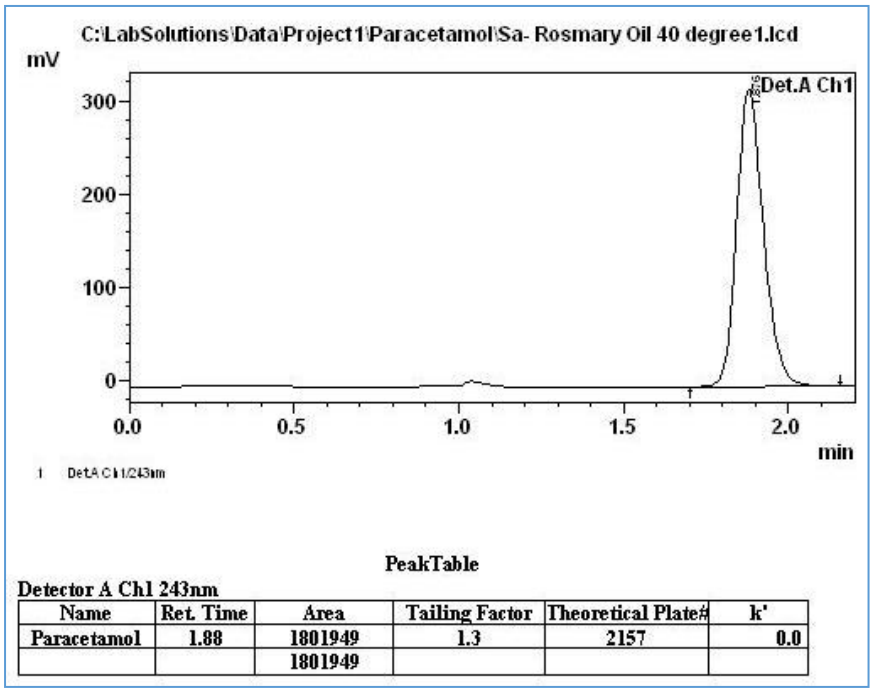


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1821583	1.3	2162	0.0
		1821583			

Thyme Oil, 40 °C

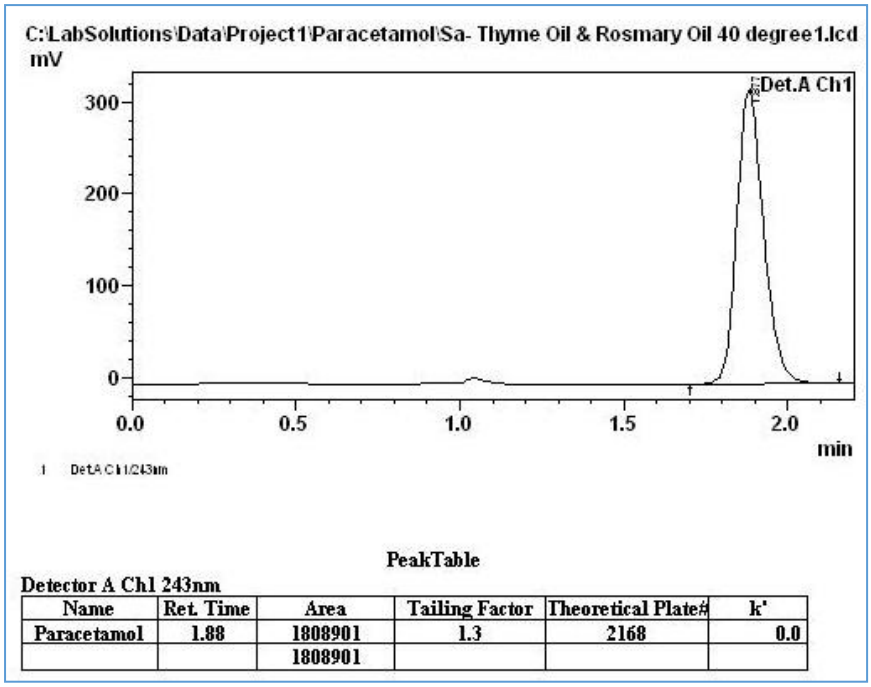


PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1801949	1.3	2157	0.0
		1801949			

Rosemary Oil, 40 °C



PeakTable

Detector A Ch1 243nm

Name	Ret. Time	Area	Tailing Factor	Theoretical Plate#	k'
Paracetamol	1.88	1808901	1.3	2168	0.0
		1808901			

Thyme Oil and Rosemary Oil, 40 °C