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DEPARTMENT OF PHARMACEUTICAL CHEMISTRY

DESIGN OF HPLC METHOD FOR SIMULTANEOUS QUANTIFICATION OF PARACETAMOL AND P-AMINOPHENOL IN PARACETAMOL FORMULATIONS: TABLETS, SYRUPS AND SUSPENSIONS.

BY

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DECLARATION

AH YAHAYA, declare that this work is the result of my own
.h and that this thesis has neither in part nor whole been presented elsewhere for another degree.

This is submitted in partial **fulfillment** of the requirement for the award of Master of Philosophy Degree (Pharmaceutical Analysis and Quality Control) at the Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi-Ghana.

The various journals, books, periodicals and the other sources I consulted for information have been duly acknowledged by reference to the authors.

ISSAH YAHAYA ..... 16/04/13 .....
(Student)

I declare that I have supervised the student in undertaking the study submitted herein and I confirm that he, ISSAH YAHAYA, has the permission to present it for assessment.

Prof. Nathaniel N-A Okine ..... 22/04/13 .....
(Supervisor)

Prof. Remmel K. Adosraku ..... 22-4-13 .....
(Head of Department)

DEDICATION

This thesis is dedicated to:

The Glory and Oneness of Almighty **ALLAH**

My lovely wife, **SHARIFATU ABDUL-SAMED**

My dear son, **RAIYAN ISSAH**

And

ISSAH YAHAYA, for being so determined to achieve success single-handedly.

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ABSTRACT

A simple isocratic reversed-phase HPLC method for the determination of paracetamol and its major degradation impurity, *p*-aminophenol; was developed and validated for use in the analysis of paracetamol formulations. Separations were performed on a C_{18} Inertsil® ODS-3 column (250mm x 4.60mm, 5µm). A mobile phase, 1% Glacial acetic acid: Methanol (85:15v/v) at flow rate of 1 ml/min was found suitable for the separation and determination of paracetamol and *p*-aminophenol. UV detection was set at 278nm. The chromatographic parameters such as retention times, capacity factor, tailing factor, number of theoretical plates, %RSD of peak area and resolution factors were determined. The retention times were 5:65±0.15mins. for paracetamol and 2:48±0.15mins for p-aminophenol respectively. The proposed RP–HPLC method was shown to be specific, linear in the range of 0.0001 – 0.001% $^{w}/_{v}$ (R^2 = 0.9990 and 0.9994 for paracetamol and *p*-aminophenol), precise at intra-day level (%RSD; 0.227 for paracetamol and 0.187 *p*-aminophenol), precise at inter-day level (%RSD; 0.225 for paracetamol and 0.218 *p*-aminophenol), accurate (recovery rates of 100.16±0.60-100.41±0.35 for paracetamol and 100.30±1.34-101.13±1.92 for *p*-aminophenol). The detection and quantitation limits were 1.65×10^{-6} %^{w}/v and 5.01×10^{-6} %^{w}/v for paracetamol and 1.499×10^{-6} %^{w}/v and 4.455×10^{-6} %^{w}/v for *p*-aminophenol.

Validation acceptance criteria were met in all cases. This method was used successfully for the quality assessment of thirty paracetamol formulations in Ghana.

In all the thirty paracetamol formulations (tablets, syrups and suspensions), not a single one has shown high level of p-aminophenol.

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CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The search for chemical substances for the cure of ailments and diseases is as old as mankind. Throughout the ages, human beings have searched for substances which will not only cure their ailments but also change their perceptions on medicines in a number of ways. Thus, over the ages, humankind have and continue to use drugs and chemical substances for a variety of reasons, such as; to relieve pain, reduce stress, etc.

Paracetamol is one of the most commonly used drugs, for relieve of pain and to reduce stress, in the world.

In Ghana, over the years, paracetamol has been used as analgesic either alone or in combination with other drugs in pharmaceutical formulations (tablets, syrups, suspensions, etc.). A combination of paracetamol and caffeine, and paracetamol and other active ingredients are commercially available in tablet dosage form.

Handling and storage of paracetamol formulations have not been the best in Ghana. Some people place it close to the windscreens of their private cars, on top of their refrigerators or fridges (which exposes them to both light and heat) and on their hall tables (which exposes them to moisture) at home, and others in hand bags.

In Northern Ghana, temperatures could reach as high as 40 to 50°C, which may affect the stability of drugs. Therefore, there is the need to conduct some stability studies on paracetamol formulations to ascertain whether they maintain their physicochemical properties at temperature as high as 50°C.

1.1.1 Justification of the Study

- Paracetamol is being increasingly used for therapeutic purposes.
- The development of a simple, precise and accurate method for the determination of paracetamol.
- The various methods reported for the determination of paracetamol in body fluids and pharmaceutical preparations include spectroscopy, liquid chromatography, capillary electrophoresis and enzyme based assay methods.

1.1.2 Objectives of the research

The main objectives of the research are:

1. To design a sensitive, isocratic HPLC method, with UV detection that can be used to detect both Paracetamol and p-Amiophenol in the pure form.
2. To employ the designed method in the quantification and quality control of paracetamol and its main degradation, *p*-aminophenol in paracetamol.
3. To simultaneously quantify both paracetamol and p-aminophenol in each batch of paracetamol formulations sampled in the Kumasi metropolis using the HPLC method developed.
4. To identify the presence of p-aminophenol in the paracetamol formulations sampled, and ascertain whether the level, if detected, conform to the acceptable level (**50ppm or 0.005%$^{w}/_{w}$**).
5. To monitor the stability of paracetamol in the paracetamol tablets using the method that has been developed.

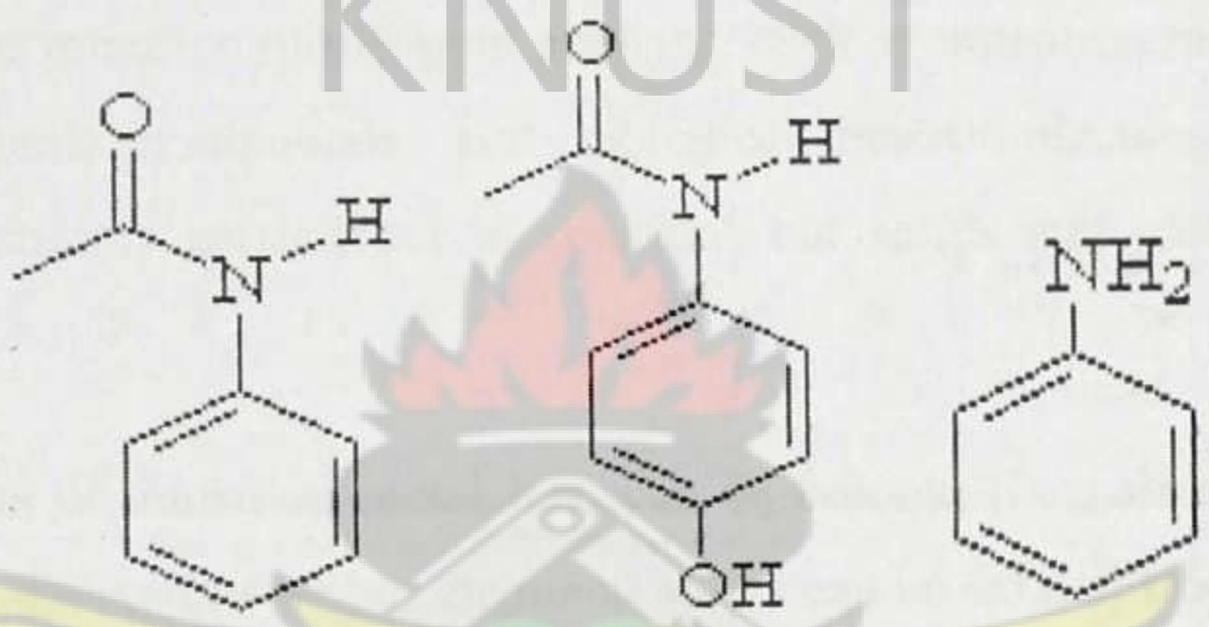
1.2 Literature Review

Paracetamol, **N-4-hydroxyphenyl acetamide**. In the US Pharmacopoeia it is known as **acetaminophen**. Paracetamol is a widely used over-the-counter analgesic and antipyretic. It is commonly used for the relief of fever, headaches, and other minor aches and pains, and is a major ingredient in numerous cold and flu remedies. In combination with **non-steroidal anti-inflammatory drugs (NSAIDs)** and opioid analgesics, paracetamol is used also in the management of more severe pain (such as postoperative pain).

Paracetamol was first discovered to have both analgesic and antipyretic properties in the late nineteenth century. Prior to this, cinchona bark, which was also used to make the anti-malaria drug quinine, had been used to treat fevers. As cinchona became scarce, people began to look for cheaper synthetic alternatives. Two of these alternative compounds were **acetanilide and phenacetin**, developed in 1886 and 1887 respectively. By this time, Harmon Northrop Morse had already synthesized acetaminophen in 1878 through the reduction of p-nitrophenol with tin in glacial acetic acid. In 1893, the white, odourless crystalline compound with a bitter taste that became known as paracetamol was discovered. While generally safe for use at recommended doses (1,000mg per single dose for adults and up to 4,000 mg per day for adults, also, up to 2,000mg per day if drinking alcohol),[9] acute overdoses of paracetamol can cause potentially fatal liver damage and, in rare individuals, a normal dose can do the same. The risk is heightened by alcohol consumption [9]. Paracetamol toxicity is the foremost cause of acute liver failure in the Western world, and accounts for most drug overdoses in the United States, the United Kingdom, Australia and New Zealand [10-13].

Paracetamol is derived from coal tar, and is part of the class of drugs known as "aniline analgesics"; it is the only such drug still in use today. It is the active metabolite of phenacetin, once popular as an analgesic and antipyretic in its own right, but unlike phenacetin and its combinations, paracetamol is not considered to be carcinogenic at therapeutic doses [14]. The words acetaminophen and paracetamol both come from chemical names for the compound: N-**acet**yl-para-**aminophen**ol and **para-acet**yl-**am**ino-phenol.

The onset of analgesia is approximately eleven minutes after oral administration of paracetamol,[15] and its half-life is one to four hours. The analgesic properties of paracetamol were discovered by accident when a similar molecule (acetanilide) was added to a patient's prescription about 100 years ago [11]. But since acetanilide is toxic in moderate doses, chemists modified its structure to try and find a compound that was less harmful but which still retained the analgesic properties, which is also known as acetaminophen in the US and paracetamol (from ***para*-acet**yl-**am**ino-phen**ol**) in the UK.



Acetanilide Paracetamol Aniline

In fact, in the body, the original compound, acetanilide is partially converted into a mixture of paracetamol and aniline. The paracetamol provides the analgesic properties, but the aniline is toxic. Paracetamol has a very similar structure to aspirin, and because of this they are recognised by the same enzyme. This enzyme is responsible for the biosynthesis of prostoglandins, which are involved in the dilation of blood vessels that causes the pain experienced in a headache. Reduction of the amount of prostoglandin, therefore, helps prevent headaches and other pain.

1.2.1 Ultraviolet–visible Spectroscopy

Ultraviolet–visible spectroscopy or ultraviolet-visible spectrophotometry (UV-Vis or UV/Vis) refers to absorption spectroscopy or reflectance spectroscopy in the ultraviolet-visible spectral region. This means it uses light in the visible and adjacent (near-UV and near-infrared ranges. The absorption or reflectance in the visible range affects the perceived color of the chemicals involved. [16]

Principle of Ultraviolet-Visible Absorption

Molecules containing π-electrons or non-bonding electrons can absorb the energy in the form of ultraviolet or visible light to excite these electrons to higher anti-bonding molecular orbitals.[17] The more easily excited the electrons the longer the wavelength of light it can absorb.

Applications

Ultraviolet–visible spectroscopy is routinely used in analytical chemistry for the quantitative determination of different analytes, such as transition metal ions, highly conjugated organic compounds, and biological macromolecules. Spectroscopic analysis is commonly carried out in solutions but solids and gases may also be studied.

- Solutions of transition metal ions can be colored (i.e., absorb visible light) because d electrons within the metal atoms can be excited from one electronic state to another. The colour of metal ion solutions is strongly affected by the presence of other species, such as certain anions or ligands. For instance, the colour of a dilute solution of copper sulfate is a very light blue; adding ammonia intensifies the colour and changes the wavelength of maximum absorption (λ_{max}).
- Organic compounds, especially those with a high degree of conjugation (e.g. DNA, RNA, protein), also absorb light in the UV or visible regions of the electromagnetic spectrum. The solvents for these determinations are often water for water-soluble compounds, or ethanol for organic-soluble compounds. (Organic solvents may have significant UV absorption; not all solvents are suitable for use in UV spectroscopy. Ethanol absorbs very weakly at most wavelengths.) Solvent polarity and pH can affect the absorption spectrum of an organic compound. Tyrosine, for example, increases in absorption maxima and molar extinction coefficient when pH increases from 6 to 13 or when solvent polarity decreases.
- While charge transfer complexes also give rise to colours, the colours are often too intense to be used for quantitative measurement.

The **Beer-Lambert** law states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution and the path length. Thus, for a fixed path length, UV/Vis spectroscopy can be used to determine the concentration of the absorber in a solution. It is necessary to know how quickly the absorbance changes with concentration. This can be taken from references (tables of molar extinction coefficients), or more accurately, determined from a calibration curve.

A UV/Vis spectrophotometer may be used as a detector for HPLC. The presence of an analyte gives a response assumed to be proportional to the concentration. For accurate results, the instrument's response to the analyte in the unknown should be compared with the response to a standard; this is very similar to the use of calibration curves. The response (e.g., peak height) for a particular concentration is known as the response factor.

The wavelengths of absorption peaks can be correlated with the types of bonds in a given molecule and are valuable in determining the functional groups within a molecule. The **Woodward-Fieser** rules, for instance, are a set of empirical observations used to predict λ_{max}, the wavelength of the most intense UV/Vis absorption, for conjugated organic compounds such as dienes and ketones. The spectrum alone is not, however, a specific test for any given sample. The nature of the solvent, the pH of the solution, temperature, high electrolyte concentrations, and the presence of interfering substances can influence the absorption spectrum. Experimental variations such as the slit width (effective bandwidth) of the spectrophotometer will also alter the spectrum. To apply UV/Vis spectroscopy to analysis, these variables must be controlled or accounted for in order to identify the substances present.[18]

Beer-Lambert law

The method is most often used in a quantitative way to determine concentrations of an absorbing species in solution, using the Beer-Lambert law:

$$\log_{10}\left(\frac{I_0}{I}\right) = \varepsilon c l \quad \text{or} \quad \varepsilon = \frac{A}{cl}$$

Where A is the measured absorbance, I_0 is the intensity of the incident light at a given wavelength, I is the transmitted intensity, l the pathlength through the sample, and c the concentration of the absorbing species. For each species and wavelength, ε is a **constant known as the molar absorptivity or extinction coefficient**. This constant is a fundamental molecular property in a given solvent, at a particular temperature and pressure.

The absorbance and extinction ε are sometimes defined in terms of the natural logarithm instead of the base-10 logarithm.

The Beer-Lambert Law is useful for characterizing many compounds but does not hold as a universal relationship for the concentration and absorption of all substances. A second order polynomial relationship between absorption and concentration is sometimes encountered for very large, complex molecules such as organic dyes (Xylenol Orange or Neutral Red, for example).

Practical considerations

The Beer-Lambert law has implicit assumptions that must be met experimentally for it to apply. For instance, the chemical makeup and physical environment of the sample can alter its extinction coefficient. The chemical and physical conditions of a test sample therefore must match reference measurements for conclusions to be valid.

Spectral bandwidth: A given spectrometer has a spectral bandwidth that characterizes how monochromatic the light is. If this bandwidth is comparable to the width of the absorption features, then the measured extinction coefficient will be altered. In most reference measurements, the instrument bandwidth is kept below the width of the spectral lines. When a new material is being measured, it may be necessary to test and verify if the bandwidth is sufficiently narrow. Reducing the spectral bandwidth will reduce the energy passed to the detector and will, therefore, require a longer measurement time to achieve the same signal to noise ratio.

Wavelength error: In liquids, the extinction coefficient usually changes slowly with wavelength. A peak of the absorbance curve (a wavelength where the absorbance reaches a maximum) is where the rate of change in absorbance with wavelength is

smallest. Measurements are usually made at a peak to minimize errors produced by errors in wavelength in the instrument. That is, errors due to having a different extinction coefficient than assumed.

Stray light: Another important factor is the *purity* of the light used. The most important factor affecting this is the *stray light* level of the monochromator [19]. The detector used is broadband, it responds to all the light that reaches it. If a significant amount of the light passed through the sample contains wavelengths that have much lower extinction coefficients than the nominal one, the instrument will report an incorrectly low absorbance. Any instrument will reach a point where an increase in sample concentration will not result in an increase in the reported absorbance, because the detector is simply responding to the stray light. In practice the concentration of the sample or the optical path length must be adjusted to place the unknown absorbance within a range that is valid for the instrument. Sometimes an empirical calibration function is developed, using known concentrations of the sample, to allow measurements into the region where the instrument is becoming non-linear.

As a rough guide, an instrument with a single monochromator would typically have a stray light level corresponding to about 3AU, which would make measurements above about 2AU problematic. A more complex instrument with a double monochromator would have a stray light level corresponding to about 6AU, which would therefore allow measuring a much wider absorbance range.

Absorption flattening: At sufficiently high concentrations, the absorption bands will saturate and show absorption flattening. The absorption peak appears to flatten because close to 100% of the light is already being absorbed. The concentration at which this occurs depends on the particular compound being measured. One test that can be used to test for this effect is to vary the path length of the measurement. In the Beer-Lambert law, varying concentration and path length has an equivalent effect—diluting a solution by a factor of 10 has the same effect as shortening the path length by a factor of 10. If cells of different path lengths are available, testing if this relationship holds true is one way to judge if absorption flattening is occurring.

Solutions that are not homogeneous can show deviations from the Beer-Lambert law because of the phenomenon of absorption flattening. This can happen, for instance, where the absorbing substance is located within suspended particles.[20] The deviations will be most noticeable under conditions of low concentration and high absorbance. The reference describes a way to correct for this deviation.

Measurement uncertainty sources

The above factor contributes to the measurement uncertainty of the results obtained with UV/Vis spectrophotometry. If UV/Vis spectrophotometry is used in quantitative chemical analysis then the results are additionally affected by uncertainty sources arising from the nature of the compounds and/or solutions that are measured. These include spectral interferences caused by absorption band overlap, fading of the color of the absorbing species (caused by decomposition or reaction) and possible composition mismatch between the sample and the calibration solution.[21]

Ultraviolet-visible spectrophotometer

The instrument used in ultraviolet-visible spectroscopy is called a UV/Vis **spectrophotometer**. It measures the intensity of light passing through a sample (I), and compares it to the intensity of light before it passes through the sample (I_o). The ratio I / I_o is called the *transmittance*, and is usually expressed as a percentage (%T). The absorbance, A, is based on the transmittance:

$$A = -\log\left(\frac{\%T}{100\%}\right)$$

The UV-visible spectrophotometer can also be configured to measure reflectance. In this case, the spectrophotometer measures the intensity of light reflected from a sample (I), and compares it to the intensity of light reflected from a reference material (I_o)(such as a white tile). The ratio I / I_o is called the *reflectance*, and is usually expressed as a percentage (%R).

The basic parts of a spectrophotometer are a light source, a holder for the sample, a diffraction grating in a monochromator or a prism to separate the different wavelengths of light, and a detector. The radiation source is often a Tungsten filament (300-2500 nm), a deuterium arc lamp, which is continuous over the

ultraviolet region (190-400 nm), Xenon arc lamps, which is continuous from 160-2,000 nm; or more recently, light emitting diodes (LED) [22] for the visible wavelengths. The detector is typically a photomultiplier tube, a photodiode, a photodiode array or a charge-coupled device (CCD). Single photodiode detectors and photomultiplier tubes are used with scanning monochromators, which filter the light so that only light of a single wavelength reaches the detector at one time. The scanning monochromator moves the diffraction grating to "step-through" each wavelength so that its intensity may be measured as a function of wavelength. Fixed monochromators are used with CCDs and photodiode arrays. As both of these devices consist of many detectors grouped into one or two dimensional arrays, they are able to collect light of different wavelengths on different **pixels** or groups of pixels simultaneously.

A spectrophotometer can be either *single beam* or *double beam*. In a single beam instrument (such as the Spectronic 20), all of the light passes through the sample cell. I_o must be measured by removing the sample. This was the earliest design, but is still in common use in both teaching and industrial labs.

In a double-beam instrument, the light is split into two beams before it reaches the sample. One beam is used as the reference; the other beam passes through the sample. The reference beam intensity is taken as 100% Transmission (or 0 Absorbance), and the measurement displayed is the ratio of the two beam intensities. Some double-beam instruments have two detectors (photodiodes), and the sample and reference beam are measured at the same time. In other instruments, the two beams pass through a beam chopper, which blocks one beam at a time. The detector alternates measuring between the sample beam and the reference beam in synchronism with the chopper. There may also be one or more dark intervals in the chopper cycle. In this case, the measured beam intensities may be corrected by subtracting the intensity measured in the dark interval before the ratio is taken.

Samples for UV/Vis spectrophotometry are most often liquids, although the absorbance of gases and even of solids can also be measured. Samples are typically placed in a transparent cell, known as a cuvette. Cuvettes are typically rectangular in shape, commonly with an internal width of 1 cm. (This width becomes the path length, L, in the Beer-Lambert law.) Test tubes can also be used as cuvettes in some

instruments. The type of sample container used must allow radiation to pass over the spectral region of interest. The most widely applicable cuvettes are made of high quality fused silica or quartz glass because these are transparent throughout the UV, visible and near infrared regions. Glass and plastic cuvettes are also common, although glass and most plastics absorb in the UV, which limits their usefulness to visible wavelengths.[23]

Specialized instruments have also been made. These include attaching spectrophotometers to telescopes to measure the spectra of astronomical features. UV-visible nanophotometers allow cuvetteless measurement of very small sample volumes (starting with 0.3 µl). UV-visible microspectrophotometers consist of a UV-visible microscope integrated with a UV-visible spectrophotometer.

A complete spectrum of the absorption at all wavelengths of interest can often be produced directly by a more sophisticated spectrophotometer. In simpler instruments the absorption is determined one wavelength at a time and then compiled into a spectrum by the operator. By removing the concentration dependence, the extinction coefficient (ε) can be determined as a function of wavelength.

Nanophotometry

Multiple biological applications (e.g. nucleic acid labelling) require quantitative as well as qualitative analysis of nucleic acids (DNA, RNA) and Proteins with sample volumes in a submicroliter range. This becomes possible with a specialized Nano Photometer. A drop of sample (0.03µl to 5µl) is pipetted directly onto the measuring window of the instrument. In the measuring chamber the sample is squeezed to exactly defined path lengths ranging from 0.04mm up to 2mm. This reduction of the path length compared to a measurement with standard cuvettes (path length 1cm) offers an automatic sample dilution between 1/250 and 1/5 in comparison to a standard cuvette measurement. Due to this virtual sample dilution measurements can be processed with undiluted samples. The reproducibility of the results is extremely high and samples can be retrieved after the measurement for further processing.

Microspectrophotometry

UV-visible spectroscopy of microscopic samples is done by integrating an optical microscope with UV-visible optics, white light sources, a monochromator, and a sensitive detector such as a charge-coupled device (CCD) or photomultiplier tube (PMT). As only a single optical path is available, these are single beam instruments. Modern instruments are capable of measuring UV-visible spectra in both reflectance and transmission of micron-scale sampling areas. The advantages of using such instruments is that they are able to measure microscopic samples but are also able to measure the spectra of larger samples with high spatial resolution. As such, they are used in the forensic laboratory to analyze the dyes and pigments in individual textile fibers,[24] microscopic paint chips [25] and the color of glass fragments. They are also used in materials science and biological research and for determining the energy content of coal and petroleum source rock by measuring the vitrinite reflectance. Microspectrophotometers are used in the semiconductor and micro-optics industries for monitoring the thickness of thin films after they have been deposited. In the semiconductor industry, they are used because the critical dimension of circuitry is microscopic. A typical test of a semiconductor wafer would entail the acquisition of spectra from many points on a patterned or unpatterned wafer. The thickness of the deposited films may be calculated from the interference pattern of the spectra. A map of the film thickness across the entire wafer can then be generated and used for quality control purposes.[26]

1.2.2 Titrimetry

Titrimetry also known as **titration,**[27] is a common laboratory method of quantitative chemical analysis that is used to determine the unknown concentration of an identified analyte. Because volume measurements play a key role in titration, it is also known as **volumetric analysis**. A reagent, called the *titrant* or *titrator* [28] is prepared as a standard solution. A known concentration and volume of titrant reacts with a solution of analyte or *titrand* [29] to determine concentration.

History and Etymology

The word "titration" comes from the Latin word *titulus*, meaning inscription or title. The French word *titre*, also from this origin, means rank. Titration, by definition, is the determination of rank or concentration of a solution with respect to water with a pH of 7 (the pH of pure H_2O under standard conditions).[30]

Volumetric analysis originated in late 18th-century France. Francois Antoine Henri Descroizilles developed the first burette (which was similar to a graduated cylinder) in 1791.[31] Joseph Louis Gay-Lussac developed an improved version of the burette that included a side arm, and coined the terms "pipette" and "burette" in an 1824 paper on the standardization of indigo solutions. A major breakthrough in the methodology and popularization of volumetric analysis was due to Karl Friedrich Mohr, who redesigned the burette by placing a clamp and a tip at the bottom, and wrote the first textbook on the topic, *Lehrbuch der chemisch-analytischen Titrirmethode* (*Textbook of analytical-chemical titration methods*), published in 1855.[32]

Procedure

A typical titration begins with a beaker or Erlenmeyer flask containing a precise volume of the titrand and a small amount of indicator placed underneath a calibrated burette or chemistry pipetting syringe containing the titrant. Small volumes of the titrant are then added to the titrand and indicator until the indicator changes, reflecting arrival at the endpoint of the titration. Depending on the endpoint desired, single drops or less than a single drop of the titrant can make the difference between a permanent and temporary change in the indicator. When the endpoint of the reaction is reached, the volume of reactant consumed is measured and used to calculate the concentration of analyte by where C_a is the concentration of the analyte, typically in molarity; C_t is the concentration of the titrant, typically in molarity; V_t is the volume of the titrant used, typically in dm^3; M is the mole ratio of the analyte and reactant from the balanced chemical equation; and V_a is the volume of the analyte used, typically in dm^3.[33]

Preparation techniques

Typical titrations require titrant and analyte to be in a liquid (solution) form. Though solids are usually dissolved into an aqueous solution, other solvents such as glacial

acetic acid or ethanol are used for special purposes (as in petrochemistry).[34] Concentrated analytes are often diluted to improve accuracy.

Many non-acid-base titrations require a constant pH throughout the reaction. Therefore a buffer solution may be added to the titration chamber to maintain the pH.[35]

In instances where two reactants in a sample may react with the titrant and only one is the desired analyte, a separate masking solution may be added to the reaction chamber which masks the unwanted ion.[36]

Some redox reactions may require heating the sample solution and titrating while the solution is still hot to increase the reaction rate. For instance, the oxidation of some oxalate solutions requires heating to 60 °C (140 °F) to maintain a reasonable rate of reaction.[37]

Types of titrations

There are many types of titrations with different procedures and goals. The most common types of qualitative titration are acid-base titrations and redox titrations.

Acid-base titration: Acid-base titrations depend on the neutralization between an acid and a base when mixed in solution. In addition to the sample, an appropriate indicator is added to the titration chamber, reflecting the pH range of the equivalence point. The acid-base indicator indicates the endpoint of the titration by changing color. The endpoint and the equivalence point are not exactly the same because the equivalence point is determined by the stoichiometry of the reaction while the endpoint is just the color change from the indicator. Thus, a careful selection of the indicator will reduce the indicator error. For example, if the equivalence point is at a pH of 8.4, then the Phenolphthalein indicator would be used instead of Alizarin Yellow because phenolphthalein would reduce the indicator error.[38] When more precise results are required, or when the reagents are a weak acid and a weak base, a pH meter or a conductance meter are used.

Redox titration: Redox titrations are based on a reduction-oxidation reaction between an oxidizing agent and a reducing agent. A potentiometer or a redox indicator is usually used to determine the endpoint of the titration, as when one of the constituents is the oxidizing agent potassium dichromate. The color change of the solution from orange to green is not definite; therefore an indicator such as sodium diphenylamine is used.[39] Analysis of wines for sulfur dioxide requires iodine as an

oxidizing agent. In this case, starch is used as an indicator; a blue starch-iodine complex is formed in the presence of excess iodine, signalling the endpoint.[40]
Some redox titrations do not require an indicator, due to the intense color of the constituents. For instance, in permanganometry a slight faint persisting pink color signals the endpoint of the titration because of the color of the excess oxidizing agent potassium permanganate.[41]

Gas phase titration: Gas phase titrations are titrations done in the gas phase, specifically as methods for determining reactive species by reaction with an excess of some other gas, acting as the titrant. Most commonly the gaseous analyte is ozone, which is titrated with **nitrogen oxide** according to the reaction;

$$O_3 + NO \rightarrow O_2 + NO_2$$ [41][42]

After the reaction is complete, the remaining titrant and product are quantified. This is used to determine the amount of analyte in the original sample.
Gas phase titration has several advantages over simple spectrophotometry. First, the measurement does not depend on path length, because the same path length is used for the measurement of both the excess titrant and the product. Second, the measurement does not depend on a linear change in absorbance as a function of analyte concentration as defined by the Beer-Lambert law. Third, it is useful for samples containing species which interfere at wavelengths typically used for the analyte.[43]

Complexometric titration: Complexometric titrations rely on the formation of a complex between the analyte and the titrant. In general, they require specialized indicators that form weak complexes with the analyte. Common examples are Eriochrome Black T for the titration of calcium and magnesium ions, and the chelating agent EDTA used to titrate metal ions in solution.[44]

Zeta potential titration: Zeta potential titrations are titrations in which the completion is monitored by the zeta potential, rather than by an indicator, in order to characterize heterogeneous systems, such as colloids.[45] One of the uses is to determine the iso-electric point when surface charge becomes zero, achieved by changing the pH or adding surfactant. Another use is to determine the optimum dose for flocculation or stabilization.[46]

Assay: An assay is a form of biological titration used to determine the concentration of a virus or bacterium. Serial dilutions are performed on a sample in a fixed ratio

(such as 1:1, 1:2, 1:4, 1:8, etc.) until the last dilution does not give a positive test for the presence of the virus. This value is known as the titer, and is most commonly determined through enzyme-linked immunosorbent **assay** (ELISA).[47]

Measuring the endpoint of a titration

Different methods to determine the endpoint include [48]:

Indicator: A substance that changes color in response to a chemical change. An acid-base indicator (e.g., phenolphthalein) changes color depending on the pH. Redox indicators are also used. A drop of indicator solution is added to the titration at the beginning; the endpoint has been reached when the color changes.

Potentiometer: An instrument that measures the electrode potential of the solution. These are used for redox titrations; the potential of the working electrode will suddenly change as the endpoint is reached.

pH meter: A potentiometer with an electrode whose potential depends on the amount of H^+ ion present in the solution. (This is an example of an ion-selective electrode.) The pH of the solution is measured throughout the titration, more accurately than with an indicator; at the endpoint there will be a sudden change in the measured pH.

Conductivity: A measurement of ions in a solution. Ion concentration can change significantly in a titration, which changes the conductivity. (For instance, during an acid-base titration, the H^+ and OH^- ions react to form neutral H_2O.) As total conductance depends on all ions present in the solution and not all ions contribute equally (due to mobility and ionic strength), predicting the change in conductivity is more difficult than measuring it.

Color change: In some reactions, the solution changes color without any added indicator. This is often seen in redox titrations when the different oxidation states of the product and reactant produce different colors.

Precipitation: If a reaction produces a solid, a precipitate will form during the titration. A classic example is the reaction between Ag^+ and Cl^- to form the insoluble salt AgCl. Cloudy precipitates usually make it difficult to determine the endpoint precisely. To compensate, precipitation titrations often have to be done as "back" titrations.

Isothermal titration calorimeter: An instrument that measures the heat produced or consumed by the reaction to determine the endpoint. Used in biochemical titrations, such as the determination of how substrates bind to enzymes.

Thermometric titrimetry: Differentiated from calorimetric titrimetry because the heat of the reaction (as indicated by temperature rise or fall) is not used to determine the amount of analyte in the sample solution. Instead, the endpoint is determined by *the rate of temperature change*.

Spectroscopy: Used to measure the absorption of light by the solution during titration if the spectrum of the reactant, titrant or product is known. The concentration of the material can be determined by Beer's Law.

Aperometry: Measures the current produced by the titration reaction as a result of the oxidation or reduction of the analyte. The endpoint is detected as a change in the current. This method is most useful when the excess titrant can be reduced, as in the titration of halides with Ag^{+}.

Endpoint and Equivalencepoint

Though equivalence point and endpoint are used interchangeably, they are different terms. *Equivalence point* is the theoretical completion of the reaction: the volume of added titrant at which the number of moles of titrant is equal to the number of moles of analyte, or some multiple thereof (as in polyprotic acids). *Endpoint* is what is actually measured, a physical change in the solution as determined by an indicator or an instrument mentioned above.[49] There is a slight difference between the endpoint and the equivalence point of the titration. This error is referred to as an indicator error, and it is indeterminate.[50]

Back titration

Back titration is a titration done in reverse; instead of titrating the original sample, a known excess of standard reagent is added to the solution, and the excess is titrated. A back titration is useful if the endpoint of the reverse titration is easier to identify than the endpoint of the normal titration, as with precipitation reactions. Back titrations are also useful if the reaction between the analyte and the titrant is very slow, or when the analyte is in a non-soluble solid.[51]

Particular uses of titration

Specific examples of titrations include:

1. Acid-Base Titrations

In biodiesel: Waste vegetable oil (WVO) must be neutralized before a batch may be processed. A portion of WVO is titrated with a base to determine acidity, so the rest of the batch may be properly neutralized. This removes free fatty acids from the WVO that would normally react to make soap instead of biodiesel.[52]

Kjeldahl method: A measure of nitrogen content in a sample. Organic nitrogen is digested into ammonia with sulfuric acid and potassium sulfate. Finally, ammonia is back titrated with boric acid and then sodium carbonate.[53]

Acid value: The mass in milligrams of potassium hydroxide (KOH) required to neutralize carboxylic acid in one gram of sample. An example is the determination of free fatty acid content. These titrations are achieved at low temperatures.

Saponification value: The mass in milligrams of KOH required to saponify carboxylic acid in one gram of sample. Saponification is used to determine average chain length of fatty acids in fat. These titrations are achieved at high temperatures.

Ester value (or ester index): A calculated index. Ester value = Saponification value – Acid value.

Amine value: The mass in milligrams of KOH equal to the amine content in one gram of sample.

Hydroxyl value: The mass in milligrams of KOH required to neutralize hydroxyl groups in one gram of sample. The analyte is acetylated using acetic anhydride then titrated with KOH.

2. Redox titrations

Winkler test for dissolved oxygen: Used to determine oxygen concentration in water. Oxygen in water samples is reduced using manganese (II) sulfate, which reacts with potassium iodide to produce iodine. The iodine is released in proportion to the oxygen in the sample, thus the oxygen concentration is determined with a redox titration of iodine with thiosulfate using a starch indicator.[54]

Vitamin C: Also known as ascorbic acid, vitamin C is a powerful reducing agent. Its concentration can easily be identified when titrated with the **blue dye Dichlorophenolindophenol (DCPIP)** which turns colorless when reduced by the vitamin.[55]

Benedict's reagent: Excess glucose in urine may indicate diabetes in the patient. Benedict's method is the conventional method to quantify glucose in urine using a

prepared reagent. In this titration, glucose reduces cupric ions to cuprous ions which react with potassium thiocyanate to produce a white precipitate, indicating the endpoint.[56]

Bromine number: A measure of unsaturation in an analyte, expressed in milligrams of bromine absorbed by 100 grams of sample.

Iodine number: A measure of unsaturation in an analyte, expressed in grams of iodine absorbed by 100 grams of sample.

3. Miscellaneous

Karl Fischer titration: A potentiometric method to analyze trace amounts of water in a substance. A sample is dissolved in methanol, and titrated with Karl Fischer reagent. The reagent contains iodine, which reacts proportionally with water. Thus, the water content can be determined by monitoring the potential of excess iodine.[57]

Non-aqueous titration: Non-aqueous titration is the titration of substances dissolved in non-aqueous solvents. It is the most common titrimetric procedure used in pharmacopoeial assays and serves a double purpose: it is suitable for the titration of very weak acids and very weak bases, and it provides a solvent in which organic compounds are soluble. The most commonly used procedure is the titration of organic bases with perchloric acid in *anhydrous* acetic acid. These assays sometimes take some perfecting in terms of being able to judge the endpoint precisely.

Potentiometric titration: The end point of most titrations is detected by the use of visual indicator but the method can be inaccurate in very dilute or colored solutions. However under the same conditions, a potentiometric method for the detection of the equivalence point can yield accurate results without difficulty. The electrical apparatus required consists of a potentiometer or pH meter with a suitable indicator and reference electrode. The other apparatus consists of a burette, beaker and stirrer. The actual potential of the reference electrode need not be known accurately for most purposes and usually any electrode may be used provided its potential remains constant throughout the titration. The indicator electrode must be suitable for the particular type of titration (i.e. a glass electrode for acid-base reactions and a platinum electrode for redox titrations), and should reach equilibrium rapidly. The electrodes are immersed in the solution to be titrated and the potential difference between the electrodes is measured. Measured volumes of titrant are added, with thorough (magnetic) stirring, and the corresponding values of emf (electromotive

force) or pH recorded. Small increments in volume should be added near the equivalence point which is found graphically by noting the burette reading corresponding to the maximum change of emf or pH per unit change of volume. When the slope of the curve is more gradual it is not always easy to locate the equivalent point by this method. However, if small increments (0.1 cm^3 or less) of titrant are added near the end point of the titration and a curve of change of emf or pH per unit volume against volume of titrant is plotted, a differential curve is obtained in which the equivalence point is indicated by a peak.

1.2.3 High Performance 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 contained in columns, a pump that moves the mobile phase and sample components through the column, and a detector to provide a characteristic retention time for the analyte and an area count reflecting the amount of analyte passing through the detector. 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 composition and flow rate of mobile phase used, and on the column dimensions. HPLC is a form of liquid chromatography that utilizes smaller column size, smaller media inside the column, and higher mobile phase pressures compared to ordinary liquid chromatography. 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.

Operation

The sample to be separated and analyzed is introduced, in a discrete small volume, into the stream of mobile phase percolating through the column. The components of

the sample move through the column at different velocities function of specific physical or chemical interactions with the stationary phase. The velocity of each component depends on the nature of each analyte, on the nature of the stationary phase (column) and the composition of the mobile phase. The time at which a specific analyte elutes (emerges from the column) is called the retention time. The retention time under particular conditions is considered an identifying characteristic of a given analyte. The use of smaller particle size column packing materials requires the use of higher operational pressure ("backpressure") and typically improves chromatographic resolution (i.e. the degree of separation between consecutive analytes emerging from the column). Common mobile phases used include any miscible combination of water with various organic liquids (the most common are acetonitrile and methanol). Some HPLC techniques use water free mobile phases (see Normal Phase HPLC below). The aqueous component of the mobile phase may contain buffers, acids (such as trifluoroacetic acid which acts as an ion pairing agent) or salts to assist in the separation of the sample components. The composition of the mobile phase is kept constant in isocratic elution mode. This mode is effective in the separation of sample components that are fairly similar in their affinity for the stationary phase.

A further refinement of HPLC is to vary the mobile phase composition during the analysis; *gradient elution*. A typical gradient profile in reversed phase chromatography might start at 5% acetonitrile and progress linearly to 95% acetonitrile over 5–25 minutes. The composition of the mobile phase depends on the intensity of interactions between analytes and stationary phase (e.g. hydrophobic interactions in reversed-phase HPLC). Depending on their affinity for the stationary and mobile phases, analytes partition between the two during the separation process taking place in the column. 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/acetonitrile gradient, more hydrophobic components will elute (come off the column) late, once the mobile phase gets more concentrated in acetonitrile (i.e. in a mobile phase of higher eluting strength).

The choice of mobile phase components, additives (such as salts or acids) and gradient conditions depend on the nature of the column and sample components. Often a series of trial runs are performed on the sample in order to find the HPLC method which gives the best separation.

Types Chromatography

1. Partition chromatography

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 fibers 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 in HPLC, this method separates analytes based on polar differences. Hydrophilic Interaction Chromatography most often uses a bonded polar stationary phase and water miscible, high organic concentration, mobile phases. Partition HPLC has been used historically on unbonded silica or alumina supports. Each works effectively for separating analytes by relative polar differences. HILIC bonded phases have the advantage of separating acidic, basic and neutral solutes in a single chromatogram.[58]

The polar analytes diffuse into a stationary water layer associated with the polar stationary phase and are thus retained. Retention strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends on the functional groups in the analyte molecule which promote partitioning but can also include coulombic (electrostatic) interaction and hydrogen donor capability.Use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times

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2. Normal-phase chromatography

Also known as normal-phase HPLC (NP-HPLC), or adsorption chromatography, this method separates analytes based on adsorption to a stationary surface chemistry and by polarity. It was one of the first kinds of HPLC that chemists developed. NP-HPLC uses a polar stationary phase and a non-polar, non-aqueous mobile phase, and works

effectively for separating analytes readily soluble in non-polar solvents. The analyte associates with and is retained by the polar stationary phase. Adsorption strengths increase with increased analyte polarity, and the interaction between the polar analyte and the polar stationary phase (relative to the mobile phase) increases the elution time. The interaction strength depends not only on the functional groups in the analyte molecule, but also on steric factors. The effect of sterics on interaction strength allows this method to resolve (separate) structural isomers.

The use of more polar solvents in the mobile phase will decrease the retention time of the analytes, whereas more hydrophobic solvents tend to increase retention times. Very polar solvents in a mixture tend to deactivate the stationary phase by creating a stationary bound water layer on the stationary phase surface. This behavior is somewhat peculiar to normal phase because it is most purely an adsorptive mechanism (the interactions are with a hard surface rather than a soft layer on a surface).

Partition and NP-HPLC fell out of favor in the 1970s with the development of reversed-phase HPLC because of a lack of reproducibility of retention times as water or protic organic solvents changed the hydration state of the silica or alumina chromatographic media. Recently, partition chromatography it has become popular again with the development of HILIC bonded phases which improve reproducibility, and with a better understanding of the range of usefulness of the technique. Adsorption chromatography is still widely used for structural isomer separations in both column and thin-layer chromatography formats on activated (dried) silica or alumina supports.

3. Displacement chromatography

The basic principle of displacement chromatography is: A molecule with a high affinity for the chromatography matrix (the displacer) will compete effectively for binding sites, and thus displace all molecules with lesser affinities.[59] There are distinct differences between displacement and elution chromatography. In elution mode, substances typically emerge from a column in narrow, Gaussian peaks. Wide separation of peaks, preferably to baseline, is desired in order to achieve maximum purification. The speed at which any component of a mixture travels down the column in elution mode depends on many factors. But for two substances to travel at different speeds, and thereby be resolved, there must be substantial differences in

some interaction between the biomolecules and the chromatography matrix. Operating parameters are adjusted to maximize the effect of this difference. In many cases, baseline separation of the peaks can be achieved only with gradient elution and low column loadings. Thus, two drawbacks to elution mode chromatography, especially at the preparative scale, are operational complexity, due to gradient solvent pumping, and low throughput, due to low column loadings. Displacement chromatography has advantages over elution chromatography in that components are resolved into consecutive zones of pure substances rather than "peaks". Because the process takes advantage of the nonlinearity of the isotherms, a larger column feed can be separated on a given column with the purified components recovered at significantly higher concentrations.

4. Reversed-phase chromatography (RPC)

Reversed phase HPLC (RP-HPLC) has a non-polar stationary phase and an aqueous, moderately polar mobile phase. One common stationary phase is silica which has been surface-modified with RMe_2SiCl, where R is a straight chain alkyl group such as $C_{18}H_{37}$ or C_8H_{17}. With such stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily (early in the analysis). An investigator can increase retention times by adding more water to the mobile phase; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase. Similarly, an investigator can decrease retention time by adding more organic solvent to the eluent. RP-HPLC is so commonly used that it is often incorrectly referred to as "HPLC" without further specification. The pharmaceutical industry regularly employs RP-HPLC to qualify drugs before their release.

RP-HPLC operates on the principle of hydrophobic interactions, which originate from the high symmetry in the dipolar water structure and play the most important role in all processes in life science. RP-HPLC allows the measurement of these interactive forces. The binding of the analyte to the stationary phase is proportional to the contact surface area around the non-polar segment of the analyte molecule upon association with the ligand on the stationary phase. This solvophobic effect is dominated by the force of water for "cavity-reduction" around the analyte and the C_{18}-chain versus the complex of both. The energy released in this process is proportional to the surface tension of the eluent (water: 7.3×10^{-6} J/cm², methanol:

2.2×10^{-6} J/cm²) and to the hydrophobic surface of the analyte and the ligand respectively. The retention can be decreased by adding a less polar solvent (methanol, acetonitrile) into the mobile phase to reduce the surface tension of water. Gradient elution uses this effect by automatically reducing the polarity and the surface tension of the aqueous mobile phase during the course of the analysis. Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) is retained longer because it is non-interacting with the water structure. On the other hand, analytes with higher polar surface area (conferred by the presence of polar groups, such as -OH, $-NH_2$, COO^- or $-NH_3^+$ in their structure) are less retained as they are better integrated into water. Such interactions are subject to steric effects in that very large molecules may have only restricted access to the pores of the stationary phase, where the interactions with surface ligands (alkyl chains) take place. Such surface hindrance typically results in less retention.

Retention time increases with hydrophobic (non-polar) surface area. Branched chain compounds elute more rapidly than their corresponding linear isomers because the overall surface area is decreased. Similarly organic compounds with single C-C-bonds elute later than those with a C=C or C-C-triple bond, as the double or triple bond is shorter than a single C-C-bond.

Aside from mobile phase surface tension (organizational strength in eluent structure), other mobile phase modifiers can affect analyte retention. For example, the addition of inorganic salts causes a moderate linear increase in the surface tension of aqueous solutions (ca. 1.5×10^{-7} J/cm² per Mol for NaCl, 2.5×10^{-7} J/cm² per Mol for $(NH_4)_2SO_4$), and because the entropy of the analyte-solvent interface is controlled by surface tension, the addition of salts tend to increase the retention time. This technique is used for mild separation and recovery of proteins and protection of their biological activity in protein analysis (hydrophobic interaction chromatography, HIC).

Another important factor is the mobile phase pH since it can change the hydrophobic character of the analyte. For this reason most methods use a buffering agent, such as sodium phosphate, to control the pH. Buffers serve multiple purposes: control of pH, neutralize the charge on the silica surface of the stationary phase and act as ion pairing agents to neutralize analyte charge. Ammonium formate is commonly added

in mass spectrometry to improve detection of certain analytes by the formation of analyte-ammonium adducts. A volatile organic acid such as acetic acid, or most commonly formic acid, is often added to the mobile phase if mass spectrometry is used to analyze the column effluent. Trifluoroacetic acid is used infrequently in mass spectrometry applications due to its persistence in the detector and solvent delivery system, but can be effective in improving retention of analytes such as carboxylic acids in applications utilizing other detectors, as it is a fairly strong organic acid. The effects of acids and buffers vary by application but generally improve chromatographic resolution.

Reversed phase columns are quite difficult to damage compared with normal silica columns; however, many reversed phase columns consist of alkyl derivatized silica particles and should never be used with aqueous bases as these will destroy the underlying silica particle. They can be used with aqueous acid, but the column should not be exposed to the acid for too long, as it can corrode the metal parts of the HPLC equipment. RP-HPLC columns should be flushed with clean solvent after use to remove residual acids or buffers, and stored in an appropriate composition of solvent. The metal content of HPLC columns must be kept low if the best possible ability to separate substances is to be retained. A good test for the metal content of a column is to inject a sample which is a mixture of 2,2'- and 4,4'- bipyridine. Because the 2,2'-bipy can chelate the metal, the shape of the peak for the 2,2'-bipy will be distorted (tailed) when metal ions are present on the surface of the silica.[59]

5. Size-exclusion chromatography

Size-exclusion chromatography (SEC), also known as *gel permeation chromatography* or *gel filtration chromatography* separates particles on the basis of size. It is generally a low resolution chromatography and thus it is often reserved for the final, "polishing" step of purification. It is also useful for determining the tertiary structure and quaternary structure of purified proteins. SEC is used primarily for the analysis of large molecules such as proteins or polymers. SEC works by trapping these smaller molecules in the pores of a particle. The larger molecules simply pass by the pores as they are too large to enter the pores. Larger molecules therefore flow through the column quicker than smaller molecules, that is, the smaller the molecule, the longer the retention time.

This technique is widely used for the molecular weight determination of polysaccharides. SEC is the official technique (suggested by European pharmacopeia) for the molecular weight comparison of different commercially available low-molecular weight heparins.

6. Ion-exchange chromatography

In ion-exchange chromatography (IC), retention is based on the attraction between solute ions and charged sites bound to the stationary phase. Ions of the same charge are excluded. Types of ion exchangers include:

Polystyrene resins – These allow cross linkage which increases the stability of the chain. Higher cross linkage reduces swerving, which increases the equilibration time and ultimately improves selectivity.

Cellulose and dextran ion exchangers (gels) – These possess larger pore sizes and low charge densities making them suitable for protein separation.

Controlled-pore glass or porous silica

In general, ion exchangers favor the binding of ions of higher charge and smaller radius.

An increase in counter ion (with respect to the functional groups in resins) concentration reduces the retention time. A decrease in pH reduces the retention time in cation exchange while an increase in pH reduces the retention time in anion exchange. By lowering the pH of the solvent in a cation exchange column, for instance, more hydrogen ions are available to compete for positions on the anionic stationary phase, thereby eluting weakly bound cations.

This form of chromatography is widely used in the following applications: water purification, preconcentration of trace components, ligand-exchange chromatography, ion-exchange chromatography of proteins, high-pH anion-exchange chromatography of carbohydrates and oligosaccharides, and others.

7. Bio-affinity chromatography

This chromatographic process relies on the property of biologically active substances to form stable, specific, and reversible complexes. The formation of these complexes involves the participation of common molecular forces such as the Van der Waals interaction, electrostatic interaction, dipole-dipole interaction, hydrophobic interaction, and the hydrogen bond. An efficient, bio-specific bond is formed by a

simultaneous and concerted action of several of these forces in the complementary binding sites.

8. Aqueous normal-phase chromatography

Aqueous normal-phase chromatography (ANP) is a chromatographic technique which encompasses the mobile phase region between reversed-phase chromatography (RP) and organic normal phase chromatography (ONP). This technique is used to achieve unique selectivity for hydrophilic compounds, showing normal phase elution using reverse-phase solvents.[60]

Parameters

Internal diameter: The internal diameter (ID) of an HPLC column is an important parameter that influences the detection sensitivity and separation selectivity in gradient elution. It also determines the quantity of analyte that can be loaded onto the column. Larger columns are usually seen in industrial applications, such as the purification of a drug product for later use. Low-ID columns have improved sensitivity and lower solvent consumption at the expense of loading capacity.

Larger ID columns (over 10 mm) are used to purify usable amounts of material because of their large loading capacity.

Analytical scale columns (4.6 mm) have been the most common type of columns, though smaller columns are rapidly gaining in popularity. They are used in traditional quantitative analysis of samples and often use a UV-Vis absorbance detector.

Narrow-bore columns (1–2 mm) are used for applications when more sensitivity is desired either with special UV-vis detectors, fluorescence detection or with other detection methods like liquid chromatography-mass spectrometry

Capillary columns (under 0.3 mm) are used almost exclusively with alternative detection means such as mass spectrometry. They are usually made from fused silica capillaries, rather than the stainless steel tubing that larger columns employ.

Particle size: Most traditional HPLC is performed with the stationary phase attached to the outside of small spherical silica particles (very small beads). These particles come in a variety of sizes with 5 μm beads being the most common. Smaller particles generally provide more surface area and better separations, but the pressure required

for optimum linear velocity increases by the inverse of the particle diameter squared.[61][62][63]

This means that changing to particles that are half as big, keeping the size of the column the same, will double the performance, but increase the required pressure by a factor of four. Larger particles are used in preparative HPLC (column diameters 5 cm up to >30 cm) and for non-HPLC applications such as solid-phase extraction.

Pore size: Many stationary phases are porous to provide greater surface area. Small pores provide greater surface area while larger pore size has better kinetics, especially for larger analytes. For example, a protein which is only slightly smaller than a pore might enter the pore but does not easily leave once inside.sudheer

Pump pressure: Pumps vary in pressure capacity, but their performance is measured on their ability to yield a consistent and reproducible flow rate. Pressure may reach as high as 40 MPa (6000 lbf/in^2), or about 400 atmospheres. Modern HPLC systems have been improved to work at much higher pressures, and therefore are able to use much smaller particle sizes in the columns (<2 μm). These "Ultra High Performance Liquid Chromatography" systems or RSLC/UHPLCs can work at up to 100 MPa (15,000 lbf/in^2), or about 1000 atmospheres. The term "UPLC" is a trademark of the Waters Corporation, but is sometimes used to refer to the more general technique.

1.3 Background

Acetanilide was the first aniline derivative serendipitously found to possess analgesic as well as antipyretic properties, and was quickly introduced into medical practice under the name of Antifebrin by A. Cahn and P. Hepp in 1886.[64] But its unacceptable toxic effects, the most alarming being cyanosis due to methemoglobinemia, prompted the search for less toxic aniline derivatives.[65] Harmon Northrop Morse had already synthesized paracetamol at Johns Hopkins University via the reduction of *p*-nitrophenol with tin in glacial acetic acid in 1877,[66, 67] but it was not until 1887 that clinical pharmacologist Joseph von Mering tried paracetamol on patients.[65] In 1893, von Mering published a paper reporting on the clinical results of paracetamol with phenacetin, another aniline derivative.[68] Von Mering claimed that, unlike phenacetin, paracetamol had a slight tendency to produce methemoglobinemia. Paracetamol was then quickly discarded in favor of

phenacetin. The sales of phenacetin established Bayer as a leading pharmaceutical company.[69] Overshadowed in part by aspirin, introduced into medicine by Heinrich Dreser in 1899, phenacetin was popular for many decades, particularly in widely advertised over-the-counter "headache mixtures," usually containing phenacetin, an aminopyrine derivative of aspirin, caffeine, and sometimes a barbiturate.[65] Von Mering's claims remained essentially unchallenged for half a century, until two teams of researchers from the United States analyzed the metabolism of acetanilide and paracetamol.[69] In 1947 David Lester and Leon Greenberg found strong evidence that paracetamol was a major metabolite of acetanilide in human blood, and in a subsequent study they reported that large doses of paracetamol given to albino rats did not cause methemoglobinemia.[70] In three papers published in the September 1948 issue of the *Journal of Pharmacology and Experimental Therapeutics*, Bernard Brodie, Julius Axelrod and Frederick Flinn confirmed using more specific methods that paracetamol was the major metabolite of acetanilide in human blood, and established it was just as efficacious an analgesic as its precursor.[71, 72, 73] They also suggested that methemoglobinemia is produced in humans mainly by another metabolite, phenylhydroxylamine. A followup paper by Brodie and Axelrod in 1949 established that phenacetin was also metabolized to paracetamol.[74] This led to a "rediscovery" of paracetamol.[65] It has been suggested that contamination of paracetamol with 4-aminophenol, the substance from which it was synthesized by von Mering, may be the cause for his spurious findings.

[69] Paracetamol was first marketed in the United States in 1953 by Sterling-Winthrop Co., which promoted it as preferable to aspirin since it was safe to take for children and people with ulcers.[69] The best known brand today for paracetamol in the United States, Tylenol, was established in 1955 when McNeil Laboratories started selling paracetamol as a pain and fever reliever for children, under the brand name Tylenol Children's Elixir—the word "tylenol" was a contraction of *para*-acetylaminophenol.[75] In 1956, 500 mg tablets of paracetamol went on sale in the United Kingdom under the trade name Panadol, produced by Frederick Stearns & Co, a subsidiary of Sterling Drug Inc. Panadol was originally available only by prescription, for the relief of pain and fever, and was advertised as being "gentle to the stomach," since other analgesic agents of the time contained aspirin, a known stomach irritant.[67] Concerns about paracetamol's safety delayed its widespread

acceptance until the 1970s, but in the 1980s paracetamol sales exceeded those of aspirin in many countries, including the United Kingdom. This was accompanied by the commercial demise of phenacetin, blamed as the cause of analgesic nephropathy and hematological toxicity [65].

The U.S. patent on paracetamol has long expired, and generic versions of the drug are widely available under the Drug Price Competition and Patent Term Restoration Act of 1984, although certain Tylenol preparations were protected until 2007. U.S. patent 6,126,967 filed September 3, 1998 was granted for "Extended release acetaminophen particles" [76].

Paracetamol is a *p*-aminophenol derivative, which is synthesized by acetylation of *p*-aminophenol and acetic anhydride. It may be hydrolyzed to *p*-aminophenol in some conditions such as high temperature, acidic or basic media. *Para-Aminophenol* is the main impurity in paracetamol preparations that may be formed during the storage of preparation or may be originated during the synthesis of paracetamol [77]. It was reported that *p*-aminophenol may cause nephrotoxicity and teratogenicity; therefore, its amount should be strictly controlled [78]. The United States Pharmacopeias (USP 2007) and British Pharmacopeias (BP 2007 Volume I and II, 6th edition) limit the amount of *p*-aminophenol in paracetamol substance at 0.005%$^{w}/_{w}$. The limits of *p*-aminophenol may vary in different products depending on the dosage forms and formulations. The monograph of paracetamol tablets in BP, *p*-aminophenol is limited to 0.1%$^{w}/_{w}$, while no impurity testing method is presented in USP31 [79,80]. The BP uses a spectrophotometric assay method for it quantitative determination of paracetamol in tablet in dosage forms. The USP31 recommends method for paracetamol determination using HPLC method. Paracetamol is a major ingredient in numerous cold and flu medications due to its analgesic and antipyretic properties. Several HPLC applications have been developed for the determination of paracetamol [81, 82].

Under high temperatures, paracetamol undergoes hydrolysis forming 4-aminophenol and acetic acid, resulting in 4-chloroacetanilide as a major side product. In total, 10 process-related impurities can occur during the synthesis of paracetamol. The European Pharmacopoeia monograph on paracetamol has adopted an isocratic method using a C8 silica-based stationary phase with a 5 µm particle size for

monitoring of impurities in paracetamol preparations, requiring a run time of 45min for analysis [83].

New methods for the monitoring of paracetamol formulations must take into account that efficient pharmaceutical Quality Assurance/Quality Control laboratories require fast method development, high efficiency, and rapid resolution. These priorities call for stable sub-2 μm particle packed columns. Compared to the more traditional columns packed with 5 μm particles, sub-2 μm columns offer shorter analysis times, improvements in resolving power, sensitivity and peak capacity.

Standard usage of paracetamol has no detrimental effect on the human body but over usage of the drug could lead to some serious side effects, such as liver disorders, skin rashes and inflammation of the pancreases [84]. Besides p-aminophenol, the primary hydrolytic degradation product of paracetamol can be present in pharmaceutical preparations as a synthetic intermediate or as a degradation product of paracetamol that can cause serious nephrotoxicity and tetragenic effects [85, 86].

However, these methods usually involve the hydrolysis of paracetamol sample to 4-aminophenol, which then required the formation of a coloured complex using an appropriate reagent, which takes a long time to perform.

Electrochemical methods based on chemically modified electrodes have attracted much attention because of quick response, high sensitivity, and selectivity in the determination of trace level analytes. The slow electron transfer kinetics on bare (unmodified) electrode is substantially changed by modifying the surface of the bare electrode which speeds up the electron transfer kinetics [87-97].

Considering the fact that p-aminophenol may potentially cause liver damage when above the acceptable limit, it is therefore important to accurately quantify it level in paracetamol formulations: tablets, syrups and suspensions.

1.4 Profile of Paracetamol and Para-Aminophenol

1.4.1 Profile of Paracetamol

Paracetamol is a white, odourless crystalline powder with a bitter taste, soluble in 70 parts of water (1 in 20 boiling water), 7 parts of alcohol (95%), 13 parts of acetone, 40 parts of glycerol, 9 parts of propylene glycol, 50 parts of chloroform, or 10 parts

of methyl alcohol. **Paracetamol has a molecular weight of 151.2g/mol and melts at about 168 °C to 172 °C with a specific gravity of 1.293** [98]. It is soluble in organic solvents such as methanol and ethanol but slightly soluble in water and ether. Its pH range is 5.5 - 6.5 based on saturated aqueous solution. It, chemically N-(4-Hydroxyphenyl) acetamide, is derived from the interaction of p-aminophenol and an aqueous solution of acetic anhydride. Acetaminophen is a non-prescription analgesic and antipyretic drug similar to aspirin. But acetaminophen is not an NSAID (Non-steroidal Anti-inflammatory Drug) as it doesn't participate in the inflammatory response as it can not inhibit cyclooxygenases in the presence of peroxides. There are high levels of peroxides in platelets. Prostaglandin which reduces blood coagulation is produced through cyclooxygenase pathway. In result, NSAIDs including aspirin have detrimental effects on the stomach lining, where prostaglandins serve a protective role, but paracetamol has almost no adverse effects on the stomach or esophagus. Taking large doses of acetaminophen for a long time may impair liver function to some liver damage. Paractamol (**para-acet**yl-**am**ino-phen**ol**) is another name of acetaminophen (N-**acet**yl-para-**aminophen**ol). It is also used as an intermediate for pharmaceuticals (as a precursor in penicillin) and azo dyes, stabilizer for hydrogen peroxide, photographic chemicals. Paracetamol consists of a benzene ring core, substituted by one hydroxyl group and the nitrogen atom of an amide group in the *para* (1,4) pattern. The amide group is acetamide. It is an extensively conjugated system, as the lone pair on the hydroxyl oxygen, the benzene pi cloud, the nitrogen lone pair, the p-orbital on the carbonyl carbon, and the lone pair on the carbonyl oxygen are all conjugated. The presence of two activating groups also makes the benzene ring highly reactive toward electrophilic aromatic substitution. As the substituents are ortho,para-directing and para with respect to each other, all positions on the ring are more or less equally activated. The conjugation also greatly reduces the basicity of the oxygens and the nitrogen, while making the hydroxyl acidic through delocalisation of charge developed on the phenoxide anion. Its pain relief (analgesic) and fever relief (antipyretic) effects are similar to those of aspirin and it works in a similar, though not identical, way. Unlike aspirin, however, increasing the dose does not result in clinically useful anti-inflammatory activity.

Recent research has shown the presence of a new, previously unknown cyclooxygenase enzyme COX-3, found in the brain and spinal cord, which is selectively inhibited by paracetamol, and is distinct from the two already known cyclooxygenase enzymes COX-1 and COX-2. It is now believed that this selective inhibition of the enzyme COX-3 in the brain and spinal cord explains the effectiveness of paracetamol in relieving pain and reducing fever without having unwanted gastrointestinal side effects. Paracetamol may be easily prepared in the laboratory by nitrating phenol with sodium nitrate, separating the desired *p*-nitrophenol is separated from the *ortho*- byproduct, and reducing the nitro group with sodium borohydride. The resultant *p*-aminophenol is then acetylated with acetic anhydride. Industrial preparation of paracetamol usually proceeds from nitrobenzene. A one-step reductive acetamidation reaction can be mediated by thioacetate.

Many methods have been described for the assay of paracetamol including titrimetry, chromatography, electrochemistry and spectrophotometry [98]. The British pharmacopoeia method which involves titrimetry is based on the ability of cerium (IV) sulphate which is a strong oxidizing agent to oxidize the amine (4-aminophenol) to an iminoquinone. After complete oxidation of the amine has been achieved, it would further oxidize the ferrion indicator to ferrin to establish a colour change for detection. This method of assay involves the acid catalyzed hydrolysis of the paracetamol (amide) to an amine and a carboxylic acid and titrating the amine with an oxidizing agent such as ammonium cerium (IV) sulphate using iron (II) complex (ferroin) to determine the end point. For accurate research purposes high performance liquid chromatography is favoured for its sensitivity, precision and specificity.

Some of the brand names of paracetamol include Panadol, Disprol and Medinol. Paracetamol is available in a tablet, capsule, liquid suspension, suppository, intravenous, and intramuscular form. The common adult dose is 500 mg to 1000 mg. The recommended maximum daily dose, for adults, is 4000 mg. In recommended doses, paracetamol generally is safe for children and infants, as well as for adults. Side-effects of paracetamol are rare when taken at the recommended dose. However, skin rashes, blood disorders, liver and a swollen pancreas have occasionally happened in people taking the drug on a regular basis for a long time.

Excessive use of paracetamol can damage multiple organs, especially the liver and kidney. **In both organs, toxicity from paracetamol is not from the drug itself but from one of its metabolites.**

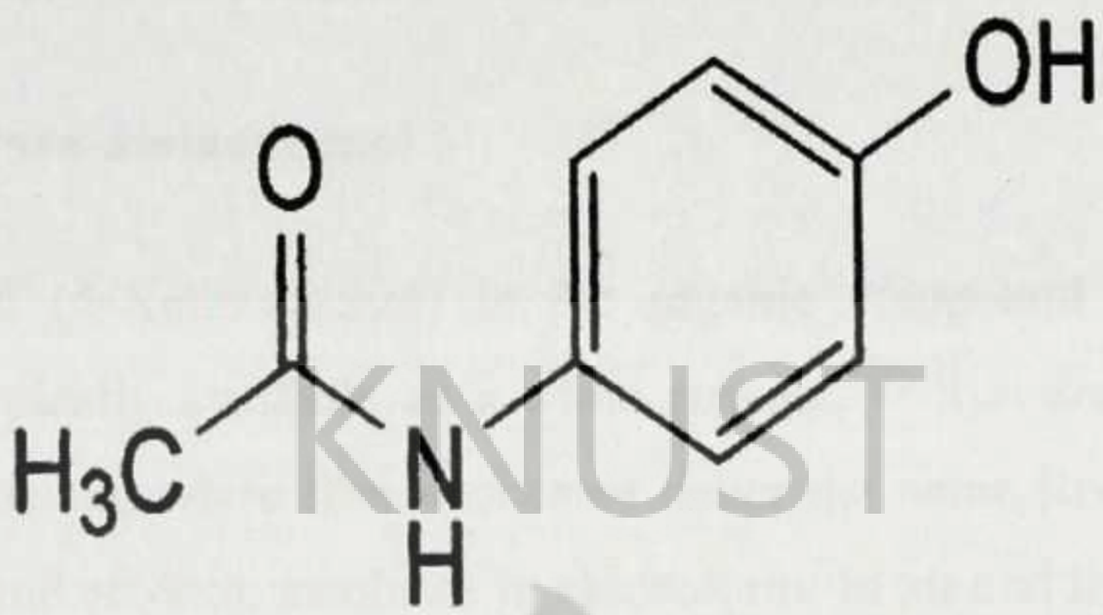


Figure 1.1: Structure of Paracetamol [98]

- **Definition:** *N*-(4-Hydroxyphenyl) acetamide.
- **Chemical Formula:** $CH_3CONHC_6H_4OH$
- **Synonyms:** 4'-hydroxyacetanilide; Tylenol; Paracetamol; Paracetamolo; Paracetamole; P-acetamido-Phenol; 4'-hydroxyacetanilide; n-(p-Hydroxyphenyl)-Acetamide; N-(4-hydroxyphenyl)-Acetamide; P-acetamidophenol; 4-Acetamidophenol; Acetaminofen; Acetaminophen; P-Acetaminophenol; N-acetyl-p-aminophenol; P-Acetylamino Phenol; P-hydroxyacetanilide; Paracetamol;4-hydroxy Acetanilide; 4-hydroxyanilid Kyseliny Octove; N-(4-hydroxyphenyl) Acetamide
- **Molecular weight**: 151.2g/mol[98]
- **Derivation**: p-aminophenol, acetic anhydride[98]
- **Appearance**: White crystalline powder[98]
- **ASSAY**: 99.0-101.0%[98]
- **Melting Point**: 168 °C to 172 °C[98]
- **p-Aminophenol**: 50ppm max[98]
- **Solubility in:**
  - acetone : easily soluble[98]
  - ether and benzene : hardly soluble[98]
  - water: Slightly soluble[98]
- **Saturated solution:** slight acidity[98]

- **Specific gravity:** 1.293[98]
- **pH range:** 5.5 - 6.5 based on saturated aqueous solution[98]
- **Ionization Constant, pKa :** 9.71[98]
- **Oral administration / 1day:** 300-500mg[98]
- **Drug Activity Classification:** Analgesic; antipyretic[98]

1.4.2 Profile of Para-aminophenol

Para-aminophenol (4-Aminophenol) is the organic compound with the formula $H_2NC_6H_4OH$. Typically available as a white powder,[99] it is commonly used as a developer in black and white film, marketed under the name Rodinal. It is slightly soluble in water and alcohol, insoluble in chloroform. In air and light, the colour will be darkening.

Para-aminophenol is the primary hydrolytic product of paracetamol which has been limited at a very low level in the drug substance by the Europeans, United States, Britain and Germany Pharmacopoeias. The low level of p-aminophenol is required to ensure paracetamol drug safety [100].

4-aminophenol is a widely used industrial chemical and a metabolite of common household analgesic such as paracetamol. However, 4-aminophenol is a fundamental material for the production of paracetamol as one of the most produced pharmaceuticals worldwide. Since it occurs in paracetamol in only trace amounts, and also shows both biochemical and environmental risks, a very sensitive method of determination is needed. It is a harmful compound for a human organism because it increases body temperature remains active for a long time [100].

Reflecting on its slight hydrophilic character, the white powder is moderately soluble in alcohols and can be recrystallised from hot water. In the presence of a base, it oxidizes readily. The *N*-methyl and *N*,*N*-dimethyl derivatives are of commercial value. It is an organic chemical intermediate and it is mainly applied in pesticides, dyes, and some chemical industries. The compound is one of three isomeric aminophenols, the other two being 2-aminophenol and 3-aminophenol.

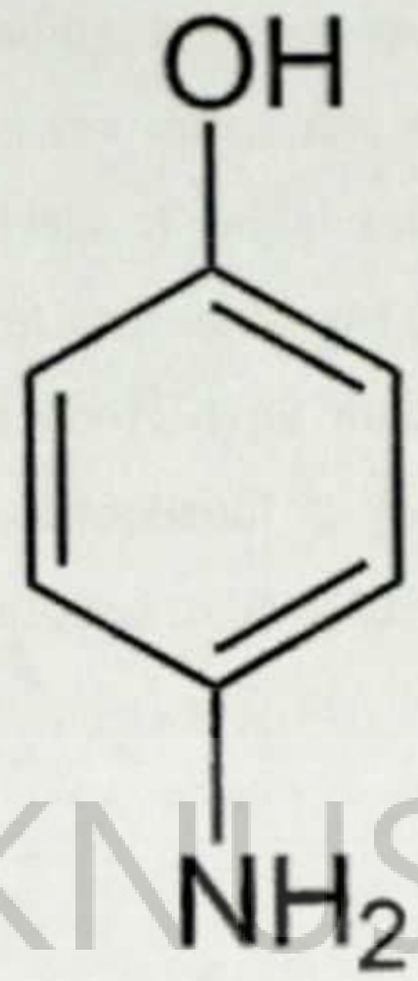


Figure 1.2: Chemical structure of Para-aminophenol

- **Molecular formula** = C_6H_7NO
- **Molar mass** = 109.13 g/mol
- **Density** = 1.13g/cm^3
- **Melting point** = 188 –190°C[98]
- **Boiling point** = 284°C[98]
- **Solubility in water** = 1.5 g/100 ml (25 °C) [98]

1.5 Synthesis of Para-aminophenol

It is produced from phenol by nitration followed by reduction with iron. Alternatively, the partial hydrogenation of nitrobenzene affords phenylhydroxylamine, which rearranges primarily to 4-aminophenol [101]:

$$C_6H_5NO_2 + 2H_2 \rightarrow C_6H_5NHOH + H_2O$$

$$C_6H_5NHOH \rightarrow HOC_6H_4NH_2$$

Also, the catalytic hydrogenation of *p*-nitrophenol to *p*-aminophenol was investigated in a laboratory-scale batch-slurry reactor. Pt/C catalyst (1%) was chosen for optimization of reaction conditions and kinetic studies because of its higher catalytic activity compared to that of other heterogeneous transition metal catalysts. The average catalytic activity and initial rate of hydrogenation was found to increase with increase in the solvent polarity. To investigate the intrinsic kinetics of the

reaction, the effect of catalyst loading, agitation speed, *p*-nitrophenol concentration, and hydrogen partial pressure on the initial rate of hydrogenation was studied at different temperatures. The analysis of initial rate data indicated that the mass-transfer resistances were not significant under the prevailing reaction conditions. A simple Langmuir–Hinschelwood (L–H)-type model was found to represent the kinetics of hydrogenation of *p*-nitrophenol to *p*-aminophenol satisfactorily. The apparent energy of activation was found to be 61kJ/mol.

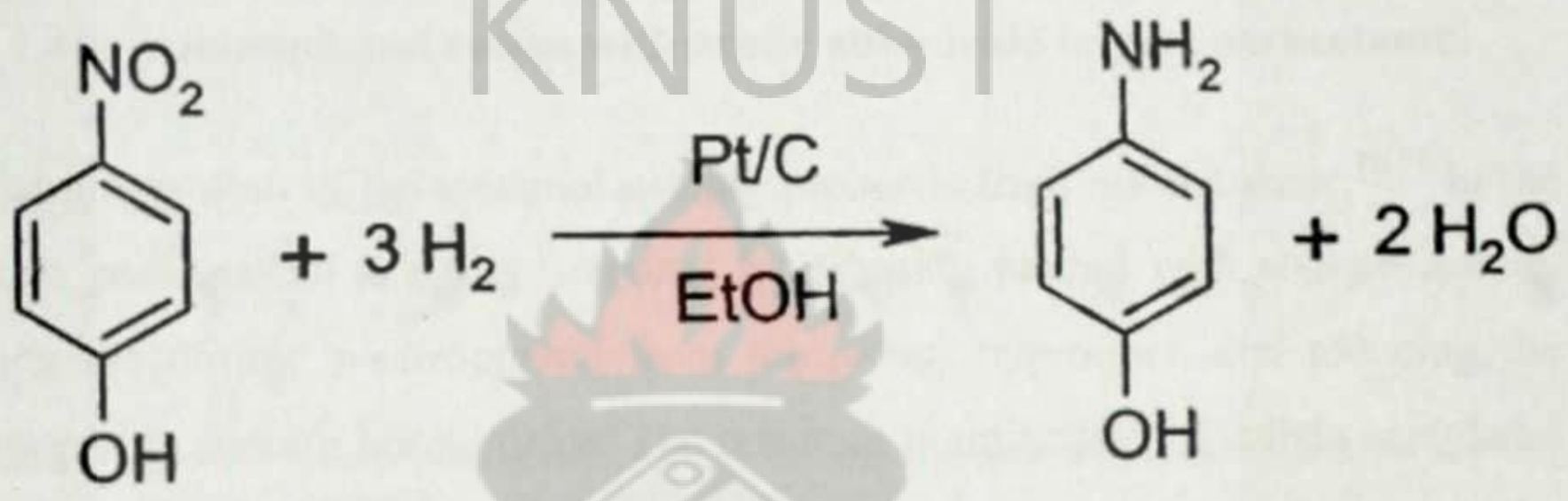
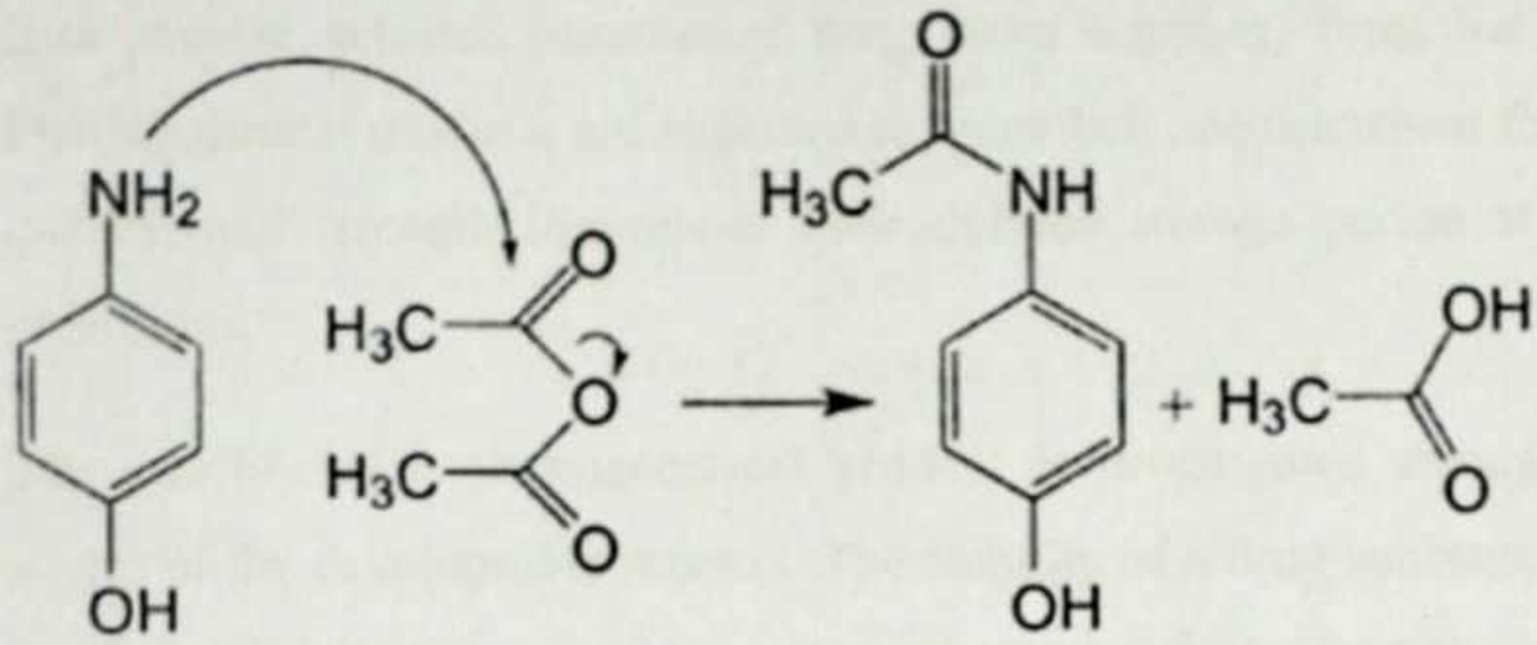


Figure 1.3: Catalytic Hydrogenation of p-nitrophenol to p-aminophenol

1.6 Synthesis of Paracetamol

Para-Aminophenol reacts with acetic anhydride to give paracetamol.[102]

Para-Aminophenol may be obtained by the amide hydrolysis of paracetamol. Para-Aminophenol prepared this way, and related to the commercially available Metol, has been used as a developer in photography by hobbyists.[103] This reaction is also used to determine paracetamol in urine samples: After hydrolysis with hydrochloric acid, *p*-aminophenol reacts in ammonia solution with a phenol derivate, e.g. salicylic acid, to form an indophenol dye under oxidization by air.[104]



p-aminophenol Acetic anhydride Paracetamol Acetic acid

Figure 1.4: p-Aminophenol reacts with acetic anhydride to give paracetamol

Industrial preparation of paracetamol usually proceeds from nitrobenzene.[105] In the laboratory, paracetamol is easily prepared by nitrating phenol with sodium nitrate, separating the desired *p*-nitrophenol from the *ortho*- byproduct, and reducing the nitro group with sodium borohydride. The resultant *p*-aminophenol is then acetylated with acetic anhydride.[106] In this reaction, phenol is strongly activating, thus the reaction requires only mild conditions (cf. the nitration of benzene):

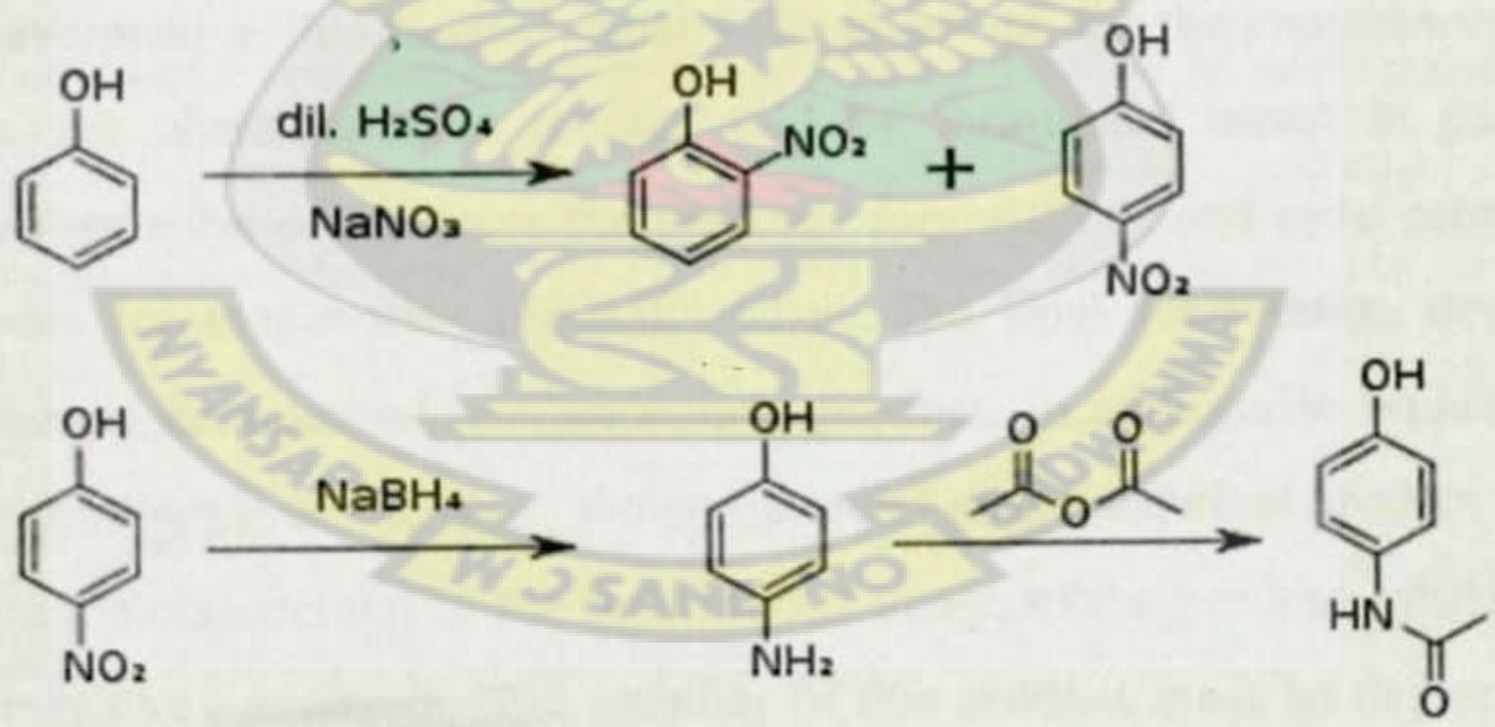


Figure 1.5: Industrial preparation of paracetamol

1.7 Stability of Pharmaceutical Products

Stability of a pharmaceutical product may be defined as the capability of a particular formulation, in a specific container/closure system, to remain within its physical, chemical, microbiological, therapeutic, and toxicological specifications. Assurances that the packaged products will be stable for its anticipated shelf life must come from an accumulation of valid data on the drug I its commercial package. These stability

data involve selected parameters that, taken together, form the stability profile. Pharmaceutical products are expected to meet their specifications for identity, purity, quality, and strength throughout their defined storage period at specific storage conditions.

The stability of a pharmaceutical product is investigated throughout the various stages of the development process. The stability of a drug substance is first assessed in the formulation stage. At this stage, pharmaceutical scientists determine the drug substance and its related salts stability/compatibility with various solvents, buffered solutions, and excipients considered for formulation development. Optimization of a stable formulation of a pharmaceutical product is built upon the information obtained from the preformultion stage and continues during the formulation development stages.

Typically, the first formulation development stage is the preparation of a "first in human" formulation which is often a non-elegant formulation optimized for short-term dose-ranging clinical studies. The second major formulation development stage occurs to support Phase II and early Phase III clinical studies. The pharmaceutical product developed at this stage is usually the prototype for the commercial product. Therefore, the pharmaceutical product will be formulated based in part on the stability information obtain from the previous formulations and must meet stability requirements for longer-term clinical studies. The final formulation development stage is for the commercial pharmaceutical product. In addition to building on the clinical requirements of the drug, the commercial pharmaceutical product must also incorporate the commercial or the final market image of the product, which includes the container closure system. The stability of this product must be demonstrated to the appropriate regulatory agencies in order to assign an expiration date for the product.

Once a pharmaceutical product has gained regulatory approval and is marketed, the pharmacist must understand the proper storage and handling of the drug. In some cases, a pharmacist may need to prepare stable compounded preparations from this product. It is the responsibility of the pharmacist, via the information of the manufacturer, to instruct the patient in the proper storage and handling of the drug

product. The impact of a drug product with a poor stability profile could delay approval, affect the safety and efficacy of the drug, and/or cause product recall.

The USP 26 defines the stability of a pharmaceutical product as "extent to which a product retains, within specified limits, and throughout its period of storage and use (i.e. its shelf-life), the same properties and characteristics that it possessed at the time of its manufacture." There are five types of stability that must be considered for each drug as shown in the table below.

Type of Stability	**Conditions Maintained Throughout the Shelf-Life of the Drug Product**
Chemical	Each active ingredient retains its chemical integrity and labeled potency, within the specified limits.
Physical	The original physical properties, including appearance, palatability, uniformity, dissolution, and suspendability are retained.
Microbiological	Sterility or resistance to microbial growth is retained according to the specified requirements. Antimicrobial agents that are present retain effectiveness within the specified limits.
Therapeutic	The therapeutic effect remains unchanged.
Toxicological	No significant increase in toxicity occurs.

Stability of a drug also can be defined as the time from the date of manufacture and packaging of the formulation until its chemical or biological activity is not less than the predetermined level of labeled potency and its physical characteristics have not changed appreciably or deleteriously. Although there are exceptions, 90% of the labeled potency generally is recognized as minimum acceptable potency level. Expiration dating is defined, therefore as the time in which a drug product in a specific packaging configuration will remain stable when stored under recommended conditions.

An expiration date, which is expressed traditionally in terms of months and year, denotes the last day of the month. The expiration date should appear on the

immediate container and the outer retail package. However, when single-dose containers are packaged in individual cartons, the expiration date may be placed on the individual carton instead of the immediate product container. If a dry product is to be reconstituted at the time of dispensing, expiration dates are assigned to both the dry mixture and the reconstituted product. Tamper-resistant packaging is to be used where applicable.

One type of time-related stability study failure is a decrease in therapeutic activity of the preparation to below labeled content. A second type of stability failure is the appearance of a toxic substance, formed as a degradation product upon storage of the formulation. However, it is possible, though remote, for both types of stability failures to occur simultaneously within the same pharmaceutical product. Thus, the use of stability studies with the resulting application of expiration dating to pharmaceuticals is an attempt to predict the approximate time at which the probability of occurrence of a stability failure may reach an intolerable level. This estimate is subject to the usual Type 1 or alpha error (setting the expiration too early so that the product will be destroyed or recalled from the market appreciably earlier than actually is necessary) and Type 2 or beta error (setting the date too late so that the failure occurs in an unacceptably large proportion of cases). Thus, it is obligatory that the manufacturer clearly and succinctly define the method for determining the degree of change in a formulation and the statistical approach to be used in making the shelf-life prediction. An intrinsic part of the statistical methodology must be the statements of value for the two types of error. For the safety of the patient a Type 1 error can be accepted, but not a Type 2 error.

Regulatory Requirement for Stability

Stability study requirements and expiration dating are covered in the current Good Manufacturing Practices (cGMP)[110], the USP[111], and the FDA guidelines.[112]

1. Good Manufacturing Practices

The GMPs [110] state that there shall be a written testing program designed to assess the stability characteristics of drug products. The results of such stability testing shall be used to determine appropriate storage conditions and expiration dating. The latter is to ensure that the pharmaceutical product meets applicable standards o identity,

strength, quality, and purity at time of use. These regulations, which apply to both human and veterinary drugs, are updated periodically in light of current knowledge and technology.

2. Compendia

The compendia also contain extensive stability and expiration dating information. Included are a discussion of stability consideration in dispensing practices and the responsibilities of both the pharmaceutical manufacturer and the dispensing pharmacist. It is now required that product labeling of official articles provide recommended storage conditions and an expiration date assigned to the specific formulation and package. Official storage conditions as defined by the USP26 [111] are as follows: *Cold* is any temperature not exceeding 8°C, and *refrigerator* is a cold place where the temperature is maintained thermostatically between 2 and 8°C. A *freezer* is a cold place maintained between -25 and -10°C. *Cool* is defined as any temperature between 8 and 15°C, and *room temperature* is that temperature prevailing in a working area. *Controlled room temperature* is that temperature maintained thermostatically between 20 and 25°C. *Warm* is any temperature between 30 and 40°C, while *excessive heat* is any heat above 40°C. Should freezing subject a product to a loss of potency or to destructive alteration of the dosage form, the container label should bear appropriate instructions to protect the product from freezing. When no specific storage instructions are given in USP monograph, it is understood that the product's storage conditions shall include protection from moisture, freezing, and excessive heat.

As is noted above in USP 26, the definition of controlled room temperature was a "temperature maintained thermostatically between 20 and 25°C." This definition was established to harmonize with international drug standard efforts. The usual or customary temperature range is defined as 20 to 25°C, with the possibility of encountering excursions in the 15 to 30°C range and with the introduction of the **mean kinetic temperature** (MKT).

3. FDA Guidelines

Food and Drug Authority (FDA) Guidelines provide recommendations for:

1. The design of stability studies to establish appropriate expiration dating periods and product storage requirements.
2. The submission of stability information for investigational new drug, biological, new drug applications, and biological product license applications.

Thus, the guidelines represent a framework for the experimental design and data analysis as well as the type of documentation needed to meet regulatory requirements in the drug-development process.

Table 1.2.1 Stability Protocols

Conditions	Minimum Time Period at Submission
Long-term testing 25°C ±2°C/60%±5% RH	12 months
Accelerated testing 40°C ±2°C/75%±5% RH	6 months
Alternative testing* 30°C ±2°C/65%±5% RH	12 months

*Required if significant change occurs during 6-month storage under conditions of accelerated testing

Product Stability

Many factors affect the stability of a pharmaceutical product and include the stability of the active ingredient(s), the potential interaction between the active and the inactive ingredients, the manufacturing process, the dosage form, the container-liner-closure system, and the environmental conditions encountered during shipment, storage and handling, and length of time between manufacture.

Classically, pharmaceutical product stability evaluations have been separated into studies of chemical (including biochemical) and physical stability of formulations. Realistically, there is no absolute division between these two arbitrary divisions. Physical factors, such as heat, light, and moisture, may initiate or accelerate chemical reactions, while every time a measurement is made on a chemical compound. Physical dimensions are included in the study.

In this treatment, physical and chemical stability are discussed along with those dosage form properties that can be measured and are useful in predicting shelf-life.

The effect of various physical and chemical phenomena of pharmaceuticals also treated.

Knowledge of physical stability of a formulation is very important for three primary reasons. First, a pharmaceutical product must appear fresh, elegant, and professional, for as long as it remains on the shelf. Any changes in physical appearance such as colour fading or haziness ca cause the patient or consumer to lose confidence in the product. Second, since some products are dispensed in multiple-dose containers, uniformity of dose content o the active ingredient over time must be ensured. A cloudy solution or broken emulsion can lead to a non-uniform dosage pattern. Third, the active ingredient must be available to the patient throughout the expected shelf-life of the preparation. A breakdown in the physical system can lead to non-availability or "dose dumping" of the medication to the patient. In the case of metered-dose inhaler pulmonary aerosols, particle aggregation may result in inadequate lung deposition of the medication.

The chemical causes of drug deterioration have been classified as incompatibility, oxidation, reduction, hydrolysis, racemization, and other mechanisms. In the latter category, decarboxylation, deterioration of hydrogen peroxide and hypochlorites, and the formation of precipitates have been included.

Pharmaceutical Dosage Forms

As the various pharmaceutical dosage forms present unique stability problems, they are discussed separately.

1. Tablets

Stable tablets retain their original size, shape, weight, and colour under normal handling and storage conditions throughout their shelf-life. In addition, the *in vitro* availability of the active ingredients should not change appreciably with time.

Excessive powder or solid particles a the bottom of the container, cracks o chips on the face of a tablet, or appearance of crystals on the surface of tablets or on container walls are indications of physical instability of uncoated tablets. Hence, the effect of mild, uniform, and reproducible shaking and tumbling of tablets should be studied.

The recommended test for such studies is the determination of tablet friability as described in the USP. Packaged tablets also should be subjected to cross-country shipping tests as well as to various drop tests.

Tablet hardness (or resistance to crushing or fracturing) can be assessed by commercially available hardness testers. As results will vary with the specific make of the test apparatus used, direct comparison of results obtained on different instruments may not necessarily be made. Thus, the same instrument should be used consistently throughout a particular study.

Colour stability of tablets can be followed by appropriate colorimeter or reflectometer with heat, sunlight, and intense artificial light employed to accelerate the colour determination. Caution must be used in interpreting the elevated temperature data, as the mechanism for degradation at that temperature may differ from that at lower temperature. It is not always proper to assume that the same changes will occur at elevated temperatures as will be evidenced later at room temperature. Cracks, mottling, or tackiness of the coating indicates evidence of instability of coated tablets.

For tablets containing the more insoluble active ingredients, the results of dissolution tests are more meaningful than disintegration results for making bioavailability predictions. Dissolution-rate tests should b run in an appropriate medium such as artificial gastric and/or intestinal fluid at 37°C. When no significant change (such as a change in the polymorphic form of the crystal) has occurred, an unaltered dissolution-rate profile of a tablet formulation usually indicates constant *in vivo* availability.

Uniformity of weight, odour, texture, drug and moisture contents, and humidity effect also are studied during a tablet stability test.

2. Gelatin Capsules

Hard gelatin capsules are the type use by pharmaceutical manufacturers in the production of the majority of their products. The pharmacist in the extemporaneous compounding of prescriptions may also se hard gelatin capsules. Soft gelatin capsules are prepared from shells o geltin to which glycerin or a polyhydric alcohol such as sorbitol has been added to render the gelatin elastic or plastic-like. Gelatin is

stable in air when dry but is subject to microbial decomposition when it becomes moist or when it is maintained in aqueous solution. Normally, hard gelatin capsules contain between 13% and 16% moisture. If stored a high humidity environment capsule shells may soften, stick together, or become distorted and lose their shape. On the other hand, in an environment of extreme dryness gelatin capsules may harden and crack under slight pressure. Gelatin capsules should be protected from sources of microbial contamination.

Encapsulated products, like all other dosage forms, must be packaged properly. Because moisture may be absorbed or released by gelatin capsules depending on the environmental conditions, capsules offer little physical protection to hygroscopic or deliquescent materials enclosed within a capsule when stored in an area of high humidity. It is not uncommon to find capsules packaged in containers along with a packet of desiccant material as a precautionary measure.

Both hard and soft gelatin capsules exposed to excessive heat and moisture may exhibit delayed or incomplete dissolution due to cross-linking of the gelatin in the capsule shell. The cross-linking of gelatin capsules is an irreversible chemical reaction. Cross-linking may also occur in capsules that are exposed to aldehydes and peroxides. Although cross-linked capsules may fail dissolution due to pellicle formation, digestive enzymes will dissolve the capsules. For hard or soft gelatin capsule that do not conform to the dissolution specification, the dissolution test may be repeated with the addition of enzymes. Where water or a medium with a pH less than 6.8 is specified as the, medium in the individual monograph, the same medium specified may be used with the addition of purified pepsin that results in an activity of 750,000 units or less per 1000mL. For media with a pH of 6.8 or greater, pancreatin can be added to produce not more than 1750 USP units of proteas activity per 1000mL.

3. Suspensions

A stable suspension can be redispersed homogeneously with moderate shaking and can be poured easily through its shelf-life, with neither the particle-size distribution and the crystal form, nor the physiological availability of the suspended active ingredient changing appreciably with time. Most stable pharmaceutical suspensions are flocculated; that is, the suspended particles are bonded together physically to

form a loose, semi rigid structure. The particles are said to uphold each other while exerting no significant force on the liquid. Sedimented particles of a flocculated suspension can be redispersed easily at any time with only moderate shaking.

In non-flocculated suspensions, the particles remain as individuals unaffected by neighbouring particles and are affected only by the suspension vehicle. These particles, which are smaller and lighter, settle slowly, but once they have settled, often form a hard, difficult-to-disperse sediment. Non-flocculated suspensions can be made acceptable by decreasing the particle size of the suspended material or by increasing the density and viscosity of the vehicle, thus reducing the possibility of settling.

When studying the stability of suspension, first determine with differential manometer if the suspension is flocculated. If the suspension is flocculated, the liquid will travel the same distance in the two side arms. With non-flocculated suspensions, the hydrostatic pressures in the two arms are unequal; hence, the liquids will be at different levels.

The history of settling of the particles of a suspension may be followed by a Brookfield viscometer fitted with a Helipath attachment. This instrument consists of a rotating T-bar spindle that descends slowly into the suspension as it rotates. The dial reading on the viscometer is a measure of the resistance that the spindle encounters at various levels of the sedimented suspension. This test must be run only on fresh, undisturbed samples.

An electronic particle counter and sizer, such as a Coulter counter, or a microscope may be used to determine changes in particle-size distribution. Crystal form alterations may be detected by microscopic, near-IR or Raman examination and, when suspected, must be confirmed by x-ray powder diffraction.

All suspensions should be subjected to cycling temperature conditions to determine the tendency for crystal growth to occur within the suspension. Shipping tests, i.e., transporting bottles across the country by rail or truck are also used to study the stability of suspensions.

4. Solutions

A stable solution retains its original clarity, colour, and odour throughout its shelf-life. Retention of clarity of a solution is a main concern of a physical stability program. As visual observation alone under ordinary light is a poor test of clarity, a microscope light should be projected through a diaphragm into the solution. Undissolved particles will scatter the light, and the solution will appear hazy. While the Coulter counter also can be used, light-scattering instruments are the most sensitive means of following solution clarity.

Solutions should remain clear over a relatively wide temperature range such as 4 to 47°C. At the lower range an ingredient may precipitate due to its lower solubility at that temperature, while at the higher temperature the flaking of particles from the glass container or rubber closures may destroy homogeneity. Thus, solutions should be subjected to cycling temperature conditions.

The stability program for solutions also should include a study of pH changes, especially when the active ingredients are soluble salt of insoluble acids or bases. Among other tests are observations for changes in odour, appearance, colour, taste, light stability, redispersibility, suspendibility, pourability, viscosity, isotonicity, gas evolution, microbial stability, specific gravity, surface tension, and pyrogen content, in the case of parenteral products.

When solutions are filtered, the filter medium may absorb some of the ingredients fom the solution. Thus, the same type of filter should be used for preparing the stability samples as will be used to prepare the production-size batches.

For dry-package formulations reconstituted prior to use, the visual appearance should be observed on both the original dry material and on the reconstituted preparation. The colour and odour of the cake, the colour odour of the solution, the moisture content of the cake, and the rate of reconstitution should be followed as a part of its stability profile.

5. Emulsions

A stable emulsion can be redispersed homogeneously to its original state with moderate shaking and can be poured at any stage of its shelf life. Although most of

the important pharmaceutical emulsions are of the oil in water (O/W) type, many stability test methods can be applied to either an O/W or water in oil (W/O) emulsion.

Two simple tests are used to screen emulsion formulations. First, heating to 50 to 70°C and observing its gross physical stability either visually or by turbidimetric measurements can determine the stability of an emulsion. Usually the emulsion that is the most stable to heat is the one most stable at room temperature. However, this may not be true always, because an emulsion at 60°C may not be the same as it is at room temperature. Second, the stability of the emulsion can be estimated by the coalescence time test. Although this is only a rough quantitative test, it is useful for detecting gross differences in emulsion stability at room temperature.

Emulsions also should be subjected to refrigeration temperatures. An emulsion stable at room temperature has been found to be unstable at 4°C. It was reasoned that an oil-soluble emulsifier precipitated at the lower temperature and disrupted the system. An emulsion chilled to the extent that the aqueous base crystallizes is damaged irreversibly.

The ultracentrifuge also is used to determine emulsion stability. When the amount of separated oil is plotted against the time of centrifugation, a plateau curve is obtained. A linear graph results when the oil flotation (creaming) rate is plotted versus the square of the number of centrifuge revolutions per minute. The flotation rate is represented by the slope of the line resulting when the log distance of emulsion water boundary from the rotor center is plotted against time for each revolution per minute.

For stability studies, two batches of an emulsion should be made at one time on two different sizes of equipment. One should be a bench-size lot and the other a larger, preferably production-size, batch. Different types of homogenizers produce different results, and different sizes of the same kind of homogenizer can yield emulsions with different characteristics.

6. Ointments

Ointments have been defined as high viscosity suspensions of active ingredients in a non-reacting vehicle. A stable ointment is one that retains its homogeneity throughout is shelf-life period. The main stability problems observed in ointments

are bleeding and changes in consistency due to aging or changes in temperature. When fluid components such as mineral oil separate at the top of an ointment, the phenomenon is known as bleeding and can be observed visually. Unfortunately, as there is no known way to accelerate this event, the tendency to bleed cannot be predicted.

An ointment that is too soft is messy to use, while one that is very stiff is difficult to extrude and apply. Hence, it is important to be able to define quantitatively the consistency of an ointment. This may be done with a penetrometer, an apparatus that allows a pointed weight to penetrate into the sample under a measurable force. The depth of the penetration is a measure of the consistency of an ointment. Consistency also can be measured by the Helipath attachment to a high-viscosity viscometer or by a Burrell Severs rheometer. In the latter instrument, the ointment is loaded into a cylinder and extruded with a measured force. The amount extruded is a measure or the consistency or the ointment.

Ointments have a considerable degree of structure that requires a minimum of 48 hours to develop after preparation. As rheological data on a freshly made ointment may be erroneous, such tests should be performed only after the ointment has achieved equilibrium. Slight changes in temperature (1 or 2°C) can affect the consistency of an ointment greatly; hence, rheological studies on ointments must be performed only at constant and controlled temperatures.

Among the other tests performed during the stability study of an ointment are a check of visual appearance, color, odor, viscosity, softening range, consistency, homogeneity, particle-size distribution, and sterility. Undissolved components of an ointment may change in crystal form or in size with time. Microscopic examination or x-ray diffraction measurement may be used to monitor these parameters.

In some instances it is necessary to use an ointment base that is less than ideal, to achieve the required stability. For example, drugs that hydrolyze rapidly are more stable in a hydrocarbon base than in a base containing water, even though they may be more effective in the latter.

7. Transdermal Patches

A typical transdermal patch consists of a protective backing, a matrix containing active drug , an adhesive that allows the patch to adhere at the skin, and a release liner to protect the skin adhering adhesive. Therefore, the transdermal patch must deliver drug as labeled, adhere properly to both the backing and to the patient's skin. In addition, the transdermal patch must be pharmaceutically elegant through the shelf life of the product. For a transdermal patch, this means that the release line peels easily with minimal transfer of adhesive onto the release liner and that the adhesive does not ooze from the sides of the patch. Therefore, the typical stability related tests for transdermal patches are, appearance, assay, impurities, and drug release USP<724> and, backing peel force.

Metered-Dose Aerosols Drug Products

A metered dose inhalation product consists of an aerosol can containing a propellant, a drug and a mouthpiece used to present an aerosolized drug to the patient. There are many drug contact components in a metered-dose inhalation product. Therefore, the drug may be in contact with materials that could allow plasticizer leach into the drug. The typical stability related tests for metered-dose aerosols include appearance, assay, impurities, plume geometry, emitted dose, particle size distribution of the emitted dose, and number of doses per unit. In addition, stability studies on leachables may be required. Shelf life of metered-dose aerosols drug products may also be dependent on the orientation that the drug product is stored. Typically most canisters type product are tested at least in the upright orientation.

Dry-Powdered Inhalation Products

A dry powdered inhalation product consists of drug with excipients delivered in a dry powdered form. The delivery system for a dry-powdered inhalation product may be a separate device or integrated with the active. A dry-powdered dosage must reproducibly deliver a specific amount of drug at a particle size that can be deposited into the lungs. Particles to large will get trapped in the throats and particles too small will just be carried out of the lungs on the next expiration. The typical stability related tests for dry powder inhalation products include appearance, assay,

impurities, emitted dose, particle size distribution of the emitted dose, and water content.

8. Nasal Inhalation Products

A nasal inhalation product consists of drug with excipients delivered from a delivery system. The delivery system for a nasal inhalation product may be a separate device or integrated with the active amount of drug at a particle size and plume that can be deposited in the nasal membrane. Particles too large will not be absorbed into nasal membrane or run out of the nose; and poor spray pattern will deposit the drug ineffective in the nasal cavity. The typical stability related tests for nasal inhalation products include appearance, assay, impurities, spray content uniformity, particle(droplet) size distribution of the emitted dose, spray pattern or/ and plume geometry, leachables, weight loss and preservative content. Sterility and microbial testing may be required periodically for stability testing.

Incompatibility

Typically, physicochemical stability is assessed at the preformulation stage of development. A drug substance candidate is treated with acid, base, heat, light, and oxidative conditions to assess its inherit chemical stability. Binary mixtures of the drug substance with individual recipients are also investigated at the preformulation stage. These tests are performed to determine the drug substance sensitivity to degrade or react with common pharmaceutical excipients. The most common reactions observed for drug substance from these test include: hydrolysis, epimerization (racemization), decarboxylation, dehydration, oxidation, polymerization, photochemical decomposition, and addition. All drug substances have the potential to degrade by at least one of the reactions mentioned above. With an understanding of the stability/ reactivity of a drug substance in the preformulation stage, it is possible to formulate the drug product to minimize drug decomposition. Numerous examples are described in other sections of this book, and the literature is replete with illustrations.

While undesirable reactions between two or more drugs are said to result in a physical, chemical, or therapeutic incompatibility, physical incompatibility is

somewhat of a misnomer. It has been defined as a physical or chemical interaction between two or more ingredients that leads to a visibly recognizable change. The latter may be in the form of a gross precipitate, haze, or color change.

On the other hand, a chemical incompatibility is classified as a reaction in which a visible change is not necessarily observed. Since there is no visible evidence of deterioration, this type of incompatibility requires trained, knowledgeable personnel to recognize it.

A therapeutic incompatibility has been defined as an undesirable pharmacological interaction between two or more ingredients that leads to

1. Potentiation of the therapeutic effects of the ingredients
2. Destruction of the effectiveness of one or more of the ingredients
3. Occurrence of a toxic manifestation within the patient.

Reaction Kinetics

An understanding of reaction kinetics is important in determining the shelf life of a product.

Chemical Reactions

The most frequently encountered chemical reactions, which may occur within a pharmaceutical product, are described below.

1. Oxidation-Reduction

Oxidation is a prime cause of product instability, and often, but not always, the addition of oxygen or the removal of hydrogen is involved. When molecular oxygen is involved, the reaction is known as auto-oxidation because it occurs spontaneously, though slowly, at room temperature.

Oxidation, or the loss of electrons from an atom, frequently involves free radicals and subsequent chain reactions. Only a very small amount of oxygen is required to initiate a chain reaction. In practice, it is easy to remove most of the oxygen from a container, but very difficult to remove it all. Hence, nitrogen and carbon dioxide frequently are used to displace the headspace air in pharmaceutical containers to help minimize deterioration by oxidation.

As an oxidation reaction is complicated, it is difficult to perform a kinetic study on oxidative processes within a general stability program. The redox potential, which is constant and relatively easy to determine, can, however, provide valuable predictive information. In many oxidative reactions, the rate is proportional to the concentration of the oxidizing species but may be independent of the concentration of the oxygen present. The rate is influenced by temperature, radiation, and the presence of a catalyst. An increase in temperature, leads to acceleration in the rate of oxidation. If the storage temperature of a preparation can be reduced to 0 to 5°C, usually it can be it can be assumed that the rate of oxidation will be at least halved.

The molecular structures most likely to oxidize are those with a hydroxyl group directly bonded to an aromatic ring (eg, phenol derivatives such as catecholamines and morphine), conjugated dienes (eg, vitamin A and unsaturated free fatty acids), heterocyclic aromatic rings, nitroso and nitrite derivatives, and aldehydes (eg, flavorings). Products of oxidation usually lack therapeutic activity. Visual identification of oxidation, for example, the change from colorless epinephrine to its amber colored products, may not be visible in some dilutions or to some eyes.

Oxidation is catalyzed by pH values that are higher than optimum, polyvalent heavy metal irons (eg, copper and iron), and exposure to oxygen and UV illumination. The latter two causes of oxidation justify the use of antioxidant chemicals, nitrogen atmospheres during ampul and vial filling, opaque external packaging, and transparent amber glass or plastic containers.

Trace amounts of heavy metals such as cupric, chromic, ferrous, or ferric ions may catalyze oxidation reactions. As little as 0.2mg of copper ion per liter considerably reduces the stability of penicillin. Similar examples include the deterioration of epinephrine, phenylephrine, lincomycin, isoprenaline, and procaine hydrochloride. Adding chelating agents to water to sequester heavy metals and working in special manufacturing equipment (eg, glass) are some means used to reduce the influence of heavy metals on a formulation. Parenteral formulations should not come in contract with heavy metal ions during their manufacture, packaging, or storage.

Hydronium and hydroxyl ions catalyze oxidative reactions. The rate of decomposition for epinephrine, for example, is more rapid in a neutral or alkaline solution with maximum stability (minimum oxidative decomposition) at pH 3.4.

There is a pH range for maximum stability for any antibiotic and vitamin preparation, which usually can be achieved by adding an acid, alkali, or buffer.

Oxidation may be inhibited by the use of antioxidants, called negative catalysts. They are very effective in stabilizing pharmaceutical products undergoing a free-radical-mediated chain reaction. These substances, which are easily oxidizable, act by possessing lower oxidation potentials than the active ingredient. Thus, they undergo preferential degradation or act as chain inhibitors of free radicals by providing an electron and receiving the excess energy possessed by the activated molecule.

The ideal antioxidant should be stable and effective over a wide pH range, soluble in its oxidized form, colorless, nontoxic, nonvolatile, nonirritating, effective in low concentrations, thermostable, and compatible with the container-closure system and formulation ingredients.

The commonly used antioxidants for aqueous systems include sodium sulfite, sodium metabisulfite, sodium bisulfate, sodium thiosulfate, and ascorbic acid. For oil systems, ascorbyl palmitate, hydroquinone, propyl gallate, butylated hydroxyt oluene, butylated hydroxyanisole, and alpha-tocopherol are employed.

Synergists, which increase the activity of antioxidants, are generally organic compounds that complex small amounts of heavy metal ions. These include the ethylenediamine tetraacetic acid (EDTA) derivatives, dihydroethylglycine, and citric, tartaric, gluconic, and saccharic acids. EDTA has been used to stabilize ascorbic acid, oxytetracycline, penicillin, epinephrine, and prednisolone.

Reduction reaction is much less common than oxidative processes in pharmaceutical practice. Examples include the reduction of gold, silver, or mercury salts by light to form the corresponding free metal.

2. Hydrolysis

Drugs containing esters (eg. Cocaine physostigmine, aspirin, tetracaine, procaine and methyldopa), amides (eg. Dibucaine), imides (eg, amobarbital), imines (eg, diazepam), and lactam (eg, penicillins, cephalosporins), functional groups are among those prone to hydrolysis.

Hydrolysis reactions are often pH dependent and are catalyzed by either hydronium ion or hydroxide ions (specific-acid or specific-base catalysis, respectively). Hydrolysis reactions can also be catalyzed by either a Bronsted acid or a Bronsted base (genera-acid or general-base catalysis, respectively). Sources of Bronsted acid or base include buffers and some excipients. Sometimes, it is necessary to compromise between the optimum pH for stability and that for pharmacological activity. For example, several local anesthetics are most stable at a distinctly acid pH, whereas for maximum activity they should be neutral or slightly alkaline. Small amounts of acids, alkalines, or buffers are used to adjust the pH of a formulation. Buffers are used when small changes in pH are likely to cause major degradation of the active ingredient.

Obviously, the amount of water present can have a profound effect on the rate of a hydrolysis reaction. When the reaction takes place fairly rapidly in water, other solvents sometimes can be substituted. For example, barbiturates are much more stable at room temperature in propylene glycol- water than in water alone.

Modification of chemical structure may be used to retard hydrolysis. In general, as it is only the fraction of the drug in solution that hydrolyzes, a compound may be stabilized by reducing its solubility. This can be done by adding various substituents to the alkyl or acyl chain of aliphatic or aromatic esters or to the ring of an aromatic ester. In some cases less-soluble salts or esters of the parent compound have been found to aid product stability. Steric and polar complexations have also been employed to alter the rate of hydrolysis. Caffeine reduces the rate of hydrolysis and thus promotes stability by complexation with local anesthetice such as benzocaine, procaine, or tetracaine.

Esters and β-lactams are the chemical bonds are the most likely to hydrolyze in the presence of water. For example, the acetyl ester in aspirin is hydrolyzed to acetic acid and salicylic acid in the presence of moisture, but in a dry environment the hydrolysis of aspirin is negligible. The aspirin hydrolysis rate increases in direct proportion to the water vapor pressure in an environment.

The amide bond also hydrolyzes, though generally at a slower rate than comparable esters. For example, procaine (an ester) will hydrolyze upon autoclaving, but procainamide will not. The amide or peptide bond in peptides and proteins varies in

the labiality to hydrolysis. The lactam and azomethine (orimine) bonds in benzodiazepines are also labile to hydrolysis. The major chemical accelerators or catalysts of hydrolysis are adverse pH and specific chemicals (eg, dextrose and copper in the case of ampicillin hydrolysis).

The rate of hydrolysis depends on the temperature and the pH of the solution. A much-quoted estimation is that for each 10°C rise in storage temperature, the rate of reaction doubles or triples. As this is empiricism, it is not always applicable.

When hydrolysis occurs, the concentration of the active ingredient decreases while the concentration of the decomposition products increases. The effect of this change on the rate of the reaction depends on the order of the reaction. With zero-order reactions the rate of decomposition is independent of concentration of the ingredient. Although dilute solutions. Increasing the concentration of an active ingredient that is hydrolyzing by zero-order kinetics will slow the percentage decomposition.

With first-order reactions, which occur frequently in the hydrolysis of drugs, the rate of change is directly proportional to the concentration of the reactive substance. Thus, changes in the concentration of the active ingredient have no influence on the percentage decomposition.

The degradation of many drugs in solution accelerates or decelerates exponentially as the pH is decreased or increased over a specific range of pH values. Improper pH ranks with exposure to elevated temperature as a factor most likely to cause a clinically significant loss of drug, resulting from hydrolysis and oxidation reactions. A drug solution or suspension, for example may be stable for days, weeks, or even years in its original for mulation, but when mixed with anther liquid that changes the pH, it degrades in minutes or days. It is possible that a pH change of only one unit (eg, from 4 to 3 or 8 to 9) could decrease drug stability by a factor of ten or greater.

A pH -buffer system, which is usually a weak acid or base and its salt, is a common excipient used in liquid preparations to maintain the pH in a range that minimizes the drug degradation rate. The pH of drug solutions may also be either buffered or adjusted to achieve drug solubility. For example, pH in relation to pKa controls the fractions of the usually more soluble ionized and less soluble nonionized species of weak organic electrolytes.

3. Interionic (Ion N+ -Ion N-) Compatibility

The compatibility or solubility of oppositely charged ions depends mainly on the number of charges per ion and the molecular size of the ions. In general, polyvalent ions of opposite charge are more likely to be incompatible. Thus, an incompatibility is likely to occur upon the addition of a large ion with a charge opposite to that of the drug. As many hydrolytic reactions are catalyzed by both hydroxonium and hydroxyl ions, pH is an important factor in determining the rate of a reaction. The pH range of minimum decomposition (or maximum stability) depends on the ion having the greatest effect on the reaction. If the minimum occurs at about pH 7, the two ions are of equal effect. A shift of the minimum toward the acid side indicates that the hydroxyl ion has the stronger catalytic effect and vice versa in the case of a shift stronger effect. Thus, the minimum is often found between pH 3 and pH 4. The influence of pH on the physical stability of two-phase systems, especially emulsions, is also important. For example, intravenous fat emulsion is destabilized by acidic pH.

4. Decarboxylation

Pyrolytic solid-state degradation through decarboxylation usually is not encountered in pharmacy, as relatively high heats of activation (25 to 30 kcal) are required for the reaction. However, solid p-aminosalicylic acid undergoes pyrolytic degradation to m-aminophenol and carbon dioxide. The reaction, which follows first-order kinetics, is highly pH-dependent and is catalyzed by hydronium ions. The decarbocylation of p-aminobenzoic acid occurs only at extremely low pH values and at high temperatures.

Some dissolved carboxylic acids, such as p-aminosalicylic acid, lose carbon dioxide from the carboxyl group when heated. The resulting product has reduced pharmacological potency. β-Keto decarboxylation can occur in some solid antibiotics that have a carbonyl group on the β-carbon of a carboxylic acid or a carboxylate anion. Such decarboxylations will occur in the following antibiotics: carbenicillin sodium, carbenicillin free acid, ticarcillin sdium, and ticarcillin free acid.

5. Racemization

Racemization, or the action or process of changing from an optically active compound into a racemic compound or an optically active mixture of corresponding R (rectus)

And S (sinister) forms, is a major consideration in pharmaceutical stability. Optical activity of a compound may be monitored by polarimetry and reported in terms of specific rotation. Chiral HPLC has been used in addition to polarimetry to confirm the enantiomeric purity of a sample.

In general, racemization follows first-order kinetics and depends on temperature, solvent, catalyst, and the presence or absence of light. Racemization appears to depend on the functional group bound to the asymmetric carbon atom, with aromatic groups tending to accelerate the process.

6. **Epimerization**

Members of the tetracycline family are most likely to incur epimerization. This reaction occurs rapidly when the dissolved drug is exposed to a pH of an intermediate range (higher than 3), and it results in the steric rearrangement of the dimethylamino group. The epimer of tetracycline, epitetracycline, has little or no antibacterial activity.

Photochemical Reactions

Photolytic degradation can be an important limiting factor in the stability of pharmaceuticals. A drug can be affected chemically by radiation of a particular wavelength only if it absorbs radiation at that wavelength and the energy exceeds a threshold. Ultraviolet radiation, which has a high energy level, is the cause of many degradation reactions. Exposure to, primarily, UV illumination may cause oxidation (photo-oxidation) and scission (photolysis) of covalent bonds. Nifedipine, nitroprusside, riboflavin, and phenothiazines are very labile to photo-oxidation. In susceptible compounds, photochemical energy creates free radical intermediates, which can perpetuate chain reactions.

If the absorbing molecule reacts, the reaction is said to be photochemical in nture. When the absorbing molecules do not participate directly in the reaction, but pass their energy to other reacting molecules, the absorbing substance is said to be a photosensitizer,

Colored-glass containers most commonly are used to protect light-sensitive formulations. Yellow-green glass gives the best protection in the ultraviolet region,

while amber confers considerable protection from ultraviolet radiation but little from infrared. Riboflavin is best protected by a stabilizer that has a hydroxyl group attached to or near the aromatic ring. The photodegradation of sulfacetamide solutions may be inhibited by an antioxidant such as sodium thiosulfate or metabisulfite.

A systematic approach to photostability testing is recommended covering, as appropriate, studies such as tests on the drug substance, tests on the exposed drug product outside of the immediate pack; and if necessary, tests on the drug product in the immediate pack. ICH Q1B discusses the minimum requirements for assessing photostability. Drug substance is first assessed by exposing sample powder having a depth of not more than 3 mm to an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter. If the drug substance shows sensitivity to photodegration, then the drug product will need to be tested as well. The testing of drug product uses the same light exposure that was used to test drug substance. The drug product should be tested directly exposed to light and in its container closure system.

Ultrasonic Energy

Ultrasonic energy, which consists of vibrations and waves with frequencies greater than 20,000Hz, promotes the formation of free radicals and alters drug molecules. Changes in prednisolone, prednisone acetate, or deoxycorticosterone acetate suspensions in an ultrasonic field have been observed spectrometrically in the side chain at C-17 and in the oxo group of the A rings. With sodium alginate, in an ultrasonic field, it has been reported that above a minimum power output, degradation increased linearly with increased power.

Ionizing Radiation

Ionizing radiation, particularly gamma rays, has been used for the sterilization of certain pharmaceutical products. At the usual sterilizing dose, 2.5mRad, it seldom causes appreciable chemical degradation. In general, formulations that are in the solid or frozen state are more resistant to degradation from ionizing radiation than those in liquid form. For example, many of the vitamins are little affected by irradiation in the solid state but are decomposed appreciably n solution. On the other

hand, both the liquid- and solid or frozen state are more resistant to degradation from ionizing radiation than those in liquid form. For example, many of the vitamins are little affected by irradiation in the solid state but are decomposed appreciably in solution. On the other hand, both the liquid- and solid-state forms of atropine sulfate are affected seriously by radiation.

Prediction Shelf-Life

ICH Recommended evaluation

The shelf life of a commercial drug product must be determined in the commercial container closure at the defined storage conditions. ICH requires at least 12months stability data at the time of NDA submission. Most products require at least 24months to be commercially viable. The ICH Q1E recommends how the 12 months data may be used to predict long-term stability.

Approximations in assessing Product Stability

1. Estimation of temperature effect.

In early development, a shelf life prediction of a clinical material, especially a phase I material, may be based on a very limited amount of sample and limited amount of time to make the evaluation. One way to estimate long-term storage for a material is by extrapolating data from studies performed at elevated conditions. An understanding of potential activation energy is needed to estimate long-term stability. Many may have heard of the estimate that for every 10°C decrease in storage temperature the shelf-life doubles. This is only true; however, if the activation energy of the reaction(s) that causes degradation is 15 kcal/moles. The activation energy, Ea, for many chemical processes related to the degradation of a drug substance/ product is typically within the range of 10 to 25 kcal/ moles.

The equation below shows a way of calculating the $Q_{\Delta T}$ value that may be used to estimate the effect of temperature on shelf-life.

$$Q_{\Delta T} = \exp\left[\frac{E_a}{R}\left(\frac{\Delta T}{T+\Delta T\,(T)}\right)\right]$$

Where, $Q_{\Delta T}$ is a factor (multiplier/divisor) used to estimate the change in the reaction rate constant with change in temperature, ΔT. Ea is the activation energy established for a reaction.

An approximation for the change in reaction rate constants due to the temperature effects are shown in the table below.

Ea (kca/mole)	Q_5 (25 to 30°C)	Q_{10} (25 to 35°C)	Q_{15} (25 to 40°C)
10	1.32	1.73	2.24
15	1.52	2.27	3.36
20	1.75	2.99	5.04
25	2.01	3.93	7.55

Therefore, the old rule of thumb that a reaction rate doubles with every 10°C is only true if the reaction has an activation energy between 10 to 15 kcal/mole (Q_{10} =1.73 and 2.27, respectively). Q_{15} is useful for understanding the relationship of ICH accelerated temperature of 40°C has with controlled room temperature a 25°C. Materials made and packaged for clinical studies are usually tested at an accelerated condition in order to predict that the packaged material will be stable for the duration of the clinical study. A material stable for one month at accelerated temperature (40°C) supports that the material stored at room temperature should be stable for at least 3 months. This true only when the activation energy to the degradation process is about 15 kcal/mole (Q_{15} factor = 3.36) [in other words, a reaction at 40°C should be 3.36 times faster than the same reaction at 25°C; or the reaction will take 3.36 times longer at 25°C than at 40°C)].

The technique of estimating the shelf life of a formulation from its accumulated stability data has evolved from examining the data and making an educated guess through plotting the time-temperature points on appropriate graph paper and crudely extrapolating a regression line to the application of rigorous physical- chemical lows, statistical concepts, and computers to obtain meaningful, reliable estimates.

A simple means of estimating shelf life from a set of computer- prepared tables has been described by Lintner et al.[112] This system was developed to select the select the best prototype formulation on the basis of short-term stability data and predict both estimated and minmum shelf-life values for the formulation. It is a middle ground approach between the empirical methods and the modern, rigorous statistical

concepts. All calculations can be made readily by hand, and the estimated values can be obtained easily from appropriate tables. The system assumes that.

1. Shelf-life predictions can be made satisfactorily for lower temperatures using the classical Arrhenius model from data obtained at higher temperatures.
2. The energy of activation of the degradation reaction is between 10-20 kcal/mol (this is a safe assumption, as Kennon [114] has noted that rarely are drugs with energies of activation below 10 kcal/ mol, the error in the shelf-life prediction will be on the conservative side)
3. The rate of decomposition will not increase beyond that already observed.
4. The standard deviation of the replicated assays is known or can be estimated from the analytical data.

This concept further assumes that the degradation reaction follows zero-or pseudo-zero- order kinetics. For data corresponding to a zero, first-, or second-order degradation pattern, it is impossible to distinguish one order from anther with usual analytical procedures, when the total degraded material is not large. In addition, shelf-life calculations assuming zero-order kinetics are more conservative than those for higher orders.

This middle-ground system is useful in creating the experimental design for the stability study. The formulator has the opportunity to study various combinations of parameters to try to optimize the physical-statistical model. One can check the effect of improving the assay standard deviation, running additional replicates, using different time points, and assuming various degradation rates and energies of activation on the stability of the test formulation.

McMinn and **Lintner** later developed and reported on an information –processing system for handling product stability data.[115] this system saves the time of formulators in analyzing and interpreting their product stability data, in addition to minimizing the amount of clerical help needed to handle an ever – increasing assay load. For products such as those of vitamins, for example, where large overages are required, the statistical portions of this advanced technique aid the manufacturer to tailor the formula composition to obtain the desired and most economical expiration dating.

This system stores both physical and chemical data and retrieves the information in three different formats (one of which was designed specifically for submitting to regulatory agencies). It analyzes single-temperature data statistically by analysis of covariance and regression or multiple- temperature data by weighted or unweighted analysis using the Arrhenius relationship; provides estimates of the shelf life of the preparation with the appropriate confidence intervals; preprints the assay request cards that are used to record the results of the respective assay procedures and to enter the data into the system; and produces a 5-yr master-stability schedule as well a periodic 14- day schedule of upcoming assays.

As mentioned above, a portion of the advanced system analyzes the stability data obtained at a single temperature by analysis of covariance and regression. This analysis is based on the linear (zero- order) model.

$$Y_{ij} = \beta_i X_{ij} + \alpha_i + \varepsilon_{ij}$$

Where Y_{ij} is the percentage of label of the jth stability assay of the ith lot, X_{ij} is the time in months at which Y_{ij} was observed, β_i and α_i are the slope and intercept, respectively, of the regression line of the ith lot, and ε_{ij} is a random error associated with Y_{ij}. The random errors are assumed to be identically and independently distributed normal variables with a zero mean and a common variance.

Traditionally, extensive stability data are collected at the recommended storage temperatures (usually refrigerator and/or room temperature) to be placed on the label of the package. However, elevated-temperature data are very valuable in determining the shelf-life of a product. In practice, multiple levels of thermal stress are applied to the formulation so that appropriate shelf-life estimates can be made for normally expected marketing conditions. In case in which data from accelerated studies are used to project a tentative expiration date that is beyond the date supported by actual shelf-life studies, testing must continue until the tentative expiration date is verified.

According to the Arrhenius relationship, faster degradation occurs at the higher temperatures; hence assays for the higher data usually are run more often but for a shorter period of time. The effect simple least-squares analysis of this type of data is to force the Arrhenius equation through the low temperature data and essentially ignore the high-temperature information. Thus, much more credence is placed in the

estimates of the low temperature than is warranted. In addition, the usual confidence limits on extrapolated degradation rates at refrigerator or room temperature cannot be made validly. For these reasons, Bentley [116] presented a method based on a weighted least-squares analysis to replace the unweighted approximation. He also developed a statistical test for the validity of the Arrhenius assumption, which is computed easily from the results of the unweighted method.

To make shelf-life estimates from elevated temperature data, two storage temperatures are obviously the minimum. As the accuracy of the extrapolation is enhanced by using additional temperatures, a minimum of four different temperatures is recommended for most product stability studies. With the current of computers to do the bulk of stability calculations, including weighted least-squares analysis, the temperatures and storage conditions need not be selected for arithmetic convenience. It is not necessary to determine the mechanism of the degradation reaction. In most cases, it is necessary only to follow some property of degradation and to linearize this function. Either the amount of intact drug or the amount of a formed degradation product may be followed. It usually is impractical to determine the exact order of the reaction. With assay errors in the range of 2 to 5%, at least 50% decomposition must occur before the reaction order can be determined. As the loss with pharmaceutical generally is less, zero-order kinetics should be assumed, unless the reaction order is known from previous work.

In any case, replication of stability assay is advisable. The batches of drugs used for stability study should be representative of production run material or at least material f a known degree of purity. The quality of the excipients also should be known, as their impurities or even their moisture content can affect product stability deleteriously. Likewise, the samples of the formulation taken for the stability study must be representative of the lot.

Specific, stability-indicating assay methods must be used, to make meaningful shelf-life estimates. The reliability and specificity of the test method on the intact molecule and on the degradation products must be demonstrated.

2. Addition of Overage

The problem of declining potency in an unstable preparation can be ameliorated by the addition of an excess or overage of the active ingredient. Overages, then, are added to pharmaceutical formulations to keep the content of the active ingredient within the limits compatible with therapeutic requirements, for a predetermined period of time.

The amount of the overage depends upon the specific ingredient and the galenical dosage form. The International Pharmaceutical Federation has recommended that overages be limited to a maximum of 30% over the labeled potency of an ingredient.

1.8 Degradation products of Paracetamol

Under the conditions of high temperature, moisture and pH, paracetamol undergoes hydrolysis forming 4-aminophenol and acetic acids as major degradation products.

Aside these major degradation products, paracetamol can also undergo hydrolysis under certain conditions to yield other minor degradation products such as:

- ✓ 4-nitrophenol
- ✓ 4-hydroxy acetophenone
- ✓ Chloroxazone
- ✓ 4- hydroxy acetophenone oxime
- ✓ 4-chloroacetanilide

These hydrolytic products of paracetamol are relatively toxic to the human tissues and organ when present in relatively large quantities in paracetamol formulations [100].

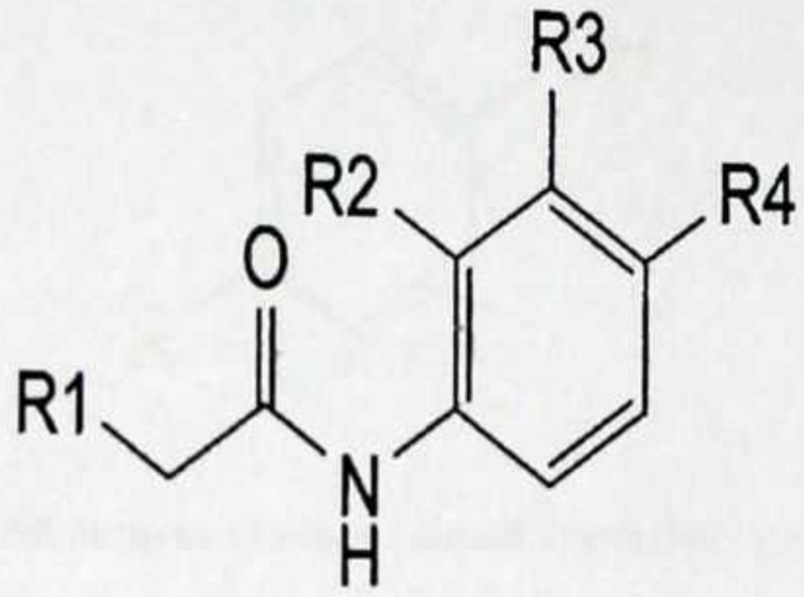


Figure 1.6: Chemical Structures of Impurities present in Paracetamol

A. R1 = R3 = R4 = H, R2 = OH: *N*-(2-hydroxyphenyl)acetamide, [98]

B. R1 = CH_3, R2 = R3 = H, R4 = OH: *N*-(4-hydroxyphenyl)propanamide, [98]

C. R1 = R2 = H, R3 = Cl, R4 = OH: *N*-(3-chloro-4-hydroxyphenyl)acetamide, [98]

D. R1 = R2 = R3 = R4 = H: *N*-phenylacetamide, [98]

E. R1 = R2 = R3 = H, R4 = O-CO-CH_3: 4-(acetylamino)phenyl acetate, [98]

F. R1 = R2 = R3 = H, R4 = Cl: *N*-(4-chlorophenyl)acetamide (chloroacetanilide), [98]

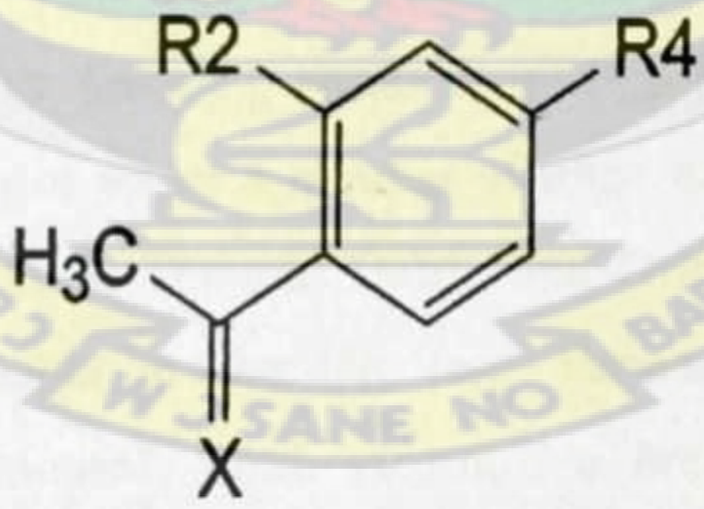


Figure 1.7: Chemical Structures of related Impurities present in Paracetamol

E. X = O, R2 = H, R4 = OH: 1-(4-hydroxyphenyl) ethanone, [98]

G. X = N-OH, R2 = H, R4 = OH: 1-(4-hydroxyphenyl) ethanone oxime, [98]

I. X = O, R2 = OH, R4 = H: 1-(2-hydroxyphenyl) ethanone, [98]

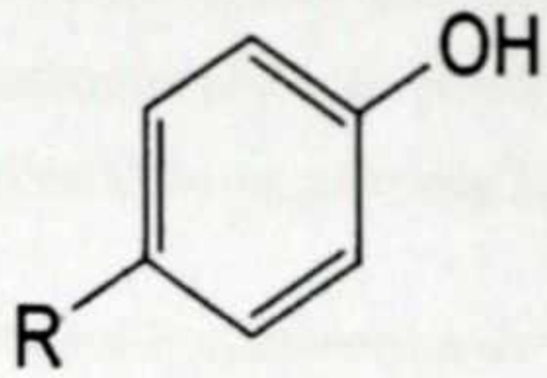


Figure 1.8: Chemical Structures of other related Impurities present in Paracetamol

F. R = NO_2: 4-nitrophenol, [98]

K. R = NH_2: 4-aminophenol. [98]

1.9 Available Dosage forms of Paracetamol

Paracetamol is available in a tablet, liquid suspension, syrup, capsule, suppository, intravenous, and intramuscular form. The common adult dose is 500 mg to 1000 mg. The recommended maximum daily dose, for adults, is 4000 mg. In recommended doses, paracetamol generally is safe for children and infants, as well as for adults,[117] although rare cases of acute liver injury have been linked to amounts lower than 2500 mg per day.[118] Panadol, which is marketed in Africa, Asia, Europe, Central America, and Australasia, is the most widely available brand of paracetamol, sold in over 80 countries. In North America, paracetamol is sold in generic form (usually labeled as acetaminophen) or under a number of trade names, for instance, Tylenol (McNeil-PPC, Inc.), Anacin-3, Tempra, Datril, and Ofirmev. While there is brand named paracetamol available in the UK (e.g. Panadol), unbranded or generic paracetamol is more commonly sold. Acamol, a brand name for paracetamol produced by Teva Pharmaceutical Industries in Israel, is one of the most widely used drugs in that country. In the Philippines, the largest-selling paracetamol brand is Biogesic, manufactured by the drug giant United Laboratories. Biogesic tablet sales reach nearly a billion units each year in the country alone, not including liquid suspension formats. The brand is also available in most of the ASEAN countries where the drug giant has market presence. In Europe, the most common brands of paracetamol are Efferalgan and Doliprane. In India, the most common brand of paracetamol is **Crocin manufactured by Glaxo SmithKline Asia. In Bangladesh the most popular brand is Napa manufactured by Beximco Pharma**. In China

paracetamol is sold over the counter as Duiyixiananjifenpian.[119] Likewise in Japan it is sold under the name Acetaminophen. In North Korea the DPRK-Swiss joint venture PyongSu Pharma markets the drug as PyongSu Cetamol.

In some formulations, paracetamol is combined with the opioid codeine, sometimes referred to as co-codamol (BAN). In the United States and Canada, this is marketed under the name of Tylenol #1/2/3/4, which contains 8–10 mg, 15 mg, 30 mg, and 60 mg of codeine, respectively. In the U.S., this combination is available only by prescription, while the lowest-strength preparation is over-the-counter in Canada, and, in other countries, other strengths may be available over the counter. There are generic forms of these combinations as well. In the UK and in many other countries, this combination is marketed under the names of Tylex CD and Panadeine. Other names include Captin, Disprol, Dymadon, Fensum, Hedex, Mexalen, Nofedol, Paralen, Pediapirin, Perfalgan, and Solpadeine. Paracetamol is also combined with other opioids such as dihydrocodeine, referred to as co-dydramol (BAN), oxycodone or hydrocodone, marketed in the U.S. as Percocet and Vicodin, respectively. Another very commonly used analgesic combination includes paracetamol in combination with propoxyphene napsylate, sold under the brand name Darvocet. A combination of paracetamol, codeine, and the calmative doxylamine succinate is marketed as Syndol or Mersyndol. The efficacy of paracetamol/codeine combinations have been questioned by recent research [120].

CHAPTER TWO

2.0 METHODOLOGY, APPARATUS AND REAGENTS

2.1 METHODOLOGY

2.1.1 Procedure/Methodology

In order to assess the purity of paracetamol and p-aminophenol reference powders, pharmacopoeial (USP 2007 and BP 2007) specifications were used.

The different USP 2007 and BP 2007 specifications of paracetamol and p-aminophenol were employed for the analysis of the reference powders.

The pharmacopoeia assessments described below were carried out in order to ensure that any observed differences among the products were not attributed to sample identity and formulation factors.

2.1.2 Identification Tests

Colour Identification Test for Paracetamol

0.1 g of pure paracetamol powder was added to 1 ml of hydrochloric acid, and heated to boiling for 3minutes. 1 ml of distilled water was added and cooled in an ice bath. No precipitate was formed. 0.05 ml of a 4.9 g/l solution of potassium dichromate was added. A violet colour developed which did not change to red and the results obtained are as recorded in **Table 3.1**.

Melting Point Determination for Pure Powders

Procedure: Small portions of finely shaped pure paracetamol were transferred into three dry capillary tubes. The powder was packed by tapping the tube on a hard surface to obtain about 5mm of the drug in the tube. These were heated slowly in a Stuart® Melting Point apparatus. The temperature at which liquefaction of the substance occurred was noted and recorded and the same procedure was repeated for the remaining two test tubes containing the pure paracetamol powder and the melting point range for each was determined. The average melting point range was then determined. **This same procedure was repeated for the pure p-aminophenol and caffeine powder and the values obtained are as recorded in Table 3.2.**

UV-Vis Absorption of Pure Paracetamol powder-BP 2007 Methodology

0.1g of pure paracetamol powder was weighed and dissolved in methanol and diluted to 100ml with the same solvent. 1.0 ml of the solution was measured and 0.5ml of a 10.3g/l solution of hydrochloric acid was added and diluted to 100ml with methanol. The solution was protected from bright light and immediately the absorbance was measured at the absorption maximum at 249 nm. The specific absorbance at the maximum is 860 to 980 and the results obtained are as recorded in **Table 3.3 and Fig. 3.1.**

UV-Vis Absorption of Pure Samples using Methanol-Water as solvent

Procedure: A standard solution of paracetamol was prepared by transferring 100mg of pure paracetamol, accurately weighed, in a 100mL volumetric flask. It was then dissolved and made to volume with methanol: water ($50:50^{v}/_{v}$) to produce $0.1\%^{w}/_{v}$. 1mL of the solution was pipetted into 100mL volumetric flask and made to volume with methanol: water ($50:50^{v}/_{v}$) to produce $0.001\%^{w}/_{v}$. The wavelength(s) of maximum absorbance of the solution in 1-cm cell was determined by scanning in the spectrophotometer in a wavelength range of 200 to 350nm [6]. **The same procedure was carried out for p-aminophenol and the results obtained are as recorded in Table 4.1 and Table 4.2, and shown in and Figures 4.1and 4.2.**

UV-Vis Absorption of a mixture of Pure Paracetamol and P-aminophenol powders

Procedure: A mixture of 100mg each of pure paracetamol and p-aminophenol, accurately weighed and uniformly mixed, was transferred in a 100mL volumetric flask. The mixture was then dissolved and made to volume with methanol: water ($50:50^{v}/_{v}$) to produce a standard solution of $0.1\%^{w}/_{v}$. 1mL of the solution was pipetted into 100mL volumetric flask and made to volume with methanol: water ($50:50^{v}/_{v}$) to produce $0.001\%^{w}/_{v}$ each of pure paracetamol and p-aminophenol. The wavelength(s) of maximum absorbance of the solution in 1-cm cell was determined by scanning in the spectrophotometer at a wavelength range of 200 to 350nm [98] and the results obtained are as recorded in **Table 4.3 and Figure 4.3.**

Identification Test for the presence of Paracetamol in Paracetamol Formulations

A quantity of paracetamol (Tablet/Syrup/Suspension) containing 0.5g of paracetamol was extracted with 20ml of methanol. The solution was the filtered and the filtrate was evaporated to dryness. A 0.1g of the dried filtrate was weighed and dissolved in methanol (50%). 0.5ml of dilute HCl was added to the sample solution and further made to 100ml volume. The absorbance of each sample solution was immediately measured at 249nm and recorded [98]. The specific absorbance of each sample solution was then determined and the results obtained are as recorded in **Table 3.4**.

Qualitative Tests for the presence of p-aminophenol in Paracetamol Formulations

Primary Aromatic Amine Test-BP Methodology

Five drops each from the freshly prepared solutions of the paracetamol samples were each dissolved in dilute HCl and then cooled to below 5°C in crushed ice. A few drops of sodium nitrite solution (prepared by dissolving 0.1g of sodium nitrite in 1ml of water) were then added to the acidified sample solutions. The solutions remained clear as the diazonium salt begins to form. Alkaline 2-naphthol solution (prepared by dissolving 0.1g of 2-naphthol in 1ml of dilute sodium hydroxide) was then added to the diazonium solution to produce an intensely coloured precipitate [98]. The remaining paracetamol formulations were subjected to the same procedure and the results obtained are as recorded in **Table 3.5**.

Limit Test for the presence of p-aminophenol in Pure Samples-BP Methodology

A mixture (1.5g Pure Paracetamol + 1.0g Pure P-aminophenol) was dissolved in sufficient methanol (50%) to produce 100ml. 1ml of a freshly prepared solution containing 1% $^{w}/_{v}$ of each sodium nitroprusside and anhydrous sodium carbonate was then added to the sample solution, mixed thoroughly and then allowed to stand in the dark for 30 minutes. A **blue** or **green** coloured solution was then observed, indicating the presence of p-aminophenol in the pure sample [98] and the results obtained are as recorded in **Table 3.6**.

Limit Test for the presence of p-aminophenol in Formulations-BP Methodology

Tablets each from the brands of paracetamol formulations were crushed individually in a mortar. 0.5g each of the dried powdered samples were individually dissolved in sufficient methanol (50%) to produce 100ml. 1ml of a freshly prepared solution containing 1% $^{w}/_{v}$ of each sodium nitroprusside and anhydrous sodium carbonate was then added to the sample solution, mixed thoroughly and then allowed to stand in the dark for 30 minutes. A blue coloured solution was then observed, indicating the presence of p-aminophenol in the paracetamol tablets [98].

For the syrup samples, an amount equivalent to 500mg (i.e. 20.83ml) each of the sample was measured. The above procedure was followed and the results obtained are as recorded in **Table 3.7**.

Uniformity of weight tests

Procedure: Twenty tablets each from the different brands of paracetamol tablets sampled were selected at random and weighed individually and then all twenty tablets weighed together using a sensitive AutoScan electronic balance already calibrated internally. The results were then tabulated. The percentage deviations per tablet were also calculated.

According to the BP 2007, no single tablet should deviate by 10% or above and not more than two tablets should deviate by 5% and the results obtained are as recorded in **Table 3.8 and summarized in Table 3.9**.

Assay of Pure Paracetamol powder Using Titration-BP Methodology

Procedure: An amount 0.300g of pure paracetamol powder was dissolved in a mixture of 10 ml of water and 30 ml of dilute sulphuric acid in a conical flask. The mixture was then boiled under a reflux condenser for an hour, cooled and diluted to 100.0 ml with water. 20.0 ml of the solution was measured and 40 ml of water, 40g of ice, and 15ml of dilute hydrochloric acid and 0.1 ml of ferroin were added. The prepared mixture was then titrated with 0.1 M cerium (IV) sulphate until a greenish-yellow colour was obtained. The procedure was further repeated without the pure paracetamol powder being examined to obtain a blank reading. The relation: 1 ml of

0.1 M cerium sulphate is equivalent to 7.56 mg of $C_8H_9NO_2$ is used to calculate the percentage content of pure paracetamol and the results obtained are as recorded in **Table 3.10**.

Assay of Paracetamol Tablets using ultraviolet -visible Method-BP Methodology

Procedure: An amount of 0.01g of pure paracetamol powder was weighed and added to 100ml of 0.1 M NaOH. Serial dilution was done with 0.1 M NaOH to obtain 0.001% $^{w}/_{v}$, 0.0008% $^{w}/_{v}$, 0.0006% $^{w}/_{v}$, 0.0004% $^{w}/_{v}$, 0.0002% $^{w}/_{v}$ and 0.0001% $^{w}/_{v}$. The absorbance readings were read and recorded at 257nm. 0.1M NaOH was used as a blank.

Twenty tablets of paracetamol were randomly selected, weighed individually and powdered. An amount of 0.1840g of the paracetamol powder was added to 50 ml of 0.1M sodium hydroxide, and diluted with 100 ml of water. It was shaken for 15 minutes and sufficient water was added to produce 200 ml. The solution was mixed and filtered. 10 ml of the filtrate was diluted to 100 ml with water. 10 ml of the resulting solution was then added to 10 ml of 0.1M sodium hydroxide and dilute to 100 ml with water. The absorbance of the resulting solution was measured at the maximum of 257 nm. The procedure was repeated with 0.1M NaOH without the paracetamol powder to obtain a blank.

The various absorbance measurements obtained was used to plot a linear graph. The initial concentration was prepared by taking 878 as the value of A(1%, 1 cm) at the maximum at 257 nm. The equation from the calibration curve was used to assay the different paracetamol tablets.

This was repeated for all the other brands of paracetamol and the results obtained are as recorded in **Table 3.12**.

2.2 Development of HPLC Method of Analysis

2.2.1 Components of HPLC System

Pump: Kontron instruments 422

Sample injector: Rheodyne Inc, Syringe loading injector, 100µL sample loop.

Stationary phase (column): Keystone, Hypersil BDS C18, 250 x 4.6 mm, 5µm, 120Å.

Detector: Applied Biosystems 783A Programmable Absorbance Detector connected to software; PowerChrom 280 with hp desktop computer.

Syringe: SGE 100µl syringe

Syringe filter: Acrodisc LC 13, PVDF 0.45µm (Gelman)

Materials: Reference paracetamol and p-aminophenol and caffeine powders, measuring cylinders, deionized water, beakers, pipettes, test tubes, volumetric flasks, funnels, membrane filters, pH meter.

Reagents: Methanol (BDH), distilled water, Glacial acetic acid.

Mode of elution: Isocratic

Mode of HPLC: Reverse Phase Liquid Chromatography (RPLC)

2.2.2 Method Development Considerations

Various literatures on paracetamol and p-aminophenol were evaluated for their physicochemical properties as well as their interaction in the formulations (tablets, syrups and suspensions). The solubility properties, structural elucidation, pka, stability levels in solution, UV absorption profiles in acidic, basic, neutral and organic solutions with their respective wavelength of maximum absorption, and their ionization potentials in solution were some of the details searched for. Based on the differences in the above physicochemical properties of the drugs, and upon critical examination on the physicochemical properties of the drugs, the choice of mode of HPLC was selected to be reverse phase liquid chromatography (RPLC). This was taken advantage of and a number of mobile phase combinations as well as column types were considered, with the prime aim of achieving better resolution for the drug in the minimum amount of time.

Some of the mobile phase combinations included methanol/water, methanol/glacial acetic acid and methanol/phosphate buffer in various combinations and at different pH-values and the results obtained are as shown in figure 3.3.

2.2.3 Development of a Separation Method

In the development of the chromatogram, attention was focused on the reference paracetamol and p-aminophenol. As a starting point, organic: aqueous mixture in the ratio of 30:70 was chosen. Depending on the characteristics of the chromatograms for the above-mentioned solutes, further manipulations of the mobile phase were tried to obtain a satisfactory resolution.

Selection of Mobile Phase

Various mobile phase compositions which comprise the following were investigated:

- 70: 30 mixture of 0.07% $^{v}/_{v}$ of glacial acetic acid and methanol at pH of 3.6±0.1 using a C_8 column.
- 70: 30 mixture of 0.5% $^{v}/_{v}$ of glacial acetic acid and methanol at pH of 3.6±0.1 using C_{18} column.
- 55: 45 mixture of phosphate buffer and methanol at pH of 6.4 ±0.1 using C_{18} column.
- 55: 45 mixture of water and methanol at pH of 6.7 ±0.1 using C_8 column.
- 60: 40 mixture of 0.05% $^{v}/_{v}$ of glacial acetic acid and acetonitrile at pH of 7.4 ±0.1 using C_{18} column.
- 65: 35 mixture of 0.06% $^{v}/_{v}$ of glacial acetic acid and acetonitrile at pH of 5.4 ±0.1 using C_{18} column.
- 75: 25 mixture 1% $^{v}/_{v}$ of glacial acetic acid and methanol at pH of 3.22 ±0.3 using C_{18} column.
- 80: 20 mixture of 1% $^{v}/_{v}$ of glacial acetic acid and methanol at pH of 3.22 ±0.3 using C_{18} column.
- 85: 15 mixture of 1% $^{v}/_{v}$ of glacial acetic acid and methanol at pH of 3.22 ±0.3 using C_{18} column.

The results for the above investigations are as shown in **figure 3.4 and appendix 9**. Finally, a mixture comprising methanol and 1% glacial acetic acid and methanol in the ratio of 85:15 $^{v}/_{v}$ was found to be appropriate. The pH of the final solution was always between 3.24 ± 0.02

Selection of UV Detection Wavelength Detector sensitivity

The pH of the designed mobile phase affected the choice of wavelength for the detection of solutes in this exercise. A suitable wavelength of maximum absorption was obtained for the analysis by scanning a solution containing the sample dissolved in the mobile phase using the mobile phase in the reference cell. With careful investigation, wavelength of maximum absorption of 278nm was chosen for paracetamol and p-aminophenol as well as the **caffeine internal standard and the results obtained are as shown in appendix 9.**

Selection of Absorption Unit Fraction Scale (AUFS)

The physicochemical parameters of paracetamol and p-aminophenol, as well as the caffeine internal standard, influenced the choice of this parameter. The aufs was therefore carefully chosen such as to give a quantitative detection of paracetamol and p-aminophenol. A sensitivity setting of 0.020 was found to be appropriate and the results obtained are as shown in **appendix 9**.

Selection of Flow Rate

The values were varied in order to get a rate that gave significant solute retention times in the minimum amount of time. It was also ensured that the values were not too high to introduce air bubbles in the mobile phase or on the pump or the detector or any part of the system to affect reproducibility of results. After all likely manipulations, 1.00ml/min. was used for the work.

After obtaining the optimum conditions for the separation of paracetamol and p-aminophenol, the conditions were applied to formulations (tables, syrups and suspensions) containing paracetamol and to monitor p-aminophenol levels in them and the results obtained are as shown in **appendix 9**.

2.2.4 Preparation of Stock Solutions

Preparation of Pure Paracetamol stock solution

10mg of pure paracetamol powder was accurately weighed and transferred into a 100ml volumetric flask. The mobile phase was added and shaken to dissolve, after which it was then made to the mark to constitute a 0.01%$^{w}/_{v}$ solution.

Preparation of Pure P-Aminophenol stock solution

10mg of pure p-aminophenol powder was accurately weighed and transferred into a 100ml volumetric flask. The mobile phase was added and shaken to dissolve, after which it was then made to the mark to constitute a 0.01%$^{w}/_{v}$ solution.

Preparation of Caffeine Internal Standard stock solution

1mg of pure caffeine powder was accurately weighed and transferred into a 100ml volumetric flask. The mobile phase was added and shaken to dissolve, after which it was then made to the mark to constitute a 0.001%$^{w}/_{v}$ solution.

2.2.5 HPLC Qualitative Analysis

Equal volumes of 0.01%$^{w}/_{v}$, 0.01%$^{w}/_{v}$ and 0.001%$^{w}/_{v}$ solutions of pure paracetamol powder solution, pure p-aminophenol powder solution and the caffeine internal standard solution respectively were injected into the column one at a time and their retention times recorded. These solutions were then injected together as shown in **Figures 3.7, 3.8, 3.9, 3.10 and 3.11**.

Preparation of Paracetamol samples

For the determination of *p*-aminophenol in paracetamol dosage forms, an average weight of 20 paracetamol tablets was determined. The tablets were powdered and an accurate weight of the finely powder equivalent to 10 mg of paracetamol was weighed. Then 20ml of mobile phase was added and the solution was mechanically shaken for 20 min. The solution was filtered using 20ml of 0.1M NaOH. The filtrate was then transferred into 100 ml volumetric flask. The flasks were filled with mobile phase. The prepared solutions were filtered through membrane filters before use, and the first 5ml of the filtrate was discarded. 20µl of the prepared solution, which was filtered through filters (Acrodisc LC 13, PVDF 0.45µm, Gelman), was injected, after addition of 10ml of caffeine internal standard, into the column for determination.

For the determination of *p*-aminophenol in paracetamol, in syrups and suspensions, an amount equivalent to 10 mg of paracetamol was weighed in 100 ml volumetric flask and followed the same process as in the tablet preparations and the results obtained are as shown in **Table 3.12 and Appendix 7**.

Preparation of Calibration Solutions

For paracetamol, volumes of 1.0 ml, 2.0 ml, 4.0 ml, 6.0 ml, 8.0 ml and 10.0 ml of paracetamol stock solution of concentration 0.01%$^{w}/_{v}$ were measured and put into 100ml of volumetric flask labeled A to F. These were made to the mark to obtain concentrations of 0.0001%$^{w}/_{v}$, 0.0002%$^{w}/_{v}$, 0.0004%$^{w}/_{v}$, 0.0006%$^{w}/_{v}$, 0.0008%$^{w}/_{v}$ and 0.0010%$^{w}/_{v}$ for paracetamol. 10ml of the caffeine internal standard solution was added to each flask and made up to volume with the mobile phase. This was then filtered using membrane filters and then made ready for injection.

The solutions were injected three times to generate the necessary data for the construction of the calibration graphs. The same procedure was repeated for p-aminophenol and the results obtained are as recorded in **Table 3.13 and Table 3.14, and shown in Figures 3.5 and 3.6**.

2.2.6 Procedure for Analysis

Parameter	Condition
Elution	Isocratic
Mode	Reverse Phase Liquid Chromatography (RPLC)
Stationary Phase	Hypersil BDS C_{18}, 250 × 4.6mm, 5µm, 120Å
Mobile Phase	Methanol : 1% Glacial Acetic acid ratio(15:85% $^{w}/_{v}$)
pH	3.22 – 3.25
Wavelength of detection	278nm
Flow rate	1.00ml/minute
AUFS (Sensitivity)/ Recorder range	0.020
Chart recorder speed	5mm/min
Attenuation	0
Temperature	Ambient temperature (28^{0}C)
Mean retention time of paracetamol	5.65±0.15mins.
Mean retention time of p-aminophenol	2.48±0.15mins.
Mean retention time of caffeine	16.78±0.15mins.

Selection of Products

Twenty different brands of paracetamol (sixteen local and four foreign) tablets, both Blister packed and loosed ones, and ten different brands of paracetamol Syrups/suspensions were procured. The brands under study were selected based on their availability in the metropolis.

2.2.7 Sampling Plan for drug Formulations

Hospitals

The following hospitals, because of the frequent patients' attendants, were chosen:

- Tafo Government Hospital
- Suntreso Government Hospital
- Komfo Anokye Teaching Hospital
- Atonsu Regional Hospital
- Emena Government Hospital
- University Hospital (KNUST)

Regional Medical Stores

Paracetamol formulations (tablets and syrup) were also obtained from the Regional Medical Stores.

Pharmacies

The pharmacies were classified as Classes A, B and C, due to the manner by which they kept (storage system/facility available) their drugs.

Class A pharmacies had better storage facility available, followed by Class B, and Class C in that order. The pharmacies are as shown below:

Table 2.1: Pharmacies were Sampling was done

Class A Pharmacies	Class B Pharmacies	Class C Pharmacies	Pharmacies around my area (Ayigya)
Ernest (Santasi)	Adum Area	K.O. Area	Slot-2 pharmacy
Palb	Kinapharma	Dadamens	Juliponia
Bandy	Kama	Oson's	KAK
L'apetite	Glorious	Central market square	
Lansah	Juliponia		
	KAK		

Table 2.2: Sample Size

Source	Sample Size		Standard Drugs
Local	**Tablets**	**Syrups**	Tylenol (Tablets)
Foreign	Blister; 3 blister packs (i.e. 30 tablets)	Same Brand (2 Batches, 2 bottles each)	Panadol (Tablets)
	Loose; 30 tablets	Two different brands, one each	Zinel (Tablets)

2.2.8 HPLC Stability Studies of Paracetamol Drug Samples

Sample preparations

Eight paracetamol samples both Blister packs and Loose ones (Local brands: A, B, C and D) and (Foreign brands: Q, R, S and T) were kept in an oven for 6 hours and were analyzed at different temperatures. Each brand was divided into four sets. The first set was kept in an

oven at ambient temperature (28°C), the second set was kept at a temperature of 35°C, the third set at a temperature of 40°C and the fourth was kept at a temperature of 50°C.

At the end of each time period, each tablet was powdered individually and weighed. An amount equivalent to 0.01%$^{w}/_{v}$ was prepared in a 100ml volumetric flask. 10ml of the caffeine internal standard solution was added to each flask and made up to volume with the mobile phase. This was then filtered using membrane filters and then made ready for injection and the results obtained are as recorded in **Tables 3.15, 3.16, 3.17 and 3.18, and shown in Figures 3.22 to 3.25, and Appendix 8.**

2.2.9 Validation of Analytical Method

The proposed method was validated according to International Conferences on Harmonization (ICH 2003) Guidelines for specificity and robustness, linearity, range, precision, accuracy, limit of detection, and limit of quantitation [121] for both paracetamol and *p*-aminophenol.

Specificity and Robustness

Specificity of a method can be defined as the absence of any interference at retention times of samples. Lack of information in the composition of generic formulations makes it difficult to assess selectivity by traditional analysis of comparison with a blank solution. The specificity of the method was demonstrated by injection of standard solution of paracetamol and *p*-aminophenol at concentration of 0.01%$^{w}/_{v}$.

The robustness of the new method under investigation was studied by intentionally varying amounts of respective components of the mobile phase to investigate the effect it will have on the results. Adulterants such as aspirin and chloroquine were added to the analyte but the mobile phase was still able to pick paracetamol and *p*-aminophenol. Various columns from different sources were used. Chromatographic conditions such as wavelength of absorption, AUF values, and chart speed and pH changes were all investigated and the result obtained is as shown in **Figure 3.7**.

Linearity

The linearity of an analytical method is its ability to obtain test results that are directly proportional to the sample concentration over a given range.

Acceptance Criteria: The Regression coefficient should not be less than 0.995 and the maximum deviation for the intercept should not be more than 2%

The linearity of paracetamol and *p*-aminophenol were study by preparing standard solution at five different concentrations ranging from 0.0001- 0010%$^{w}/_{v}$. Each concentration was injected in six replicates and the mean value of peak area was taken for the calibration curve and the results obtained are as shown in Figures 3.5 and 3.6.

Precision and Repeatability

The precision of the method was studied with respect to both intra-day (repeatability) and inter-day (intermediated) precisions. Repeatability was calculated from six replicate injections of three different concentrations of paracetamol and *p*-aminophenol in the same equipment on the same day. Inter-day precision was checked with the same concentrations as intra-day assay and the determination of each compound was repeated day by day during three days and the results obtained are as recorded in **Tables 3.20-3.22 and 4.4-4.6**.

Accuracy

This is the closeness in agreement of the accepted true value or a reference value to the actual result obtained.

The accuracy of the method was evaluated by conducting a recovery study of paracetamol and *p*-aminophenol at concentrations of 0.00075 %$^{w}/_{v}$ and 0.0018 %$^{w}/_{v}$ and the results obtained are as recorded in **Table 3.23**.

Limit of Detection and Quantification

LOD (k = 3.3) and LOQ (k = 10) of the method were established according to ICH definitions.

LOD and LOQ for both paracetamol and p-aminophenol were determined by the use of International Conferences on Harmonization (ICH 2003) Guidelines [121] and the results obtained are as recorded in **Tables 3.24 and 3.25**.

2.2.10 Apparatus

Equipment	Glassware	Reagents and chemicals
AutoScan Electronic Balance	50ml Burettes	Glacial acetic acid VS
Stuart® Melting Point apparatus	Pipettes: 1ml, 2ml, 3ml, 4ml, 5ml, 10ml, 20ml, 25ml.	Methanol (BDH Limited Poole, England)
Capillary tubes	Volumetric flask: 10ml, 25ml, 50ml, 100ml, 200ml, 250ml, 500ml, 1000ml.	Pure Paracetamol powder RS
Single Beam UV-Visible spectrophotometer (Cecil CE 2000)	Conical flasks (Pyrex)	Pure p-Aminophenol powder RS
Glass and quartz cuvettes.	Test tubes	Pure Caffeine powder RS
Refluxing Apparatus		Sodium nitrite (BDH Limited Poole, England)
20 mesh sieve		Sodium hydroxide (BDH Limited Poole, England)
Mortar and Pestles		Cerium (IV) sulphate
Water bath		Ferroin indicator
		Hydrochloric acid (36% $^{w}/_{v}$) (BDH Limited Poole, England)
		Sulphuric acid

Table 2.3: Profile of Paracetamol Tablets Sampled

No	Product Nam	Strength	Manufacturer	Batch No	DM*	DE**
1	Primadol	500mg	Kinapharma	10065	07/10	07/14
2	Paracetamol	500mg	Ernest Chemist	0901L	01/11	01/15
3	Paracetamol	500mg	M and G Pharmaceuticals Ltd.	PA180N	02/11	02/13
4	Letamol	500mg	Letap	6005281	03/11	03/13
5	Primadol	500mg	Kinapharma	10119	12/10	12/14
6	Primadol	500mg	Kinapharma	10062	02/11	07/14
7	Zinol[plus]	500mg	Amponsah Efah Pharmaceuticals	42002	03/09	03/12
8	Panadol	500mg	GlaxoSmith Kline	090929	06/09	05/13
9	Panadol	500mg	GlaxoSmith Kline	100583	05/10	04/14
10	Tylenol	500mg	McNeil-PPC INC.	26424	02/08	03/13
11	Tylenol	500mg	McNeil-PPC INC.	26242	02/08	02/12
12	Primadol	500mg	Kinapharma	11033	04/11	04/14
13	Paracetamol	500mg	Ernest Chemist	3615K	-	12/14
14	Primadol	500mg	Kinapharma	1012	-	03/11
15	Paracetamol	500mg	Ayrton Drugs	Pm 573	-	02/14
16	Letamol	500mg	Letap Pharmaceuticals Ltd.	6005281	-	03/13
17	Zinel	500mg	Amponsah Efah Pharmaceuticals	B25-4	02/10	04/13
18	Paracetamol	500mg	Pharmanova Ltd.	1060	-	11/14
19	Primadol	500mg	Kinapharma	10117	12/10	12/14
20	Primadol	500mg	Kinapharma	10119	12/10	12/14

DM*- Date of Manufacture

DE**-Date of Expiration

- - No data on the drug label

Table 2.4: Profiles of Paracetamol Syrups Sampled

No.	Product Name	Strength	Manufacturer	Batch No	DM*	DE**
1	Primadol	5ml(120mg	Kinapharma	1102	02/11	
2	Primadol	5ml(120mg	Kinapharma	11056	05/11	05/14
3	Primadol	5ml(120mg	Kinapharma	11032	04/11	04/14
4	Sedalyn C	5ml(120mg	KNUST	-	-	-
5	Paracetamol	5ml(120mg	KNUST	-	-	-
6	E-Panol	5ml(120mg	Ernest Chemist	0702L	02/11	02/14
7	Paracetamol	5ml(120mg	Ayrton Drugs	PX112	04/11	03/14
8	Letamol	5ml(120mg	Letap Pharmaceuticals Ltd.	0360201	02/11	01/13
9	Paracetamol	5ml(120mg	Ayrton Drugs	PX110	03/11	02/14
10	Paracetamol	5ml(120mg	Ayrton Drugs	PX107	03/11	02/14

DM* -Date of Manufacture

DE**-Date of Expiration

- - No data on the drug label

Table 2.5: Drug samples and code names

Sample Number	Name and Source	Code Name
1	Paracetamol Tablet brand A from Local Source	PTALS
2	Paracetamol Tablet brand B from Local Source	PTBLS
3	Paracetamol Tablet brand C from Local Source	PTCLS
4	Paracetamol Tablet brand D from Local Source	PTDLS
5	Paracetamol Tablet brand E from Local Source	PTELS
6	Paracetamol Tablet brand F from Local Source	PTFLS
7	Paracetamol Tablet brand G from Local Source	PTGFS
8	Paracetamol Tablet brand H from Local Source	PTHLS
9	Paracetamol Tablet brand I from Local Source	PTILS
10	Paracetamol Tablet brand J from Local Source	PTJLS
11	Paracetamol Tablet brand K from Local Source	PTKLS
12	Paracetamol Tablet brand L from Local Source	PTLLS
13	Paracetamol Tablet brand M from Local Source	PTMLS
14	Paracetamol Tablet brand N from Local Source	PTNLS
15	Paracetamol Tablet brand O from Local Source	PTOLS
16	Paracetamol Tablet brand P from Local Source	PTPLS
17	Paracetamol Tablet brand Q from Foreign Source	PTQFS
18	Paracetamol Tablet brand R from Foreign Source	PTRFS
19	Paracetamol Tablet brand S from Foreign Source	PTSFS
20	Paracetamol Tablet T from Foreign Source	PSTFS
21	Paracetamol Syrup A from Local Source	PSALS
22	Paracetamol Syrup B from Local Source	PSBLS
23	Paracetamol Syrup C from Local Source	PSCLS
24	Paracetamol Syrup D from Local Source	PSDLS
25	Paracetamol Syrup E from Local Source	PSELS
26	Paracetamol Syrup F from Local Source	PSFLS
27	Paracetamol Syrup G from Local Source	PSGLS
28	Paracetamol Syrup H from Local Source	PSHLS
29	Paracetamol Syrup I from Local Source	PSILS
30	Paracetamol Syrup J from Local Source	PSJLS

CHAPTER THREE

3.0 RESULTS AND CALCULATIONS

3.1 Sample Identification Tests

Table 3.1 Colour identification Test

Test	Observation	Inference
235.5 g of paracetamol + 1ml of hydrochloric acid and filtered. Then heated for 3 minutes to boiling + 1ml of distilled and then cooled in an ice bath.	No precipitate was formed.	Paracetamol may be present.
0.05 ml of a 4.9 g/l solution of potassium dichromate added to the solution.	A violet colour develops which does not change to red.	Paracetamol present.

Table 3.2: Melting point Determination for the pure powders

Sample	Average Melting Poin Range/°C	Expected value/°C	Remarks
Pure Paracetamol powder	169-170	168-172	passed
Pure P-aminophenol powder	188-190	188–190	passed
Caffeine	234.5-238	235-239	passed

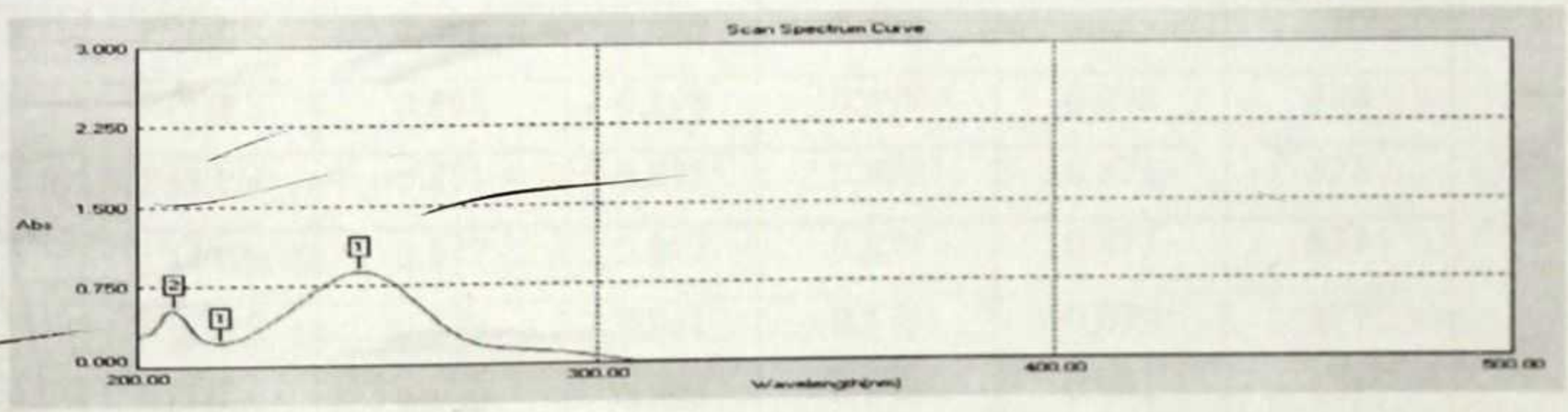


Figure 3.1: UV-Vis Spectrum of Pure Paracetamol Powder-BP Methodology

Table 3.3: Data for the UV-visible Spectrum of Pure Paracetamol Powder-BP Methodology

No.	Peak/Valley	Wavelength(nm)	Absorbance
1	Peak	248.00	0.878
2	Peak	208.00	0.529
1	Valley	218.00	0.213

Calculation of A(1%, 1cm) of Pure Paracetamol powder

From Beer-Lambert's equation: A = abc

Where: **A** is the absorbance of the solution, **a** = $A_{1cm}^{1\%}$, is the specific absorptivity of sample, **b** is the path length and **c** is the concentration of the solution in $\%^{w}/_{v}$

A = 0.878, b = 1cm, c = $0.001\%^{w}/_{v}$

Substituting these values into the Beer-Lambert's equation

$0.878 = A_{1cm}^{1\%} \times 1cm \times 0.001\%^{w}/_{v}$

Therefore $A_{1cm}^{1\%} = \frac{0.878}{1cm \times 0.001\%w/v} = 878$

Table 3.4: Identification Test for the presence of Paracetamol in Paracetamol Formulations

No.	Brand	Absorbance (A)				$A_{1cm}^{1\%}$
		1	2	3	Average	
1	PTALS	0.886	0.885	0.887	0.886	886
2	PTBLS	0.865	0.867	0.869	0.867	867
3	PTCLS	0.888	0.889	0.890	0.889	889
4	PTDLS	0.898	0.898	0.898	0.898	898
5	PTELS	0.871	0.875	0.873	0.873	873
6	PTFLS	0.877	0.877	0.877	0.877	877
7	PTGLS	0.879	0.881	0.877	0.879	879
8	PTHLS	0.890	0.893	0.890	0.891	891
9	PTILS	0.900	0.902	0.901	0.901	901
10	PTJLS	0.917	0.919	0.915	0.917	917
11	PTKLS	0.930	0.910	0.920	0.920	920

12	PTLLS	0.919	0.920	0.918	0.919	919
13	PTMLS	0.871	0.875	0.873	0.873	873
14	PTNLS	0.874	0.875	0.877	0.875	875
15	PTOLS	0.871	0.875	0.873	0.873	873
16	PTPLS	0.875	0.882	0.877	0.878	878
17	PTQFS	0.881	0.878	0.880	0.880	880
18	PTRFS	0.888	0.886	0.885	0.886	886
19	PTSFS	0.913	0.911	0.910	0.911	911
20	PTTFS	0.875	0.875	0.873	0.874	874
21	PSALS	0.881	0.880	0.883	0.881	881
22	PSBLS	0.891	0.892	0.893	0.892	892
23	PSCLS	0.885	0.885	0.884	0.885	885
24	PSDLS	0.877	0.875	0.878	0.877	877
25	PSELS	0.891	0.890	0.889	0.890	890
26	PSFLS	0.879	0.877	0.879	0.878	878
27	PSGLS	0.884	0.884	0.886	0.885	885
28	PSHLS	0.888	0.890	0.887	0.888	888
29	PSILS	0.871	0.875	0.873	0.873	873
30	PSJLS	0.874	0.875	0.876	0.875	875

Calculation of A(1%, 1cm) of Paracetamol Formulations

From Beer-Lambert's equation: A = abc

Where: **A** = the absorbance of the solution,

a = $A^{1\%}_{1cm}$, is the specific absorptivity of sample,

b = the path length and **c** is the concentration of the solution in $\%^{w}/_{v}$

For Brand PTAL, the absorbance are; 0.886, 0.885 and 0.887

Average absorbance = $\frac{0.886+ 0.885+ 0.887}{3} = 0.886$

A = 0.886, b = 1cm, c = 0.001$\%^{w}/_{v}$

Substituting these values into the Beer-Lambert's equation

$$0.886 = A_{1cm}^{1\%} \times 1cm \times 0.001\%^{w}/_{v}$$

$$\text{Therefore } A_{1cm}^{1\%} = \frac{0.886}{1cm \times 0.001\%w/v} = 886$$

The above procedure was used in finding the $A_{1cm}^{1\%}$ for the other brands.

3.2 Qualitative Test for the presence of p-aminophenol in Formulations

Table 3.5: Results of Primary Aromatic Amine Test

Brand	Observation	Inference
PTALS	Formation of coloured ppt.	Primary aromatic amine may be present
PTBLS	Formation of coloured ppt.	Primary aromatic amine may be present
PTCLS	Formation of coloured ppt.	Primary aromatic amine may be present
PTDLS	Formation of coloured ppt.	Primary aromatic amine may be present
PTELS	Formation of coloured ppt.	Primary aromatic amine may be present
PSALS	Formation of coloured ppt.	Primary aromatic amine may be present
PSBLS	Formation of coloured ppt.	Primary aromatic amine may be present
PSCLS	Formation of coloured ppt.	Primary aromatic amine may be present

Note: All the remaining brands gave a positive result for primary aromatic amine test.

Table 3.6: Results of Limit Test for the presence of p-aminophenol in Pure Samples

Test	Observation after 30 minutes	Inference
0.5g Pure Paracetamol + 10ml Methanol + 1ml of (1% Sodium Nitroprusside + 1% Sodium Carbonate)	No colour formed	p-aminophenol may be absent
0.5g Pure P-aminophenol + 10ml Methanol + 1ml of (1% Sodium	Blue colour formed	p-aminophenol may be present

Nitroprusside + 1% Sodium Carbonate)		
(1.5g Pure Paracetamol + 1.0g Pure P-aminophenol) + 10ml Methanol + 1ml of (1% Sodium Nitroprusside + 1% Sodium Carbonate)	Blue colour formed	p-aminophenol may be present

Table 3.7: Results of Limit Test for the presence of p-aminophenol in Formulations

Test	Observation after 30 minutes	Inference
0.5g of Paracetamol (PTALS) + 10ml Methanol + 1ml of (1% Sodium Nitroprusside + 1% Sodium Carbonate)	Blue colour formed	p-aminophenol may be present
0.5g of Paracetamol (PTBLS) + 10ml Methanol + 1ml of (1% Sodium Nitroprusside + 1% Sodium Carbonate)	Blue colour formed	p-aminophenol may be present
0.5g of Paracetamol (PTCLS) + 10ml Methanol + 1ml of (1% Sodium Nitroprusside + 1% Sodium Carbonate)	Green colour formed	p-aminophenol may be present
0.5g of Paracetamol (PSELS) + 10ml Methanol + 1ml of (1% Sodium Nitroprusside + 1% Sodium Carbonate)	Green colour formed	p-aminophenol may be present
0.5g of Paracetamol (PSFLS) + 10ml Methanol + 1ml of (1% Sodium Nitroprusside + 1% Sodium Carbonate)	Green colour formed	p-aminophenol may be present
0.5g of Paracetamol (PSGLS) + 10ml Methanol + 1ml of (1% Sodium Nitroprusside + 1% Sodium Carbonate)	Green colour formed	p-aminophenol may be present

Note: All the remaining brands showed presence of p-aminophenol in them.

3.3 Weight Uniformity Tests

Table 3.8: Results for Uniformity of Weight Test (Brand PTALS)

Tablet	Weight (g)	Deviation	% Deviation
1	0.6090	0.0043	0.7011
2	0.6148	-0.0015	-0.2446
3	0.6035	0.0098	1.597
4	0.6096	0.0037	0.6033
5	0.6234	-0.0101	-1.6468
6	0.6117	0.0016	0.2609
7	0.6014	0.0119	1.9403
8	0.6079	0.0054	0.8805
9	0.6113	0.0020	0.3261
10	0.6149	-0.0016	-0.2609
11	0.6262	-0.0129	-2.1034
12	0.6245	-0.0112	-1.8262
13	0.6105	0.0028	0.4565
14	0.6174	-0.0041	-0.6685
15	0.5923	0.0210	3.4241
16	0.6012	0.0121	1.9729
17	0.6260	-0.0127	-2.0708
18	0.6207	-0.0074	-1.2066
19	0.6184	-0.0051	-0.8316
20	0.6240	-0.0107	-1.7447
Total = 12.2687g			

Weight for 20 tablets of Brand PTALS = 12.266g

Calculation of Percentage Weight Deviation

Weight of 20 paracetamol tablets **(Brand PTALS)** = 12.266g

$$\text{Mean weight of 20 tablets} = \frac{12.266\text{g}}{20} = 0.6133\text{g}$$

Deviation of each tablet = mean weight – sample weight

For tablet no. 1,

Deviation = 0.6133– 0.6090 = 0.0043

Percentage deviation for a tablet 1 $= \frac{\text{Deviation}}{\text{Mean weight}} \times 100\%$

For tablet no. 1, percentage deviation $= \frac{0.0043}{0.6133} \times 100\% = 0.7011$

The same procedure was used in filling the remaining tables for weight uniformity tests of the other brands.

Table 3.9: Summary of Uniformity of weight tests of the different Paracetamol tablets

Brand	Remarks
Local Brands	All passed
Foreign Brands	All passed

3.4 Titration Results

Assay of pure Paracetamol powder

Preparation of dilute solution of H_2SO_4

98.07g of H_2SO_4 in 1000ml ≡ 1M H_2SO_4

9.807g of H_2SO_4 in 100ml ≡ 1M H_2SO_4

0.09807g of H_2SO_4 in 1ml ≡ 1M H_2SO_4

% Purity of H_2SO_4 = 98%

If 98% = 9.807g

$$100\% = \frac{100\%}{98\%} \times 9.807g$$

$$= 10.0071g$$

Specific gravity of H_2SO_4, ρ = 1.835g/ml

$$\rho = {}^{m}/_{v}$$

$$v = \frac{10.0071g}{1.835g/ml} = 5.50ml$$

5.50ml of H_2SO_4 was measured and made to 100ml with distilled water to make a dilute solution of H_2SO_4.

Preparation of 0.1M Cerium ammonium Sulphate (Ce^{4+})

632.55g of Ce^{4+} in 1000ml $\equiv$ 1M Ce^{4+}

63.255g of Ce^{4+} in 1000ml $\equiv$ 0.1M Ce^{4+}

6.3255 g of Ce^{4+} in 100ml $\equiv$ 0.1M Ce^{4+}

0.063255g of Ce^{4+} in 1ml $\equiv$ 0.1M Ce^{4+}

1ml of 0.1M cerium ammonium sulphate is equivalent to 7.56 mg of $C_8H_9NO_2$

1ml 0.1M Ce^{4+} $\equiv$ 7.56 mg of $C_8H_9NO_2$

Table 3.10: Titration of 0.1M Cerium ammonium sulphate with Pure Paracetamol powder

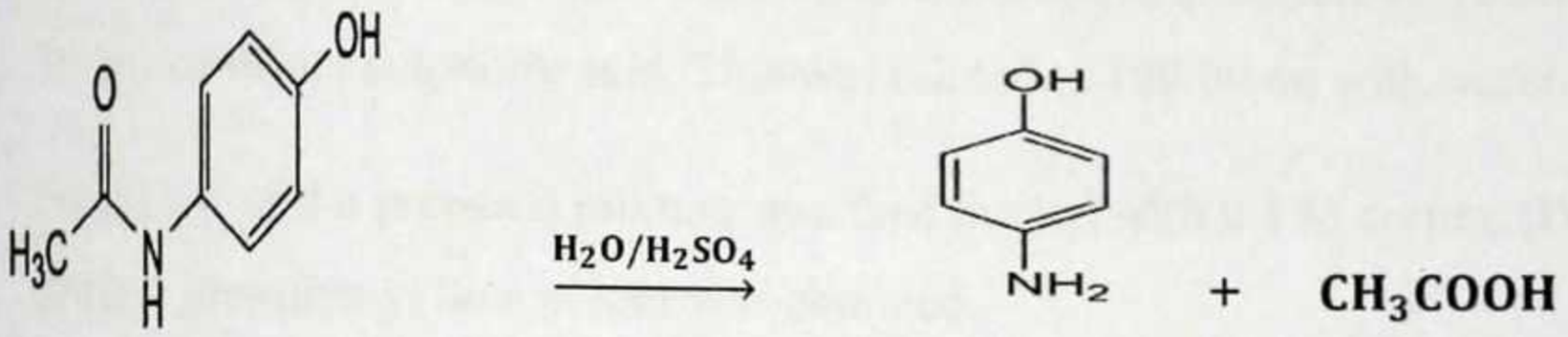
Burette readings (ml)	**1st Determination**	**2nd Determination**	**Blank Determination**
Final Readings	7.90	8.10	0.10
Initial Readings	0.00	0.00	0.00
Titre	7.90	8.10	0.10
Average titre	$\frac{7.90+8.10}{2} = \frac{15.70}{2} = 8.00$		

Blank titration = 0.1ml

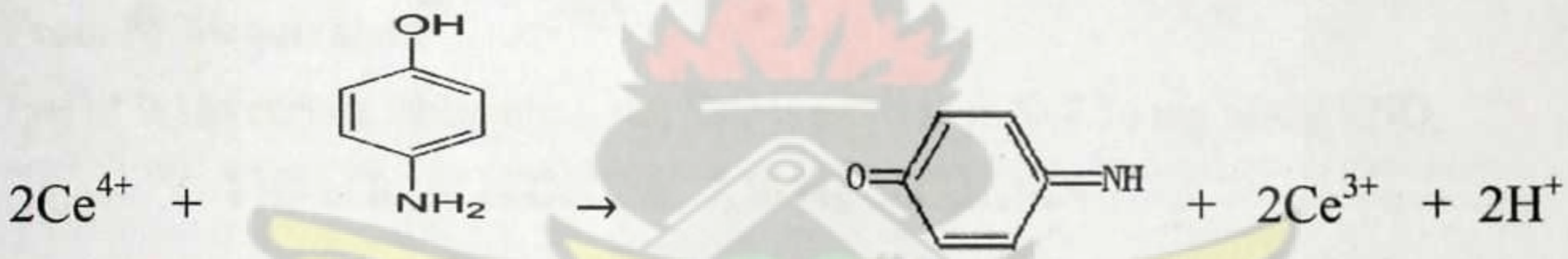
Actual volume of Ce^{4+} utilized = Average titre – blank volume (ml)

= 8.00 - 0.10 = 7.90ml

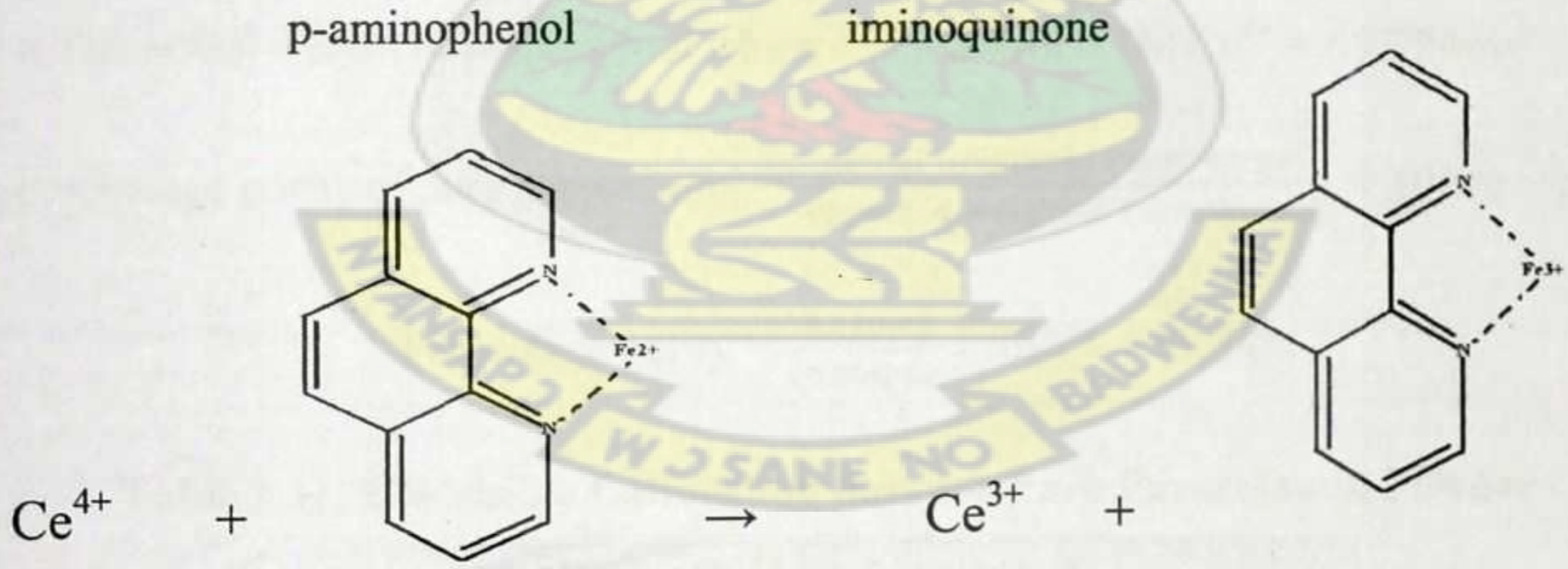
The equations for the reactions are:



Paracetamol p-aminophenol ethanoic acid



p-aminophenol iminoquinone



Ferroin (Fe^{2+}) Ferrin (Fe^{3+})

3.5 Determination of percentage content of paracetamol in pure powder

Weight of paracetamol Expected to react with 0.1M Ce^{4+}

0.300g of paracetamol was weighed and dissolved in a mixture of 10 ml of water and 30 ml of dilute sulphuric acid. This was diluted to 100.00 ml with water.

20.00 ml of the prepared mixture was then titrated with 0.1 M cerium (IV) sulphate until a greenish-yellow colour was obtained.

From the above information

100ml of the mixture contains 0.300g of paracetamol

$\therefore$ 20ml of the mixture will contain $\frac{20\text{ml}}{100\text{ml}} \times 0.300\text{g} = 0.06\text{g} = 60\text{mg}$

$\therefore$ The weight of paracetamol which was expected to react with 0.1M Ce^{4+} = 60mg

From Milliequivalent

1ml of 0.1M cerium ammonium sulphate is equivalent to 7.56 mg of $C_8H_9NO_2$

1 ml of 0.1M of Ce^{4+} = 7.56 mg of $C_8H_9NO_2$

$\therefore$ 7.90ml of $Ce^{4+} = \frac{7.90}{1\text{ml}} \times 7.56\text{ mg} = 59.724\text{mg}$

$\therefore$ The actual weight of paracetamol which reacted with 0.1M Ce^{4+} = 59.724mg

$$\text{Percentage purity of pure paracetamol} = \frac{\text{Actual weigtht of Paracetamol}}{\text{Expected weight of Paracetamol}} \times 100\%$$

$$\text{Percentage purity} = \frac{59.724\text{mg}}{60.000\text{mg}} \times 100\% = 99.54\%$$

Table 3.11: UV-visible Calibration data for Pure Paracetamol Powder

Concentration ($\%^{w}/_{v}$)	Absorbance
0.0010	0.721
0.0008	0.624
0.0006	0.501
0.0004	0.341
0.0002	0.226
0.0001	0.141

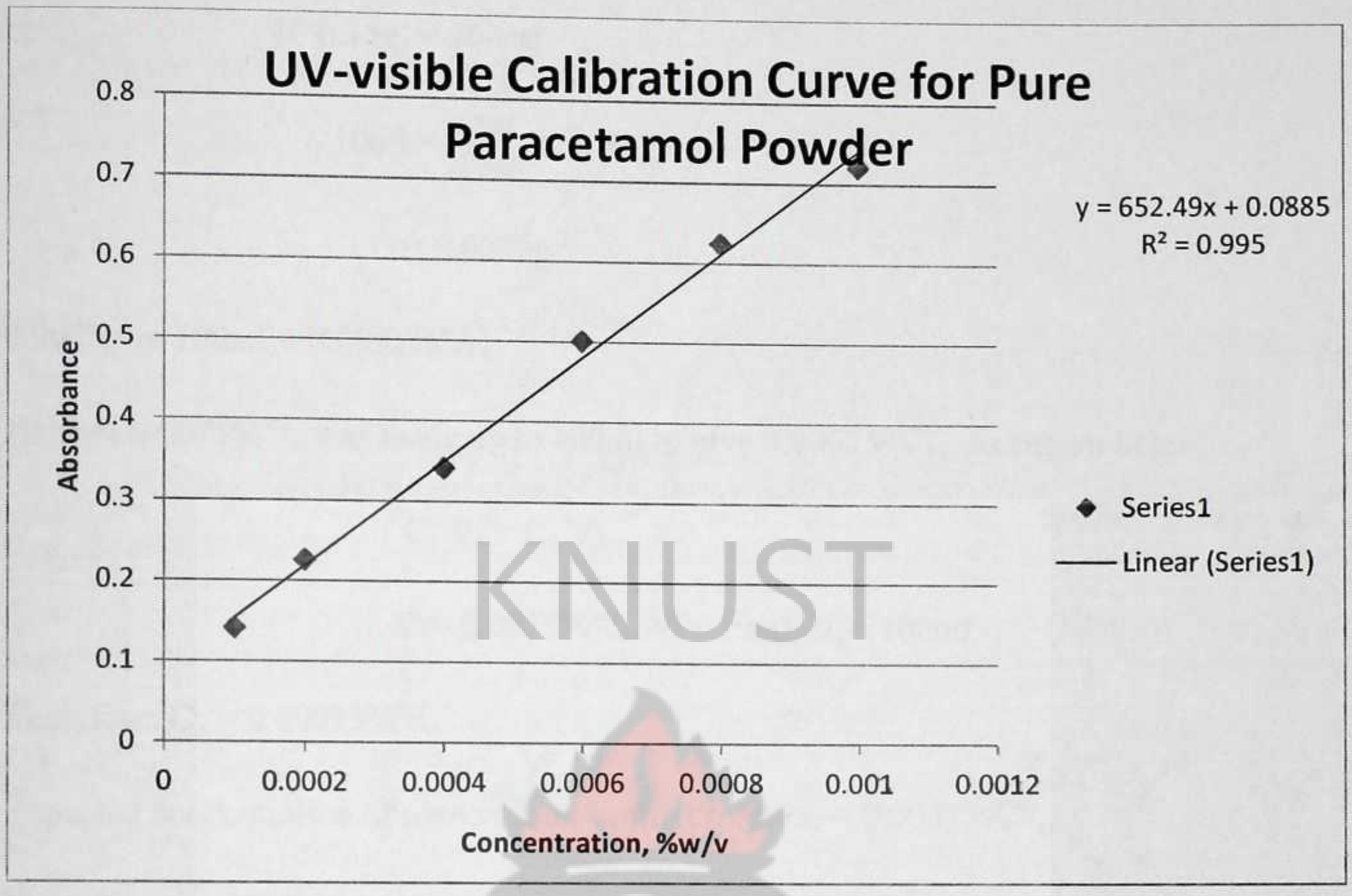


Figure 3.2: Calibration curve for pure paracetamol using UV-vis method in BP

3.6 Assay of paracetamol tablets

Preparation of 0.1M NaOH

40.00g of NaOH in 1000ml ≡ 1M NaOH

4.00g of NaOH in 1000ml ≡ 0.1M NaOH

0.40g of NaOH in 100ml ≡ 0.1M NaOH

0.040g of NaOH in 1ml ≡ 0.1M NaOH

$$99\% = 0.40g$$

$$\therefore 100\% = \frac{100\%}{99\%} \times 0.40g$$

$$= 0.4040g$$

∴ 0.4040g of NaOH was weighed and made to 100ml with distilled water to make a solution of 0.1M NaOH.

Calculations on percentage content of paracetamol tablets

Expected concentration of paracetamol in final solution

0.15g of active paracetamol = 200ml

If $0.15g = 200ml$

$$\therefore 10ml = \frac{10ml}{200ml} \times 0.15g$$

$$= 0.0075g$$

$0.0075g$ in $100ml = 0.0075\%^{w}/_{v}$,

10ml of $0.0075\%^{w}/_{v}$ was made up to 100ml to give $0.00075\%^{w}/_{v}$, as shown below

$$C_1 V_1 = C_2 V_2$$

$C_1 = 0.0075\%^{w}/_{v}$, $V_1 = 10ml$, $V_2 = 100ml$,

Therefore, $C_2 = 0.00075\%^{w}/_{v}$

Expected concentration of paracetamol in final solution = $0.00075\%^{w}/_{v}$

From Beer-lamberts law:

$A = \varepsilon cb$

Where; A = absorbance; ε = molar absorptivity; b = path length (1cm); c = concentration ($^{w}/v$)

When b = 1 (b is virtually a constant) absorbance can be expressed as:

From the calibration curve

The equation of the line: $y = 652.49x + 0.0885$

Where y (y-axis) = absorbance and x (x-axis) = concentration

$$X = \frac{y - 0.0885}{652.49}$$

For **PTALS,** absorbance (y) = 0.564

$$x = \frac{0.574 - 0.0885}{652.49}$$

$x = 0.00073\%^{w}/_{v}$

$$\text{Percentage content of PTALS} = \frac{\text{Actual concentration}}{\text{Expected concentration}} \times 100\%$$

$$= \frac{0.000744}{0.00075} \times 100\%$$

$$= 99.20\%$$

Table 3.12: Results for percentage content of paracetamol in tablets

Brand	Weight (g)	Absorbance	Percentage Content ($\%^{w}/_{v}$)
PTALS	0.1840	0.574	99.20
PTBLS	0.1722	0.578	100.03
PTCLS	0.1943	0.576	100.00
PTDLS	0.1773	0.579	100.00
PTELS	0.1685	0.589	102.67
PTFLS	0.1922	0.597	104.00
PTGLS	0.1697	0.585	101.33
PTHLS	0.1706	0.588	102.67
PTILS	0.1758	0.579	100.00
PTJLS	0.1826	0.589	102.67
PTKLS	0.1837	0.597	104.00
PTLLS	0.1772	0.585	101.33
PTMLS	0.1695	0.588	102.67
PTNLS	0.1684	0.579	100.00
PTOLS	0.1719	0.589	102.67
PTPLS	0.1694	0.597	104.00
PTQFS	0.2360	0.585	101.33
PTRFS	0.2298	0.588	102.67
PTSFS	0.1793	0.585	101.33
PTTFS	0.1806	0.588	102.67

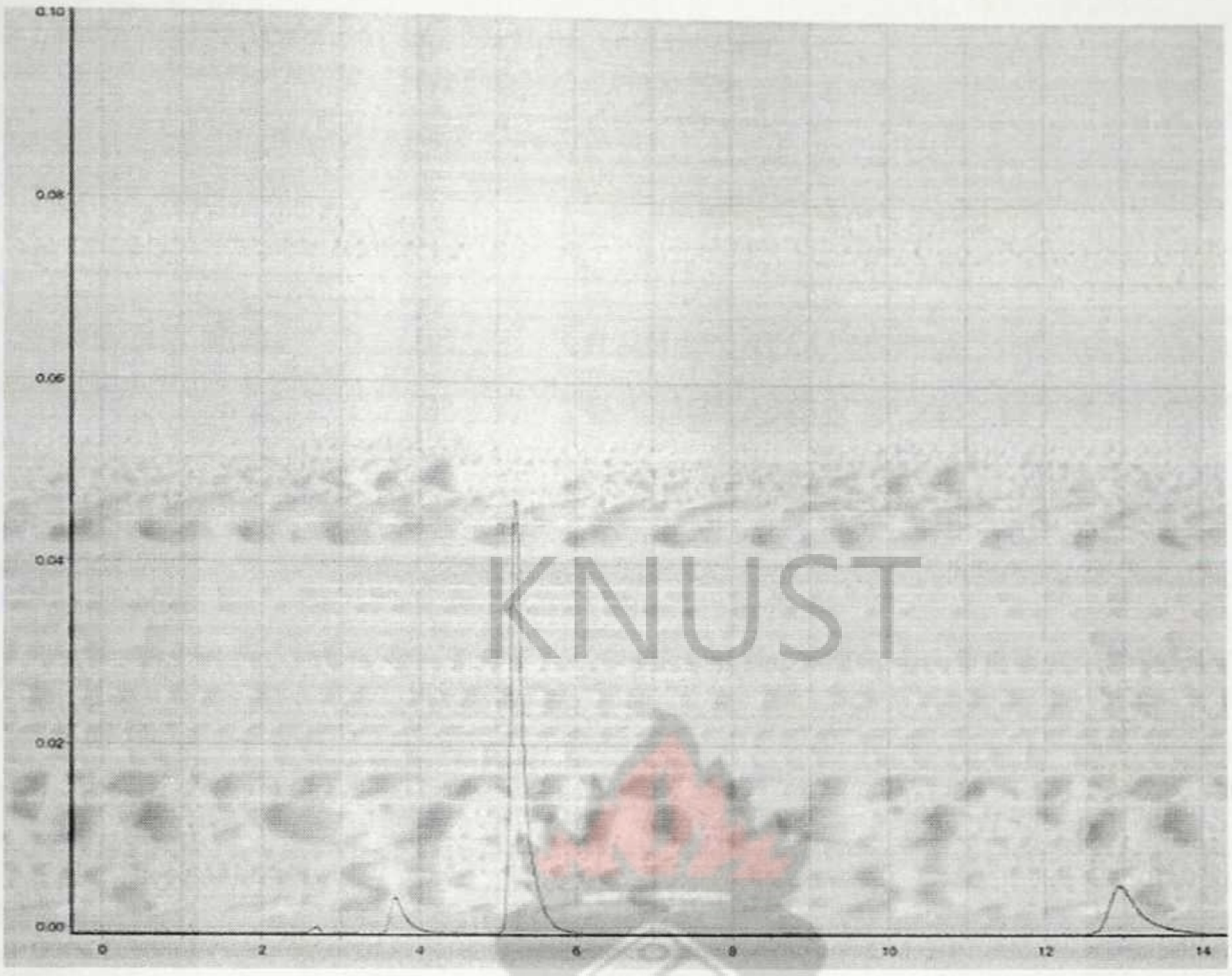


Figure 3.3: Chromatogram of HPLC Investigations

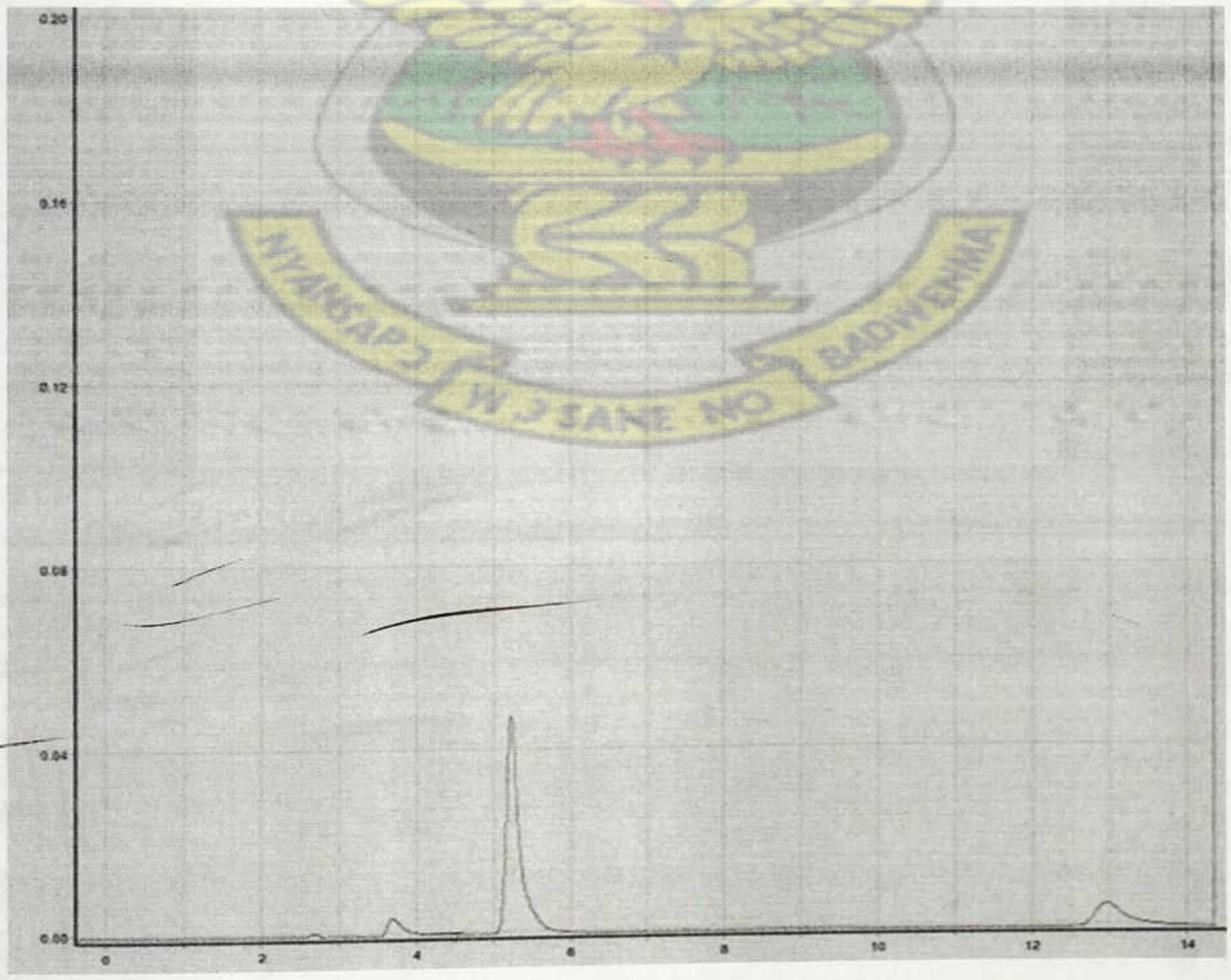


Figure 3.4: Chromatogram of HPLC Investigations

3.7 Development OF HPLC Method of Analysis

Table 3.13 Chromatographic Conditions

Parameter	Chromatographic Condition
Mobile Phase	Methanol:1% Glacial acetic acid (15% : 85% $^{v}/_{v}$)
Stationary phase	Anachem HPLC column, containing silica bonded to Octadecylsilyl groups. (S5C8-3554 250mm x 4.6mm internal diameter)
Wavelength of detection	278nm
Flow rate	1.00ml/minute
AUFS (Sensitivity)	0.020
Chart recorder speed	5mm/min
Attenuation	0
Temperature	Ambient temperature (28^{0}C)
Mean retention time of paracetamol	5.65±0.15mins.
Mean retention time of p-aminophenol	2.48±0.15mins.
Mean retention time of caffeine	16.78±0.15mins

Table 3.14: HPLC Data for Calibration Curve of Pure Paracetamol Powder

Conc. ($\%^{w}/_{v}$)	Mean Peak Area for three injections (Pure Paracetamol)	Mean Peak Area for three injections (Internal Standard)	Mean Peak Area Ratio
0.0001	0.1260	0.0886	1.422
0.0002	0.1.860	0.0887	2.097
0.0004	0.2825	0.0887	3.185
0.0006	0.3893	0.0888	4.384
0.0008	0.4775	0.0888	5.377
0.0010	0.5783	0.0893	6.476

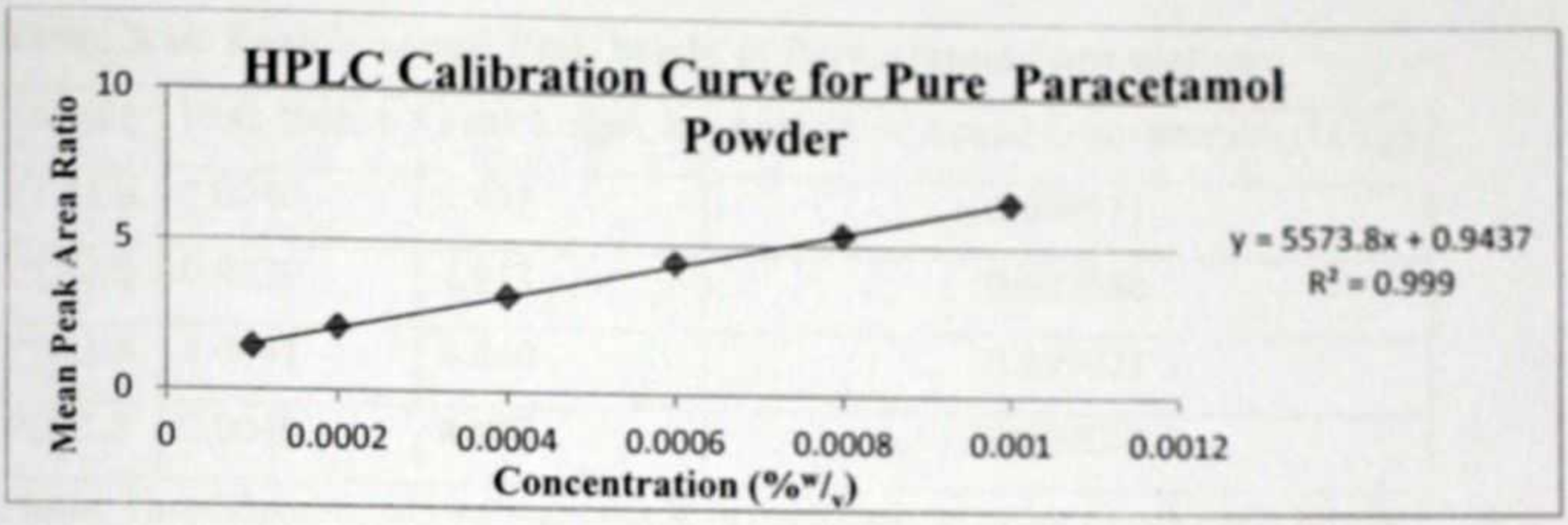


Figure 3.5: HPLC Calibration Curve for Pure Paracetamol Powde

Table 3.15: HPLC Data for Calibration Curve of Pure P-aminophenol Powder

Conc. (%$^{w}/_{v}$)	Mean Peak Area (Pure P-aminophenol)	Mean Peak Area (Internal Standard)	Mean Peak Area Ratio
0.0001	0.0963	0.0880	0.721
0.0002	0.1006	0.0885	1.137
0.0004	0.2010	0.0884	2.274
0.0006	0.3024	0.0886	3.413
0.0008	0.4026	0.0887	4.539
0.0010	0.5042	0.0889	5.672

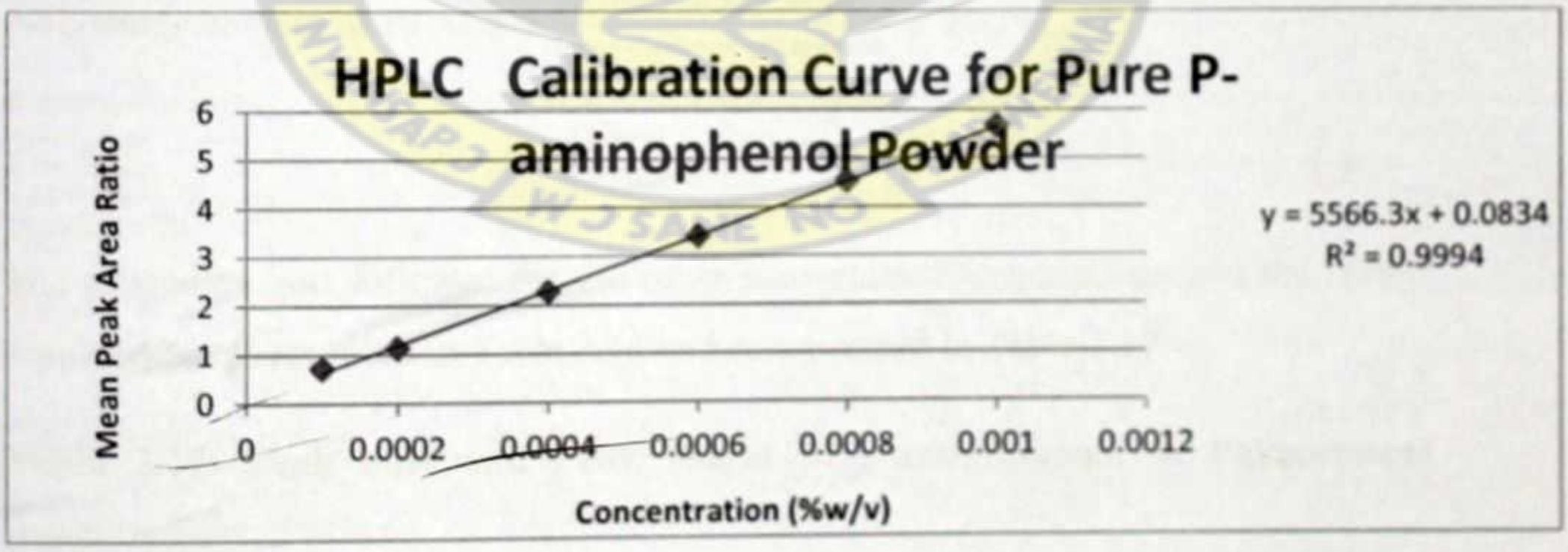


Figure 3.6: HPLC Calibration Curve for Pure P-aminophenol Powder

Table 3.16: Peak base and Peak height of Paracetamol Formulations

Sample	Peak base, b	Peak height, h	Area, A or Actual concentration ($\%^{w}/_{w}$)
PTALS	0.0040	4.955	0.009911
PTBLS	0.0039	4.913	0.009580
PSALS	0.0041	4.840	0.009921
PSBLS	0.0040	4.907	0.009813

Note: Three determinations were made in each case, in order to calculate for the mean and standard deviation.

Calculation of Percentage content of Paracetamol Formulations from the Peaks

From equation: $A = \frac{1}{2} \times b \times h$

Where: A = area of the peak = concentration of the formulation, b = peak base, h = peak height.

For Brand **PTALS**, b = 0.004, h = 4.955

$$A = \frac{1}{2} \times 0.004 \times 4.955 = 0.009911$$

Actual concentration of Brand **PTALS** = $0.009911\%^{w}/_{w}$

Expected concentration of Brand **PTALS** = $0.01000\%^{w}/_{w}$

$$\text{Percentage content of PTALS} = \frac{\text{Actual concentration}}{\text{Expected concentration}} \times 100\%$$

$$= \frac{0.00991}{0.01000} \times 100\% = 99.11\%$$

The procedure was followed for the other paracetamol formulations and the results obtained are as recorded in Table 3.16 and summarized in Table 3.17.

Table 3.17: Peak base and Peak height of p-aminophenol in Paracetamol Formulations

Sample	Peak base, b	Peak height, h	Concentration ($\%^{w}/_{w}$)	Remarks
PTALS	0.0002	0.100	0.00001	NA
PTBLS	0.0001	0.600	0.00003	NA
PSALS	0.0001	0.200	0.00001	NA
PSBLS	0.0003	0.133	0.00002	NA

Note: 1. The acceptable limit of p-aminophenol in paracetamol formulations = $0.005\%^{w}/_{w}$

2. NA = Negligible Amount

Calculation of amount of p-aminophenol in Paracetamol formulations use for Stability studies

For Brand **PTALS**, b = 0.0002, h = 0.100

$$A = \frac{1}{2} \times 0.0002 \times 0.100 = 0.00001^{w}/_{w}$$

The procedure was followed for the other paracetamol formulations, and also for determining the stability of the brands selected for the stability studies and the results obtained are as recorded in **Table 3.19 summarized in Table 3.20**.

Table 3.18: HPLC Analysis of 30 Paracetamol Formulations

NO.	Sample	%Purity of paracetamol (mean ± SD)	*p*-Aminophenol (mg)
1	PTALS	99.11 ± 0.02	NA
2	PTBLS	95.80 ± 0.01	NA
3	PTCLS	96.71 ± 0.02	NA
4	PTDLS	99.68 ± 0.03	NA
5	PTELS	97.41 ± 0.01	NA
6	PTFLS	99.68 ± 0.01	NA
7	PTGLS	97.31 ± 0.01	NA
8	PTHLS	99.57 ± 0.01	NA
9	PTILS	97.68 ± 0.01	NA
10	PTJLS	95.98 ± 0.02	NA
11	PTKLS	98.35 ± 0.02	NA
12	PTLLS	98.24 ± 0.02	NA
13	PTMLS	97.31 ± 0.01	NA
14	PTNLS	97.68 ± 0.01	NA

15	PTOLS	97.98 ± 0.02	NA
16	PTPLS	96.11 ± 0.02	NA
17	PTQFS	96.57 ± 0.01	NA
18	PTRFS	97.31 ± 0.03	NA
19	PTSFS	99.34 ± 0.02	NA
20	PTTFS	99.71 ± 0.02	NA
21	PSALS	99.21 ± 0.03	NA
22	PSBLS	98.13 ± 0.01	NA
23	PSCLS	99.13 ± 0.03	NA
24	PSDLS	99.27 ± 0.02	NA
25	PSELS	98.57 ± 0.03	NA
26	PSFLS	97.31 ± 0.02	NA
27	PSGLS	99.01 ± 0.03	NA
28	PSHLS	98.71 ± 0.02	NA
29	PSILS	98.92 ± 0.01	NA
30	PSJLS	98.73 ± 0.04	NA

NA = Negligible Amount

Table 3.19: Results for the Stability studies

Sample	Peak base, b	Peak height, h	Concentration ($\%^{w}/_{w}$)	Remarks
PTALS	0.0002	0.100	0.00001	NA
PTBLS	0.0001	0.600	0.00003	NA
PTCLS	0.0001	0.200	0.00001	NA
PTDLS	0.0003	0.133	0.00002	NA
PTQFS	0.0002	0.300	0.00003	NA
PTRFS	0.0002	0.300	0.00003	NA
PTSFS	0.0001	0.600	0.00001	NA
PTTFS	0.0003	0.133	0.00002	NA

Note: 1. The acceptable limit of p-aminophenol in paracetamol formulations = 0.005%$^{w}/_{w}$

2. NA = Negligible Amount

3.8 HPLC Qualitative Analysis of Pure Samples

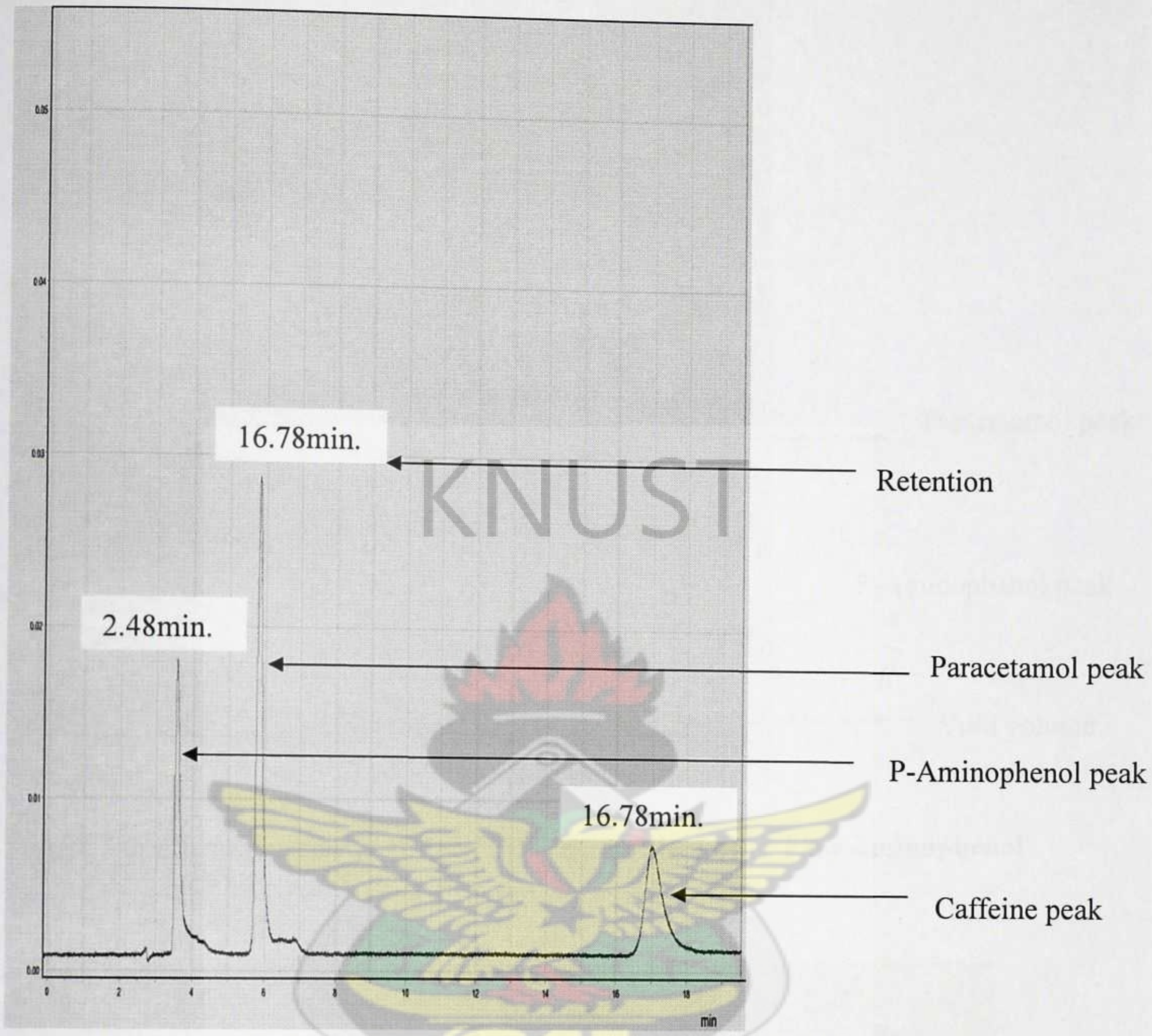


Figure 3.7: Chromatograms of Pure p-aminophenol, Paracetamol, and Caffeine

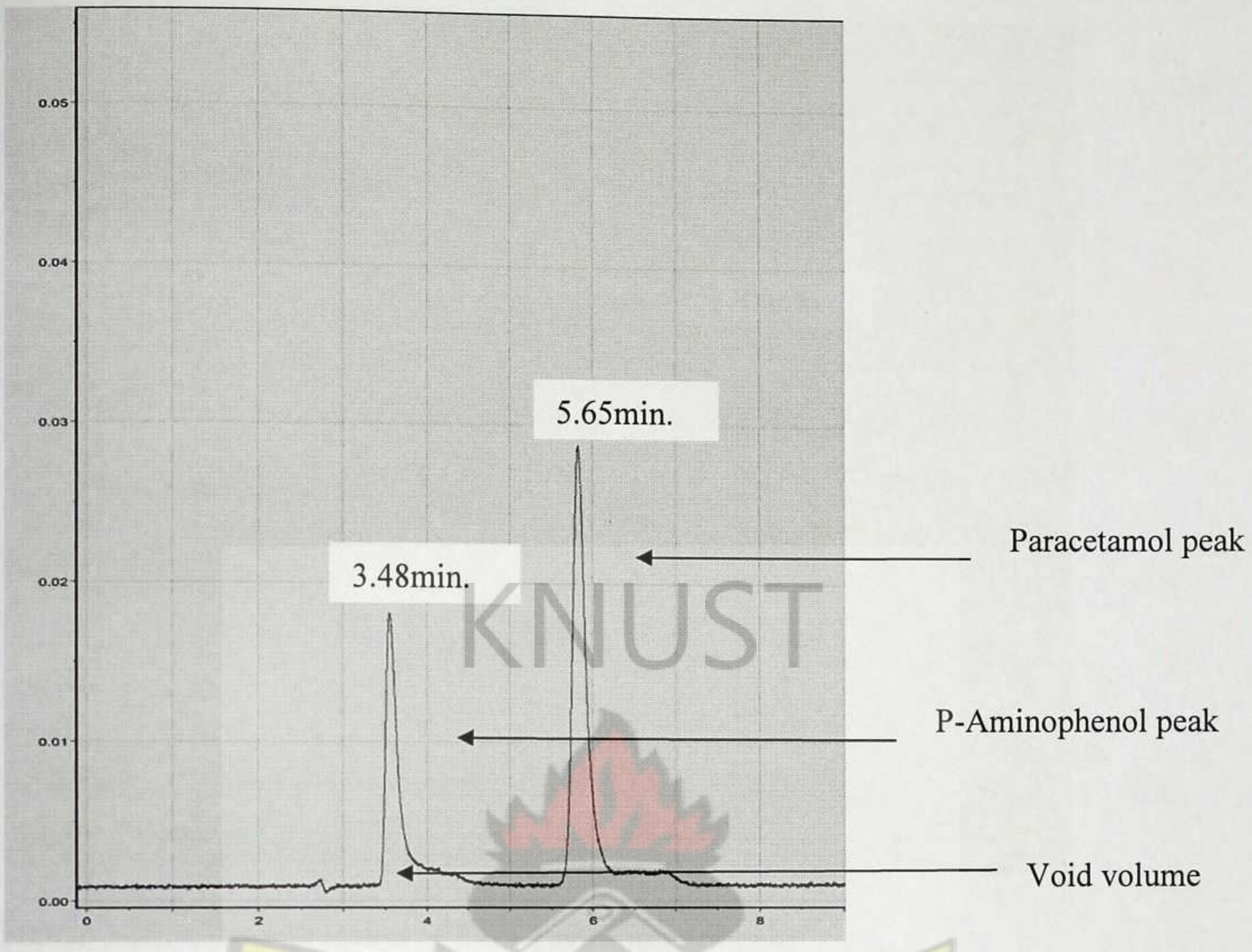


Figure 3.8: Chromatograms of Pure Paracetamol and Pure Para-aminophenol

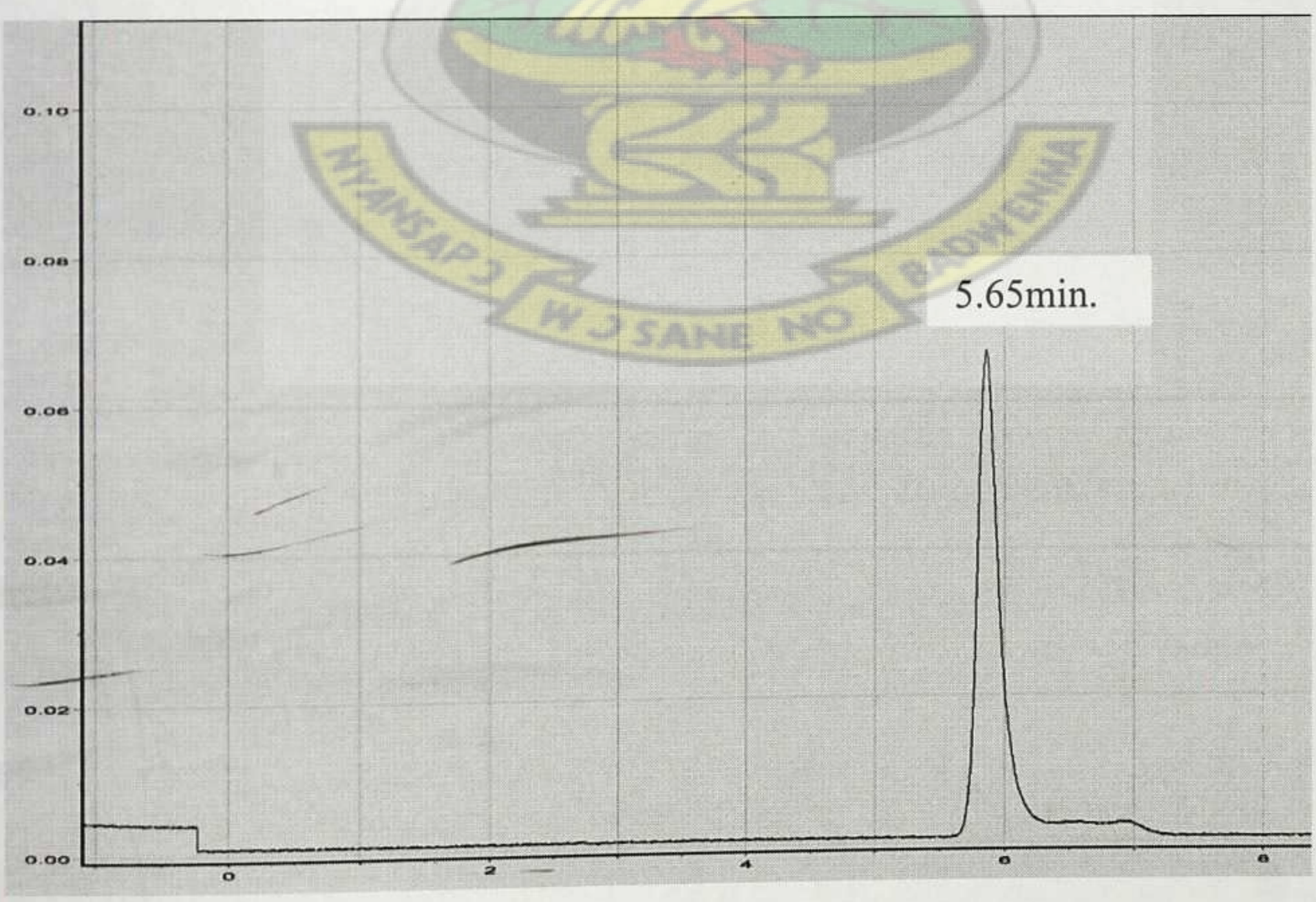


Figure 3.9: Chromatogram of Pure Paracetamol

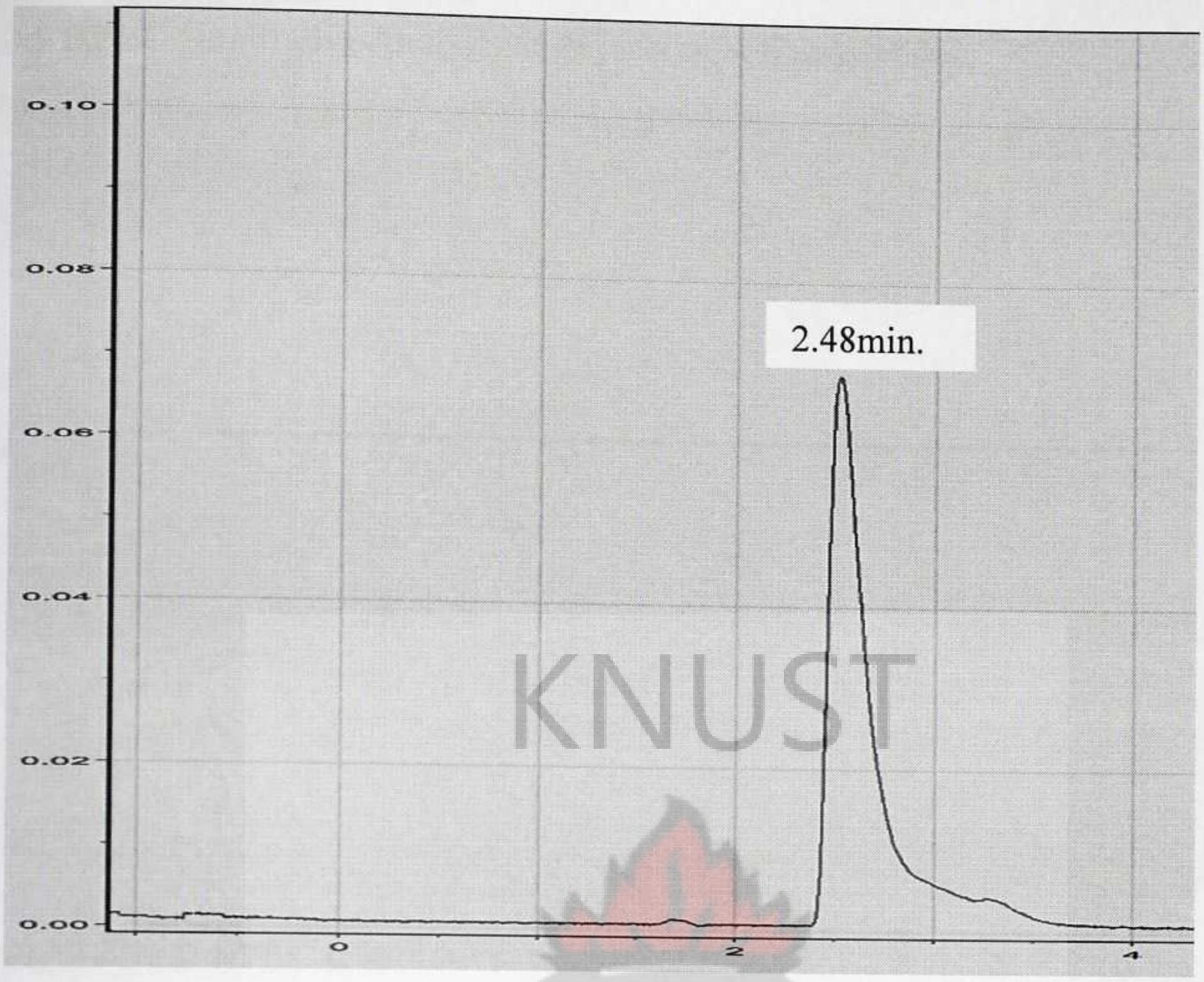


Figure 3.10: Chromatogram of Pure Para-aminophenol

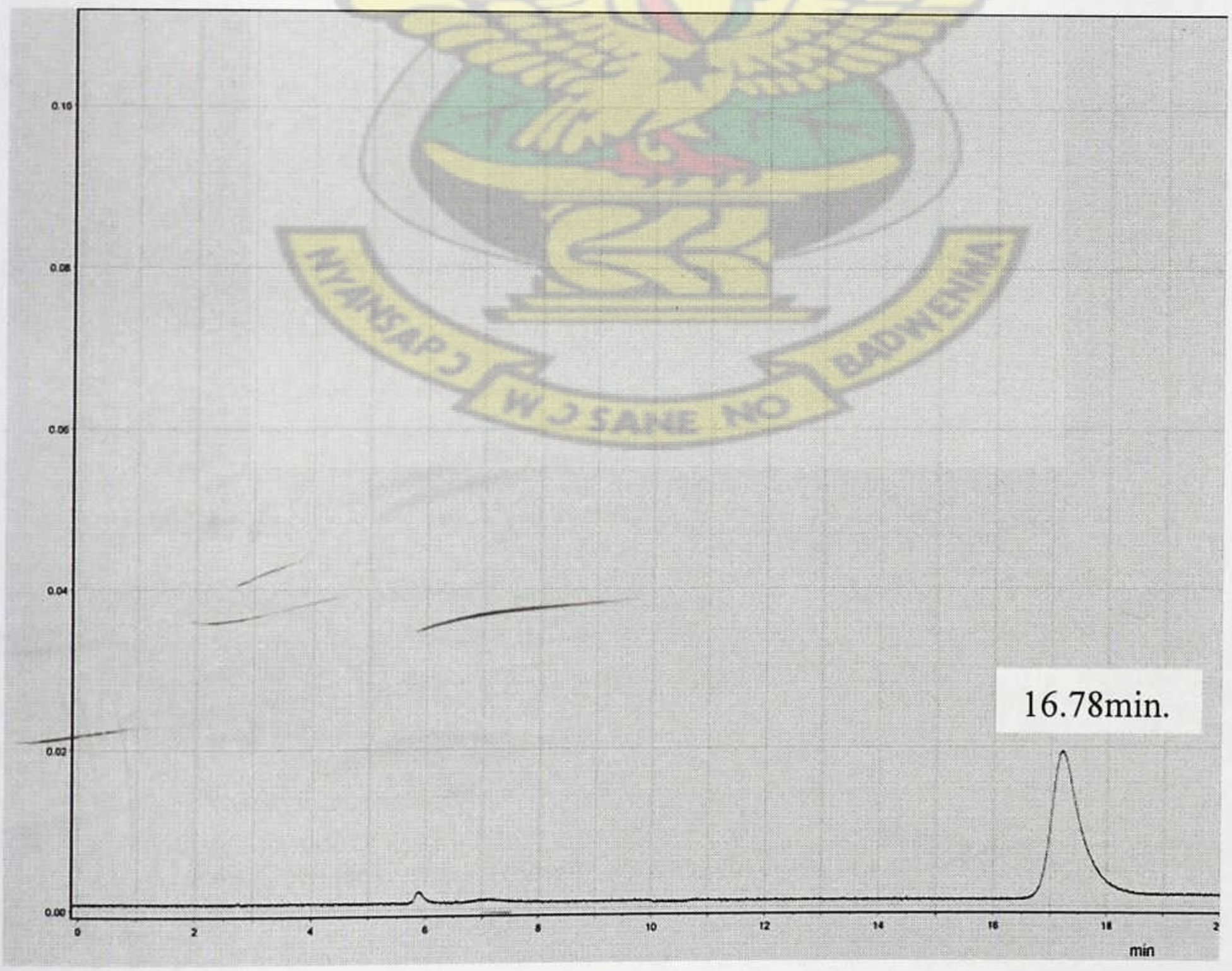


Figure 3.11: Chromatogram of Pure Caffeine

3.9 HPLC Qualitative Analysis of Paracetamol formulations

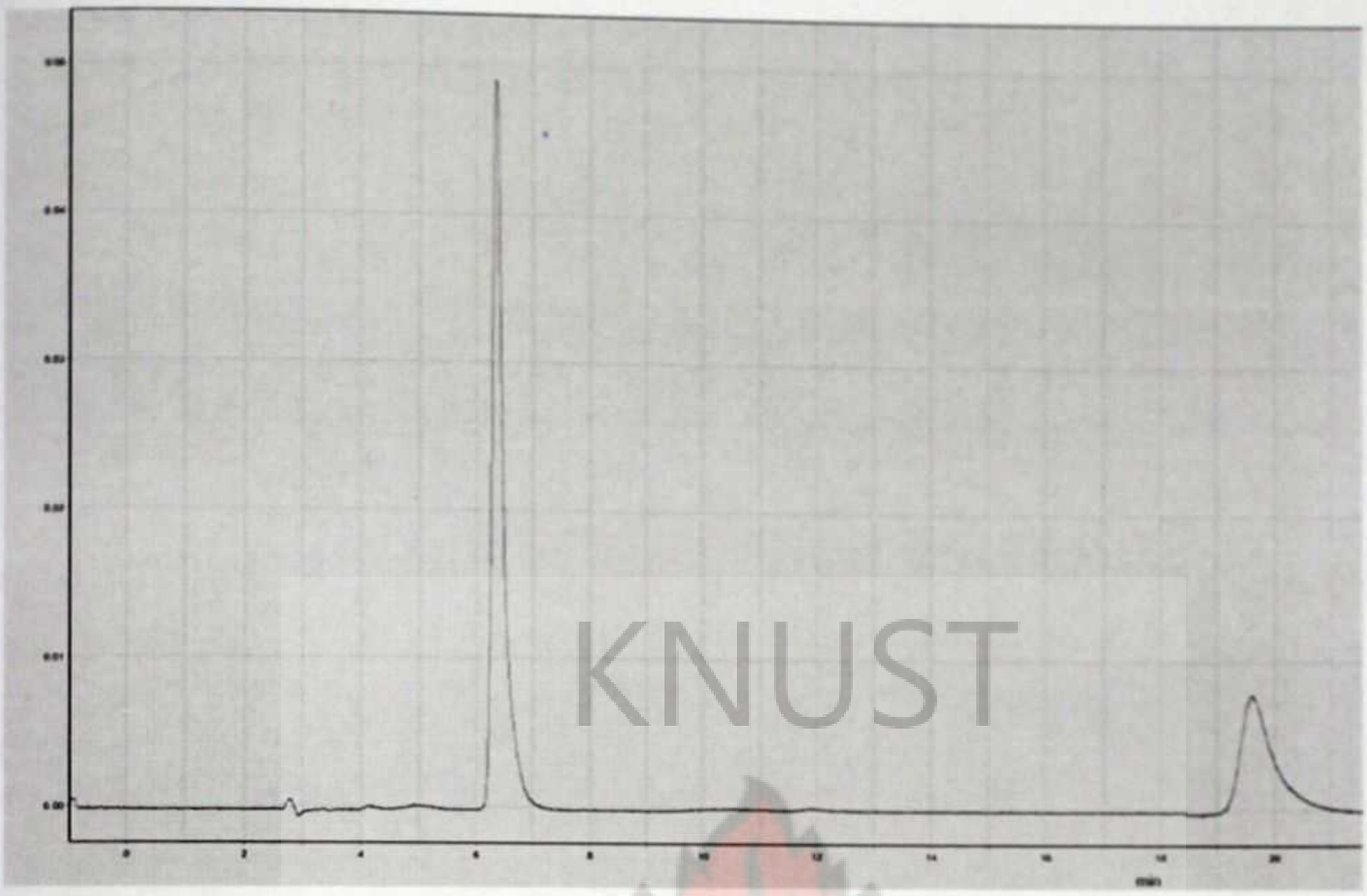


Figure 3.12: HPLC Qualitative Analysis of PTALS

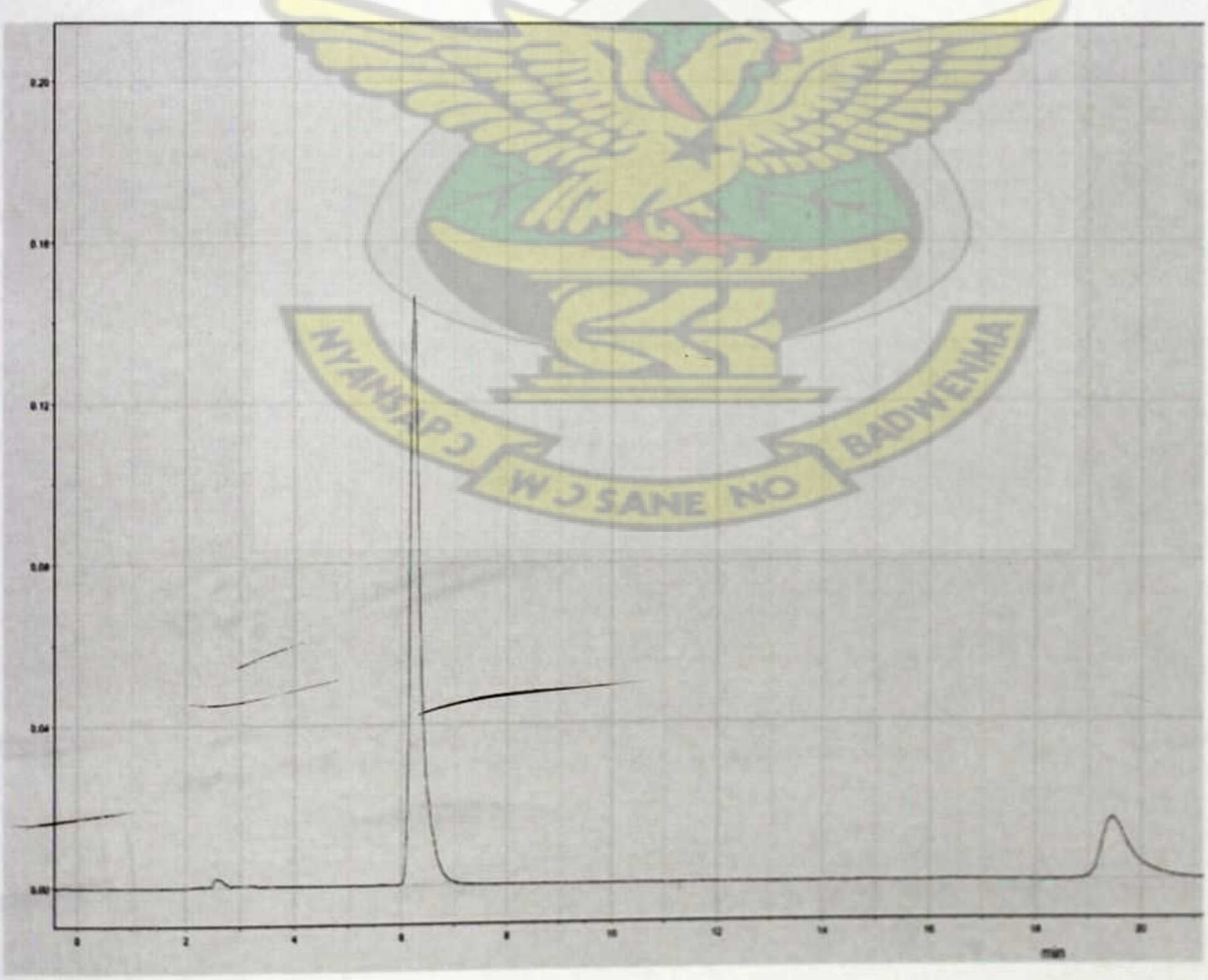


Figure 3.14: HPLC Qualitative Analysis of PTCLS

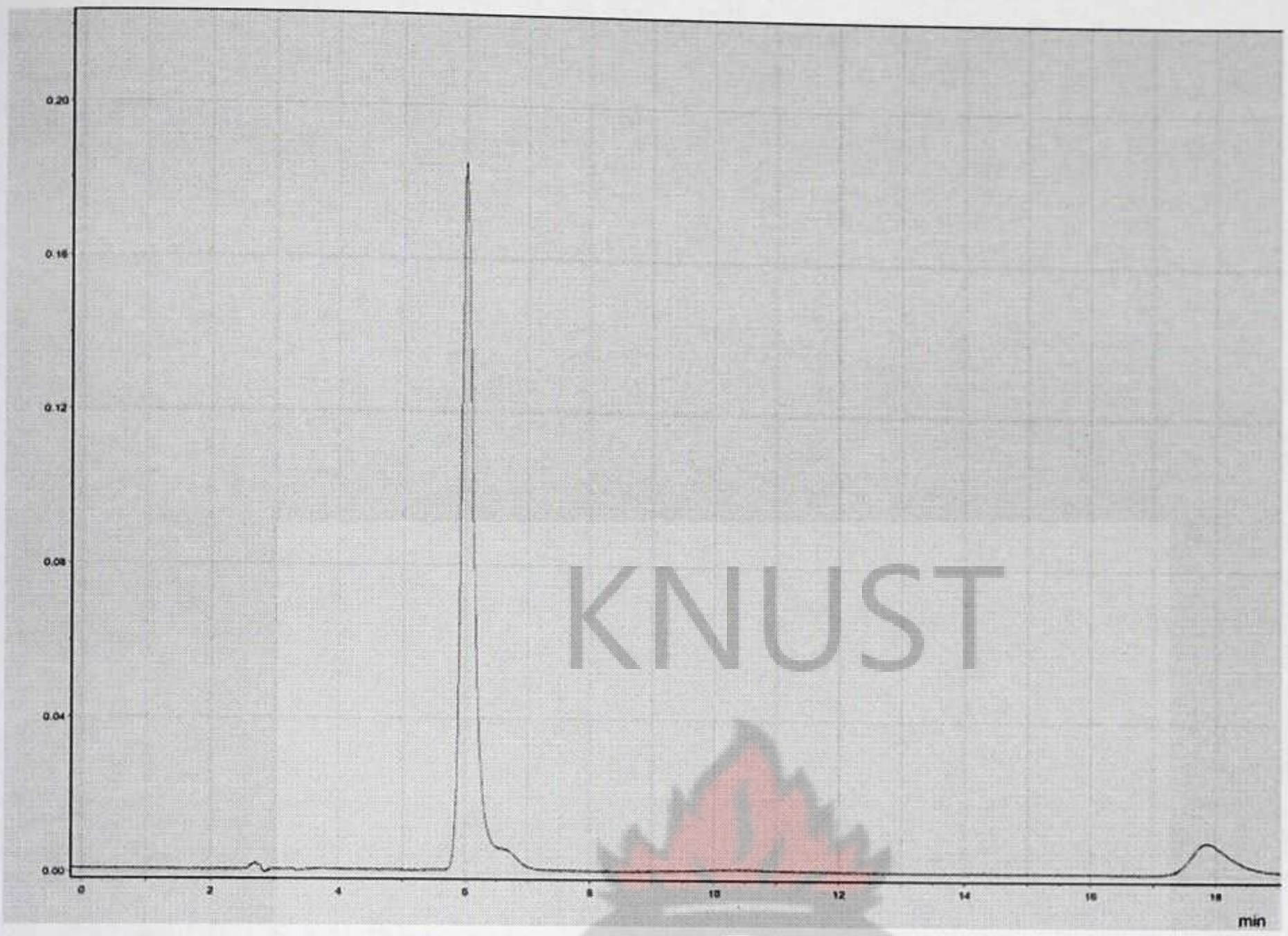


Figure 3.15: HPLC Qualitative Analysis of PTDL

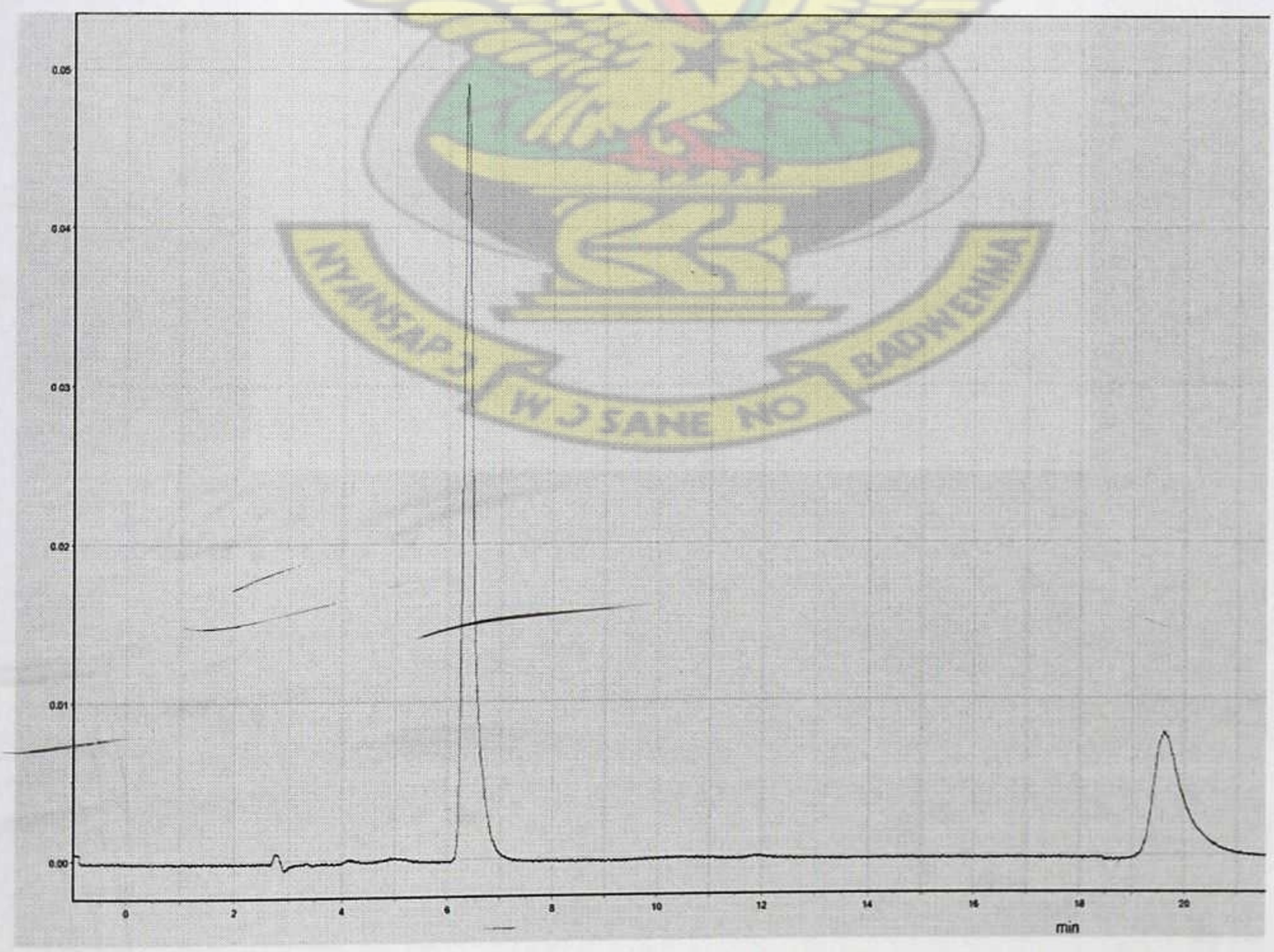


Figure 3.16: HPLC Qualitative Analysis of PTQFS

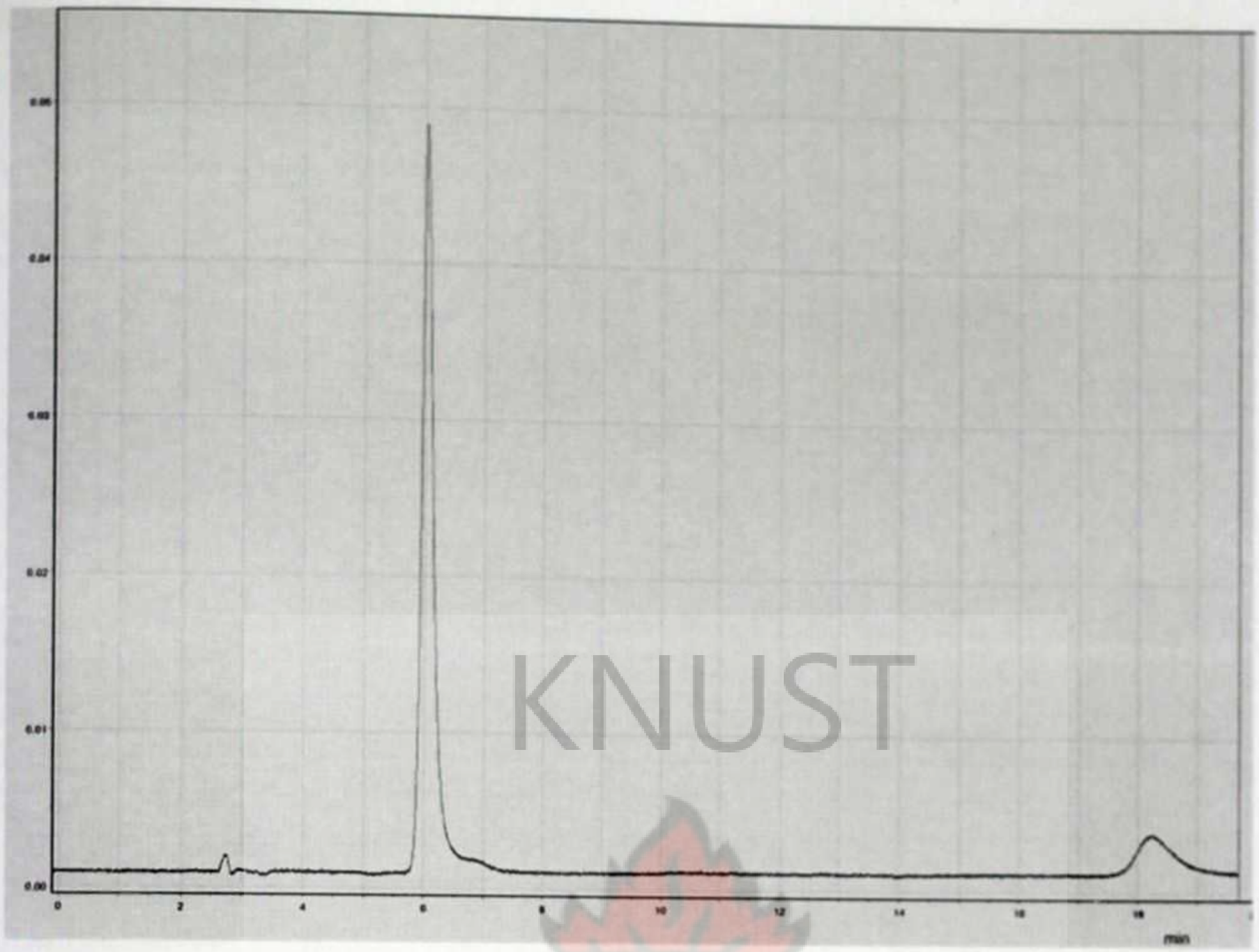


Figure 3.17: HPLC Qualitative Analysis of PTRFS

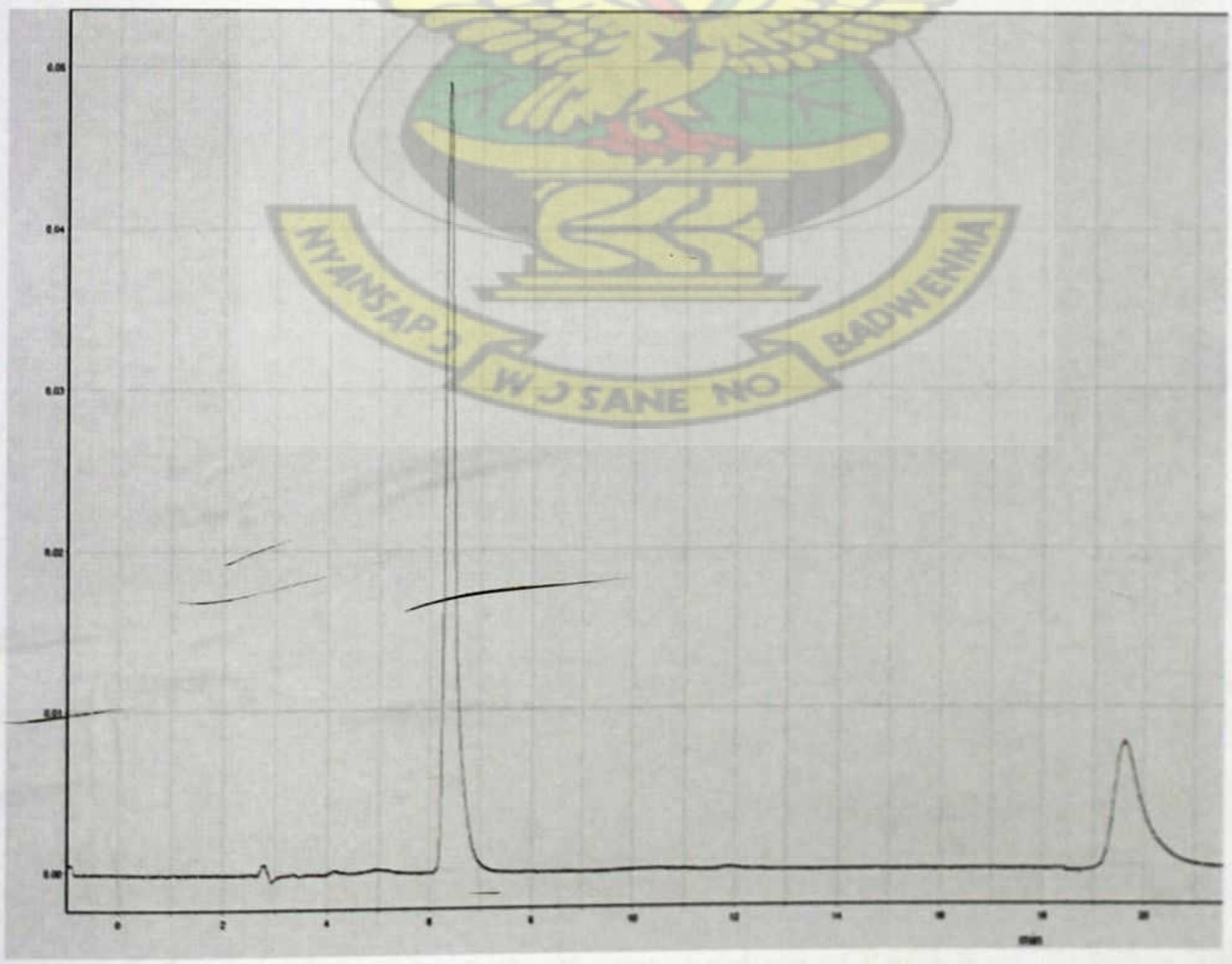


Figure 3.18: HPLC Qualitative Analysis of PSALS

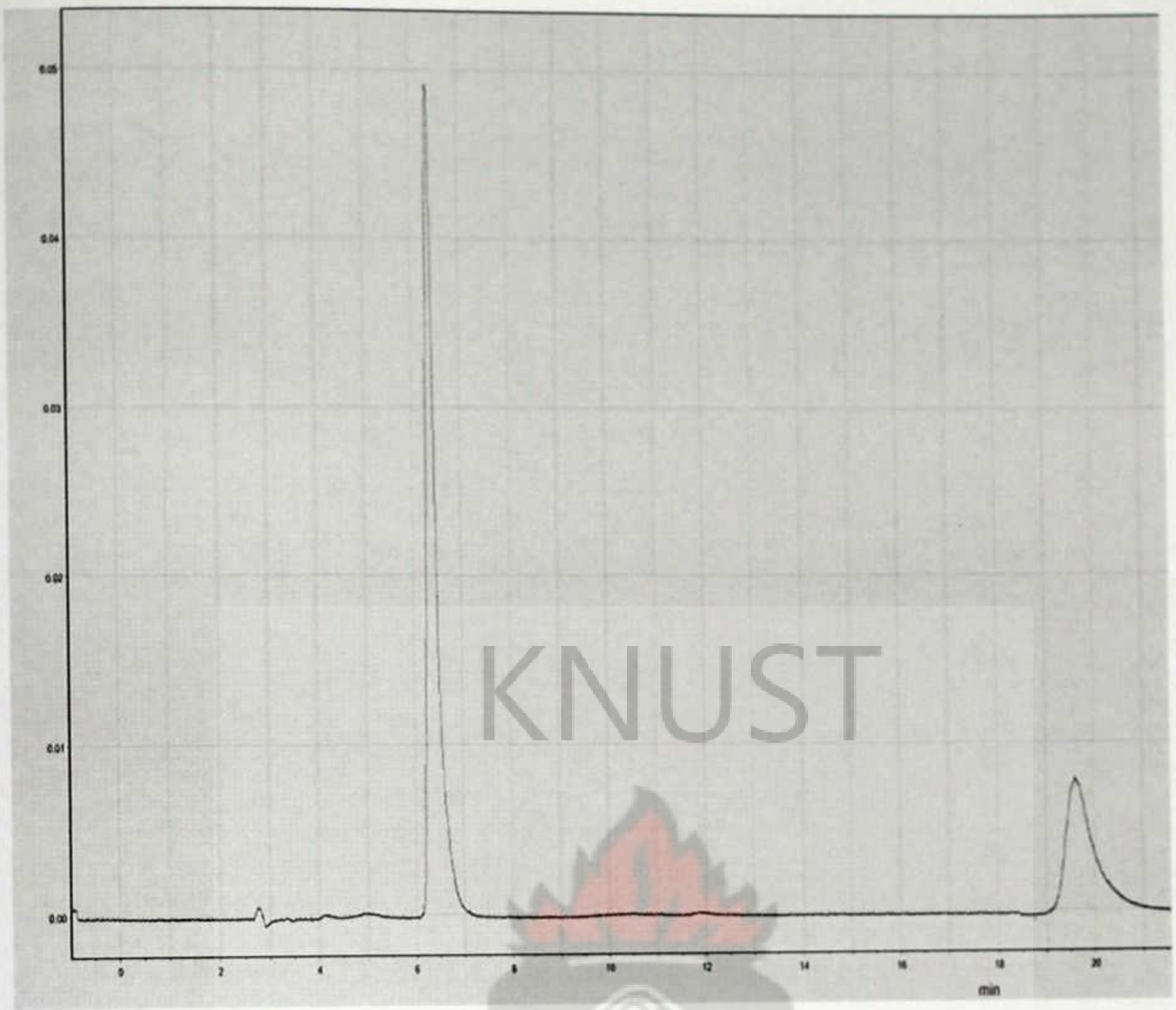


Figure 3.19: HPLC Qualitative Analysis of PSBLS

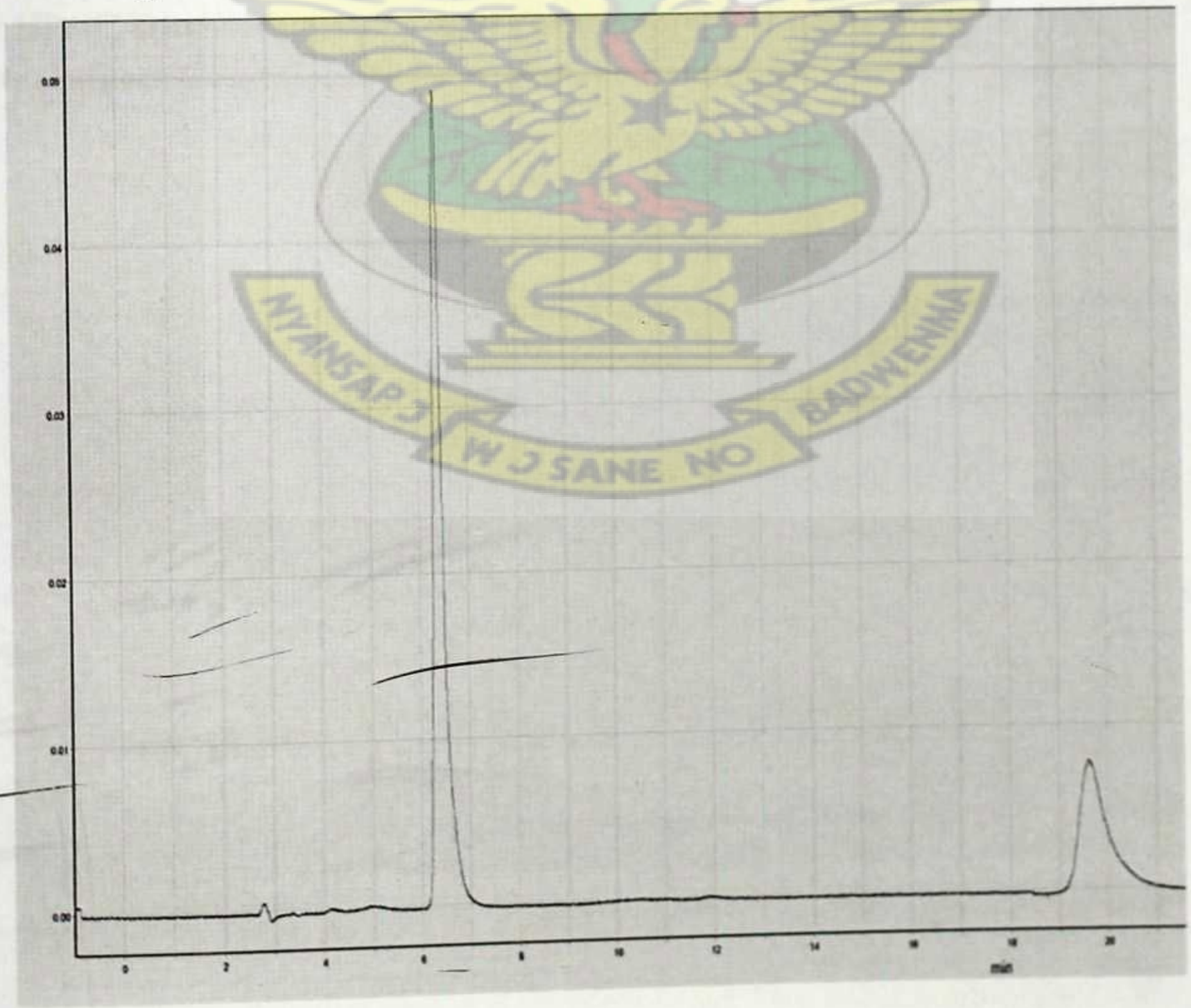


Figure 3.20: HPLC Qualitative Analysis of PSCLS

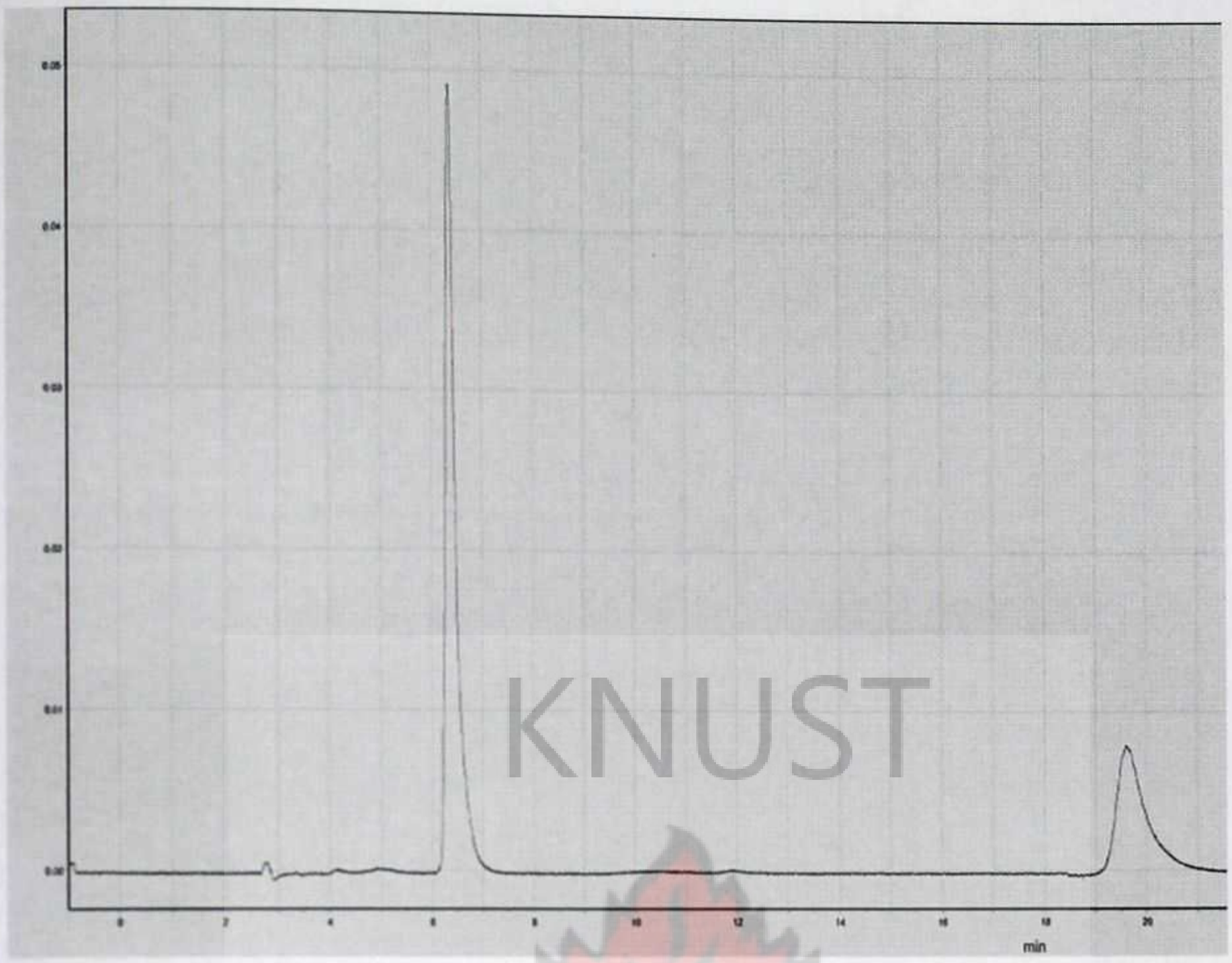


Figure 3.21: HPLC Qualitative Analysis of PSDLS

3.10 HPLC Qualitative Analysis of Paracetamol Tablets Samples at Different Temperatures Conditions to determine their stability

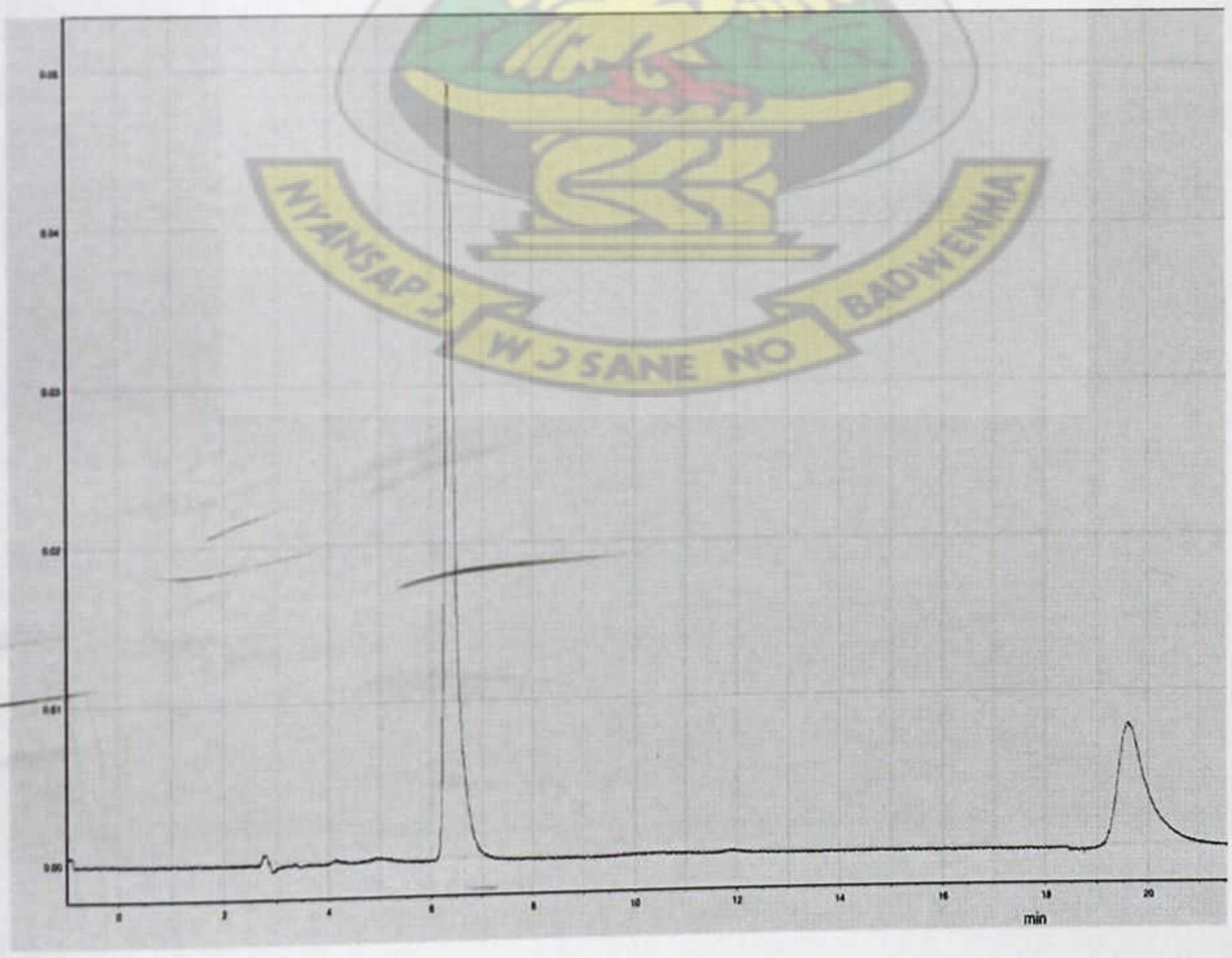


Figure 3.22: Chromatogram of Tablet Sample PTALS at 28°C

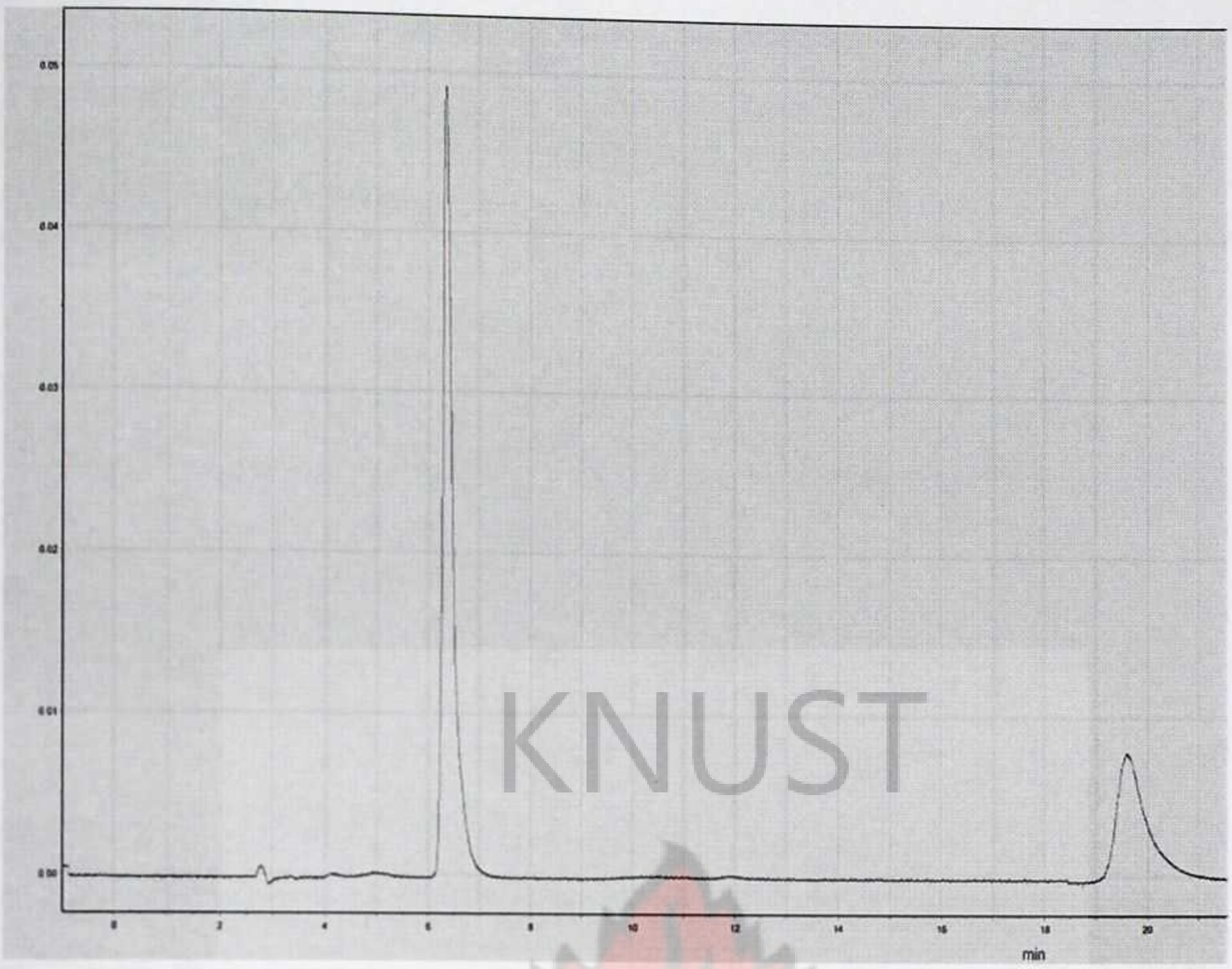


Figure 3.23: Chromatogram of Tablet Sample PTALS at 35°C

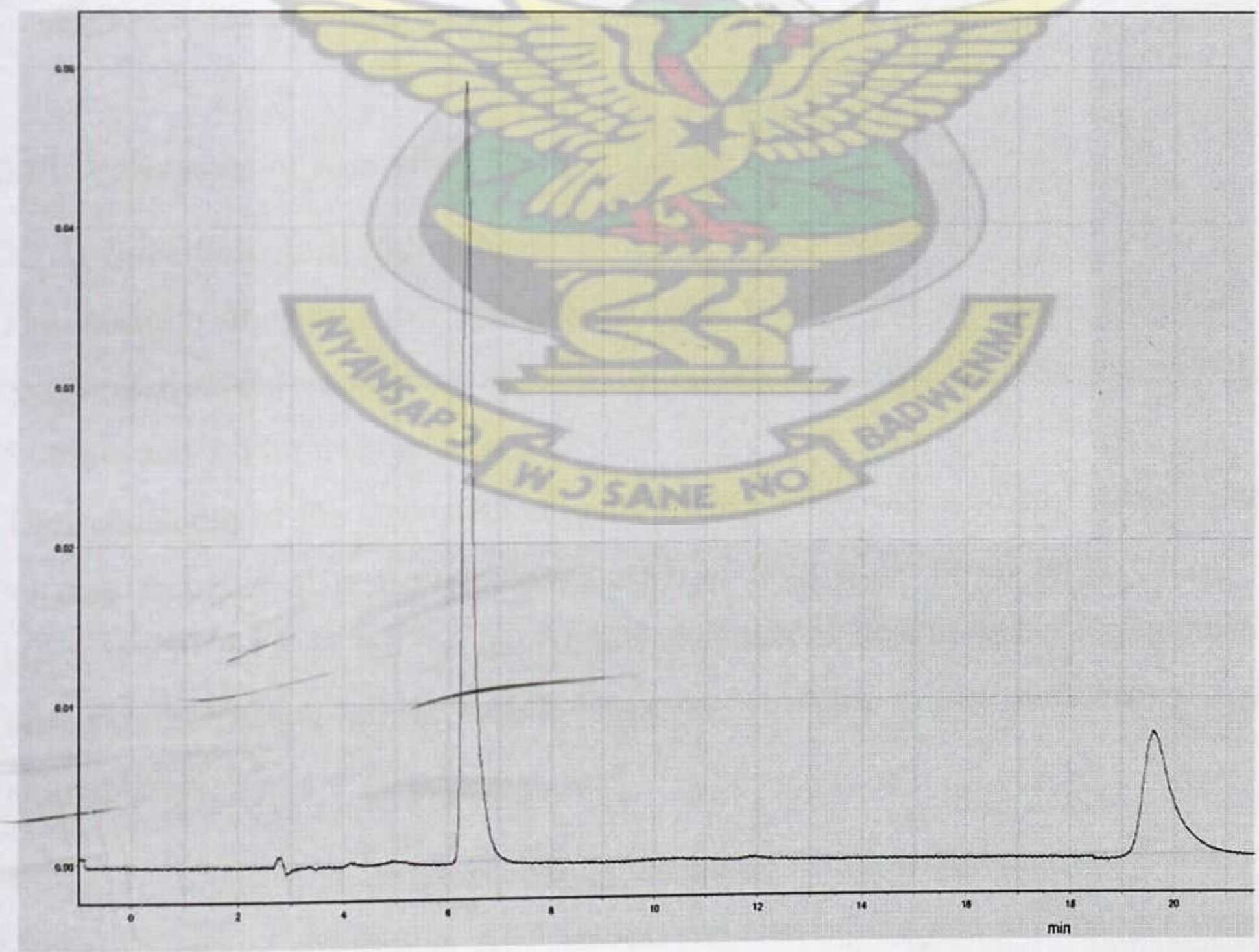


Figure 3.24: Chromatogram of Tablet Sample PTALS at 40°C

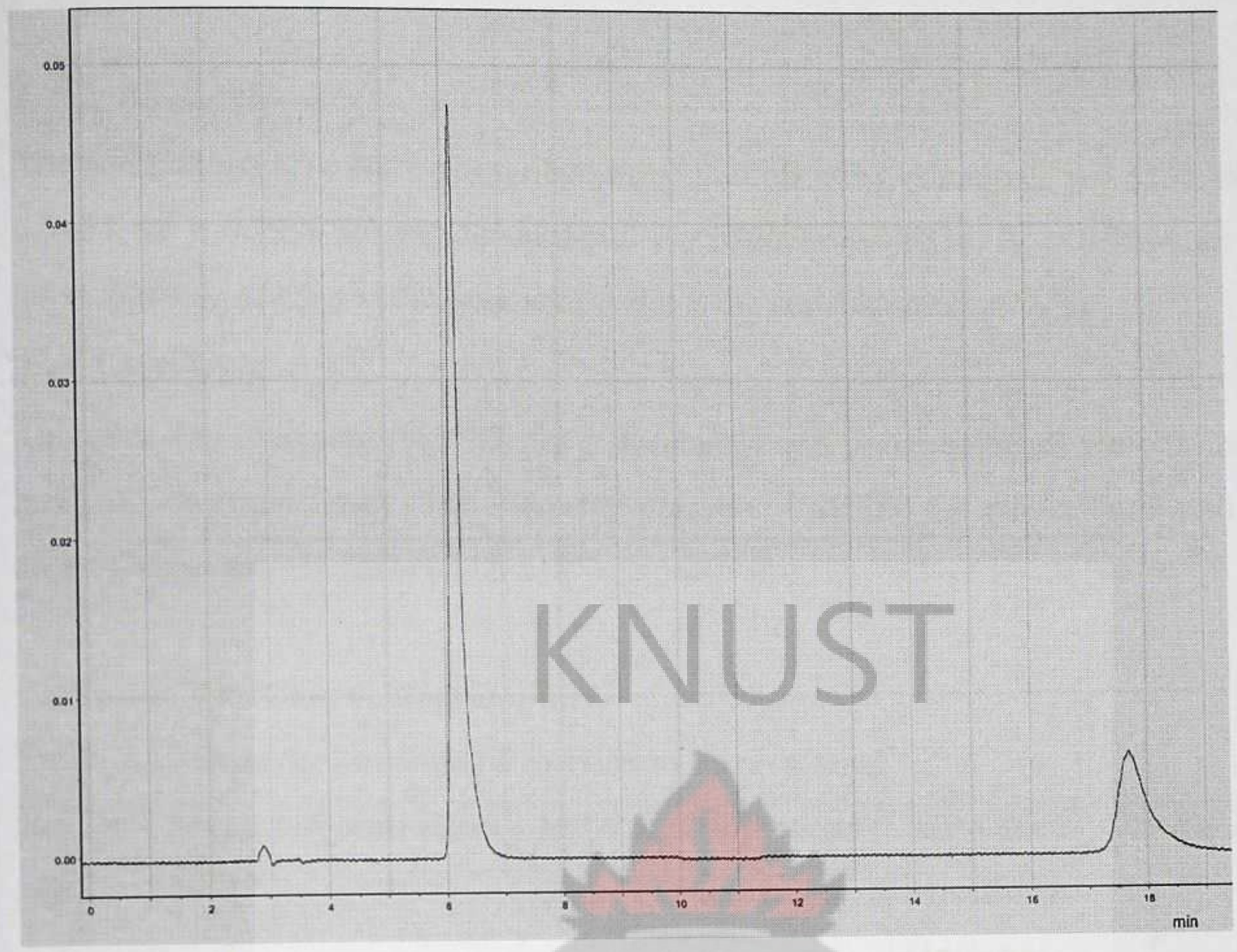


Figure 3.25: Chromatogram of Tablet Sample PTALS at 50°C

3.11 Validation of Analytical Method

3.11.1 Specificity and Robustness

The elution of peaks of paracetamol and *p*-aminophenol were presented in representative chromatograms shown in Figure 3.7 with the retention times of 5.65min and 3.48min respectively.

The robustness of the new method under investigation was studied by intentionally varying amounts of respective components of the mobile phase to investigate the effect it will have on the results. Adulterants such as aspirin and chloroquine were added to the analyte but the mobile phase was still able to pick paracetamol and *p*-aminophenol.

Various columns from different sources were used. Chromatographic conditions such as wavelength of absorption, AUF values, and chart speed and pH changes were all investigated.

3.11.2 Linearity

The calibration curve can be described using the following equations: $y = 5573.8x + 0.9437$, $R^2 = 0.9990$ for paracetamol and $y = 5566.3x + 0.0834$, $R^2 = 0.9994$ for *p*-aminophenol, where y is the peak area and x is the concentration in $\%^{w}/_{v}$.

3.11.3 Precision

The intra-day precision (%RSD) for paracetamol and *p*-aminophenol were 0.227% and 0.187% respectively. The inter-day precision (%RSD) for paracetamol and *p*-aminophenol were 0.225% and 0.218% respectively.

Intra-day Precision or Repeatability

Table 3.20 Data for Intra-day Precision of Paracetamol

No. of run	Mean concentration, $\%^{w}/_{v}$	MPA for 2 injections (Pure Paracetamol)	MPA for 2 injections (Internal Standard)	MPAR
1	0.019950	0.2774	0.1329	2.087
2	0.020000	0.2780	0.1331	2.089
3	0.019954	0.3783	0.1331	2.842
4	0.019922	0.3785	0.1332	2. 842
5	0.020016	0.4673	0.1332	3.508
6	0.020046	0.4675	0.1340	3.489

MPA = Mean Peak Area; MPAR = Mean Peak Area Ratio

Inter-day Precision or Repeatability

Table 3.21 Data for Inter-day Precision of Paracetamol

No. of run	Day 1		Day 2		Day 3	
	MPAR	Mean concentration, $\%^{w}/_{v}$	MPAR	Mean concentration ,$\%^{w}/_{v}$	MPAR	Mean concentration ,$\%^{w}/_{v}$
1	2.087	0.019950	2.090	0.0249375	2.089	0.0299160
2	2.089	0.020000	2.087	0.0250000	2.088	0.0300001
3	2.842	0.019954	2.841	0.0249400	2.840	0.029910
4	2. 842	0.019922	2.882	0.0249025	2.883	0.0298860

5	3.508	0.020016	3.500	0.0250100	3.499	0.0300150
6	3.489	0.020046	3.490	0.0250325	3.491	0.0300690

Table 3.22 Data for test of Precision of the method (student t-test) for Paracetamol

Days	STATISTICAL PARAMETERS					
	Mean concentration	Standard deviation, S	RSD (%)	t_r	t_0	Remark
Day 1	0.019981	0.000045	0.227	1.03	2.57	$t_r < t_0$ hence precise
Day 2	0.0249704	0.000046	0.184	1.58	2.57	$t_r < t_0$ hence precise
Day 3	0.0299695	0.000066	0.264	1.13	2.57	$t_r < t_0$ hence precise

Note: 1. RSD = Relative Standard Deviation

2. t_0 is the theoretical student t value for (N-1) degrees of freedom at 95% confidence level

3. t_r is the experimental t value for N = 6

4. μ_0 for day 1 = 0.0200%$^{w}/_{v}$, μ_0 for day 2 = 0.0250%$^{w}/_{v}$, μ_0 for day 3 = 0.0300%$^{w}/_{v}$

The above procedure was followed to find the intra-day and inter-day precisions for p-aminophenol pure sample as shown in Tables 4.4 and 4.5.

Tools for Statistical Analysis of Data

1. Standard deviation, S

$$S = \sqrt{\frac{\sum(x_i - x)^2}{n-1}}$$

2. Relative Standard deviation, (RSD %)

$$RSD\ \% = \frac{S}{x} \times 100\%$$

3. Student t-test, t

$$t = \frac{x - \mu_0}{S} \times \sqrt{n}$$

Where; x is the mean and S is the standard deviation, n is the number of determinations, and μ_0 is the specified value

3.11.4 Accuracy

The %recovery and SD for paracetamol were found to be 100.16±0.60% at 0.00075%$^{w}/_{v}$ and 100.41±0.35% at 0.0018%$^{w}/_{v}$, for *p*-aminophenol 101.13±1.92% and 100.30±1.34% at concentration 0.00075 %$^{w}/_{v}$ and 0.0018 %$^{w}/_{v}$, respectively.

Table 3.23: Data for Recovery of paracetamol and p-aminophenol

Compounds	Concentration (%$^{w}/_{v}$)	%Recovery (mean ± SD)
Paracetamol	0.00075	100.16 ± 0.60
	0.0018	100.41 ± 0.35
p-Aminophenol	0.00075	101.13 ± 1.92
	0.0018	100.30 ± 1.34

3.11.5 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Table 3.24: Data for LOD and LOQ determination for Paracetamol

y	x_e	x_i	(x_e-x_i)	$(x_e - x_i)^2$
0.0001	1.421	1.422	-0.001	1×10^{-6}
0.0002	2.098	2.097	0.001	1×10^{-6}
0.0004	3.183	3.185	-0.002	4×10^{-6}
0.0006	4.380	4.384	-0.004	1.6×10^{-5}
0.0008	5.373	5.377	-0.004	1.6×10^{-5}
0.0010	6.477	6.476	0.001	1×10^{-6}
$\sum(x_e - x_i)^2 = 3.9 \times 10^{-5}$				

$$\delta = [(\sum(x_e - x_i)^2)/(N-1)]^{\frac{1}{2}}$$

Where Ó is the residual standard deviation,

x_e is the estimated mean peak area ratio from the calibration graph,

x_i is experimental mean peak area ratio from the calibration table,

N is the number of calibration concentrations prepared,

y is the concentration

S is the slope

$$\delta = [(3.9 \times 10^{-5})/(6-1)]^{\frac{1}{2}} = 2.2793 \times 10^{-3}$$

According to the ICH guidelines for method validation,

LOD = Concentration yielding a signal-to-noise ratio of 3:1

LOQ = Concentration yielding a signal-to-noise ratio of 10:1

Paracetamol

$y = 5573.8x + 0.9437$

$S = 5573.3$

$\sigma = 2.2793 \times 10^{-3}$

$LOD = (3.3\sigma)/ S$

$LOD = (3.3 \times 2.2793 \times 10^{-3})/5573.8 = 1.65 \times 10^{-6}\ \%^{w}/_{v.}$

$LOQ = (10\ \sigma)/ S$

$LOQ = (10 \times 2.2793 \times 10^{-3})/5573.8 = 5.01 \times 10^{-6}\ \%^{w}/_{v.}$

Table 3.25: Data for LOD and LOQ determination for Paracetamol

y	x_e	x_i	$(x_e - x_i)$	$(x_e - x_i)^2$
0.0001	0.722	0.721	0.001	1×10^{-6}
0.0002	1.135	1.137	-0.002	4×10^{-6}
0.0004	2.273	2.274	-0.001	1×10^{-6}
0.0006	3.410	3.413	-0.003	9×10^{-6}
0.0008	4.539	4.540	0.001	1×10^{-6}
0.0010	5.676	5.672	0.004	1.6×10^{-5}
$\sum(x_e - x_i)^2 = 3.2 \times 10^{-5}$				

$\dot{O} = [(3.2 \times 10^{-5})/(6-1)]^{\frac{1}{2}} = 2.530 \times 10^{-3}$

P-aminophenol

$y = 5566.3x + 0.0834$

$\sigma = 2.530 \times 10^{-3}$

$S = 5566.3$

$LOD = (3.3 \times 2.530 \times 10^{-3}) / 5566.3 = 1.499 \times 10^{-6}\ \%^{w}/_{v.}$

$LOQ = (10 \times 2.530 \times 10^{-3}) / 5566.3 = 4.550 \times 10^{-6}\ \%^{w}/_{v}$

CHAPTER FOUR

4.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 DISCUSSIONS

Colour Identification test for Pure Paracetamol powder

When 0.1 g of pure paracetamol powder was added to 1 ml of hydrochloric acid, and heated to boiling for 3minutes and 1ml of distilled water was added and cooled in an ice bath, no precipitate was formed. Upon addition of 0.05 ml of a 4.9 g/l solution of potassium dichromate, a violet colour developed which did not change to red. This confirms the presence of pure paracetamol powder (BP 2007).

Melting point Determination for Pure powders

Pure Paracetamol powder

The reference range for the melting point of pure paracetamol is 168°C to 172°C (BP 2007).

For the determination using pure paracetamol powder, the melting point range obtained was 169°C to 170°C. The value obtained agrees with the reference range.

Pure p-aminophenol powder

The reference range for the melting point of pure p-aminophenol is 188°C to 190°C (BP 2007).

For the determination using the pure p-aminophenol powder, the melting point range obtained was 188°C to 190°C. The value obtained agrees with the reference range.

Pure Caffeine powder

The reference range for the melting point of pure caffeine is 235°C to 239°C (BP 2007). For the determination using the pure caffeine powder, the melting point range obtained was 234.5°C to 238°C. The value obtained agrees with the reference range.

Identification test for the presence of paracetamol in paracetamol formulations

All the 30 paracetamol formulations showed a positive test for the presence of paracetamol in them since all gave a specific absorbance in the range of 867-920.

This is within the range of 860-980 for 0.001%w/v solution specified in the British Pharmacopoeia. This confirms that all the paracetamol formulations investigated are actually paracetamol formulations.

Qualitative tests for the presence of p-aminiphenol in paracetamol formulations

P-aminophenol is the primary degradation product of paracetamol which is limited at a low level (500ppm or 0.005%w/v) in the drug substance by British Pharmacopoeia, 2007 employing a manual colourimetric limit test. The limit of p-aminophenol is increased to 1000ppm or 0.1%w/v for the tablet product monographs.

Primary Aromatic Amine test

All the 30 paracetamol formulations gave a positive test to the primary aromatic amine test. This gives an indication that there is a possibility of p-aminophenol being present in the tablets.

In order to ascertain the specific contaminants or inclusion within the formulations given rise to the positive result for primary aromatic amine test, a limit test for p-aminophenol was performed since it is the impurity that is under investigation.

Limit test for the presence of p-aminiphenol in paracetamol formulations

All the 30 paracetamol formulations gave a blue/green coloured precipitate when the samples were subjected to the above-mentioned test. This gives an indication that p-aminophenol is present in the paracetamol formulations investigated.

Uniformity of Weight test

According to the British pharmacopoeia for tablets which are expected to weigh more than 250mg, not more than 2 of the individual masses should deviate from the average mass by 5% and none should deviate by more than twice that percentage. Paracetamol tablet averagely weighs about 600mg and therefore falls under this criterion. Based on the results obtained for the deviations on the uniformity of weight it could be observed that the all tablets passed since not even one of the tablets fell out of the stated range and therefore tableting could be said to have been properly done to avoid overdose and under doses.

According to the BP 2007, no single tablet should deviate by 10% or above and not more than two tablets should deviate by 5%. From table 3.12, no tablet deviated by this margin. Therefore, by the BP standards, all the tablets passed the uniformity of weight test.

Assay of Pure Paracetamol powder using Titration

According to the BP 2007, pure paracetamol contains not less than 99.0% and not more than 101.0% of paracetamol using the assay method described and should be calculated with reference to the anhydrous substance. The percentages purity of the paracetamol powder assayed was 99.54% and this meets the requirement in the BP. It also confirms that the pure paracetamol powder used for the formulation was 99.54% pure.

The percentage content of the pure powder was determined to be 99.54% which clearly lies within 99% -101% stated by the British pharmacopoeia. During the assay, cerium ammonium (IV) sulphate which is a strong oxidizing agent to oxidize the amine (4-aminophenol) to an imino-quinone (p-benzoquinone). After complete oxidation of the amine has been achieved, it would further oxidize the ferrion indicator to ferrin to establish a colour change for detection. The accuracy of the percentage content obtained clearly revealed that the cerium ammonium sulphate used was able to oxidize all the 4-aminophenol present before oxidizing the ferrion to ferrin.

In the assay of the pure powder, the sample was dissolved in a mixture of water and sulphuric acid and refluxed for an hour. It is important to note that the acid added is essential for progress of the reaction in that the acid is needed to hydrolyze the paracetamol to 4-aminophenol species which then could undergo oxidation-reduction reaction with cerium ammonium (IV) sulphate used. This implies that the amount of paracetamol hydrolyzed is indirectly what would react with the cerium and would further be recorded as the percentage content of drug. This clearly reflects the importance of the sulfuric acid added.

Assay of Paracetamol tablets using UV-Vis method

According to the BP 2007, paracetamol tablet contains not less than 95.0% and not more than 105.0% of paracetamol using the assay method described and should be calculated with reference to the tablet substance. The percentage content of the

tablets was determined to be between 99.54% which clearly lies within 99.20% to 104.00% which meets the requirement stated by the British pharmacopoeia.

Development of HPLC Method of analysis

The final conditions were obtained after series of work has been done on details such as wavelength of absorption, auf value, chart speed, mobile phase composition and effects of pH.

The final mobile phase composition comprised 1%$^{v}/_{v}$ glacial acetic acid and methanol in the ratio of 85%:15%. The pH of the final solution was always in the range of 3.24 ± 0.02. It was also observed that as the pH of the mobile phase went below 3.22, the retention time of the paracetamol was prolonged. The C_{18} ODS column was employed because at the 278nm wavelength, paracetamol, p-aminphenol and caffeine were highly absorbing and they therefore elutes very well. The final method used for analysis was simple, fast, precise, accurate, reproducible and robust under changing conditions. The calibration curves of paracetamol and p-aminphenol gave a linear correlation of mean peak area ratio to the concentrations of the standard solutions prepared.

The robustness of the new method under investigations was studied by intentionally varying amounts of respective components of the mobile phase to investigate the effect it will have on the results.

Various columns from different sources were used. Chromatographic conditions such as wavelength of absorption, AUF values, and chart speed and pH effects were all investigated and the method proved to be robust.

After optimization, HPLC method was carried out on Inersil® C_{18} CDS-3, 250 mm x 4.60 mm, 5µm column using 1%1glacial acetic acid: methanol at pH 3.24 ± 0.02, in the ratio of 85:15$^{v}/_{v}$ as mobile phase at a flow rate of 1.00 ml/min. The UV wavelength at 278nm was chosen for monitoring the separation.

Typical chromatogram is illustrated in Figure 3.7. The mean retention times were 2.48±0.15minutes for *p*-aminophenol and 5.65±0.15minutes for paracetamol and 16.78±0.15minuts for the caffeine (internal standard).

Specificity of a method can be defined as absence of any interference at retention times of samples. As lack of information in the composition of generic formulations, makes it difficult to assess selectivity by traditional analysis comparison with a placebo solution. The specificity of the method was demonstrated by injection of standard solution of paracetamol and *p*-aminophenol at concentration of 0.01%$^{w}/_{v}$. The elution peaks of paracetamol and *p*-aminophenol were presented in representative chromatograms, as shown in Figure 3.7, with the retention times of 5.55 minutes and 3.50 minutes respectively.

The linearity of paracetamol and *p*-aminophenol were studies by preparing standard solution at six different concentrations ranging from 0.001%$^{w}/_{v}$ to 0.0001%$^{w}/_{v}$. Each concentration was injected in three replicates and the mean value of peak area was taken for the calibration curve.

According to the ICH guidelines, R^2 should be between 0.995 and 1 for an analytical process to be linear. The R^2 obtained from the calibration curve indicates that the method used was linear.

The calibration curve can be described using the following equations:

$y = 5573.8x + 0.9437$, $R^2 = 0.9990$ for paracetamol and $y = 5566.32x + 0.0834$, $R^2 = 0.9994$ for *p*-aminophenol, where y is the peak area and x is the concentration in %$^{w}/_{v}$.

The precision of the assay was studied with respect to both intra-day (repeatability) and inter-day (intermediated) precisions. Repeatability was calculated from five replicate injections of three different concentrations of paracetamol and *p*-aminophenol in the same equipment on the same day. Precision was checked with the same concentrations and the determination of each compound was repeated day by day during three days. The intra-day precision (%RSD) for paracetamol and *p*-aminophenol were 0.227% and 0.187% respectively. The inter-day precision (%RSD) for paracetamol and *p*-aminophenol were 0.225% and 0.218% respectively. The method could also said to be precise since none of the values exceeded the 2% limited stated in the ICH 2003 guidelines.

The accuracy of method was confirmed by determination of paracetamol and *p*-aminophenol at concentrations of 0.00075 and 0.0018%$^{w}/_{v}$. The %recovery and SD for paracetamol were found to be 100.16±0.60% at 0.00075%$^{w}/_{v}$ and 100.41±0.35%

at 0.0018%$^{w}/_{v}$, and for *p*-aminophenol 101.13±1.92% and 100.30±1.34% at concentration 0.00075%$^{w}/_{v}$ and 0.0018%$^{w}/_{v}$, respectively.

Limit of detection (LOD) and quantification (LOQ) represent the concentration of the analyte that would yield signal-to-noise ratio of 3 for LOD and 10 for LOQ, respectively. LOD and LOQ were determined calculating the signal-to-noise ratio for each compound by injecting a series of solution until the S/N ratio 3:1 for LOD and 10:1 for LOQ.

The equations of the line of the calibration graph were; $y = 5573.8x + 0.9437$, $R^2 = 0.9990$ for paracetamol and $y = 5566.32x + 0.0834$, $R^2 = 0.9994$ for *p*-aminophenol, where y is the peak area and x is the concentration in %$^{w}/_{v}$.

LOD was found to be 1.65×10^{-6} %^{w}/v and 1.499×10^{-6} %^{w}/v for paracetamol and *p*-aminophenol, respectively. LOQ was found to be 5.01×10^{-6} %^{w}/v and 4.455×10^{-6} %^{w}/v for paracetamol and *p*-aminophenol, respectively.

Stability Studies

In all the paracetamol tablets subjected to the stability studies, not even one of them showed significant amounts of *p*-aminophenol. This confirms that the paracetamol tablets in the market are doing well, especially the local ones.

Eight tablet samples were used for the analysis. These are tablets **PTALS**, **PTBLS**, **PTCLS**, **PTDLS**, **PTQFL**, **PTRFS**, **PTSFS** and **PTTFS**. Their stability at 28°C, 35°C, 40°C and 50°C after 6 hours are said to be very good.

The results also indicate that all the drug samples were well package and good manufacturing processes well followed.

HPLC Assay of paracetamol formulations

The developed HPLC method was applied for the analysis of thirty generic paracetamol formulations sampled in Kumasi metropolis. It is interestingly that the percent of the labeled amount (%LA) of paracetamol in all the products was always between 95.80 and 99.71% (Table 3.17). However, BP 2007 stated that paracetamol (both tablet and syrup) should contain not less than 95% and not more than 105% of labeled amount of paracetamol. The content of *p*-aminophenol in all thirty generic

paracetamol formulations was found to be less than the recommended limit. Therefore, the amount of *p*-aminophenol could be negligible.

4.2 CONCLUSION

An isocratic method of elution comprising a 1%$^{v}/_{v}$ of glacial acetic acid and methanol in the ratio of 85%: 15% was developed.

The simple and efficient reverse phase HPLC method was found to be accurate, precise, and linear across the analytical range.

The method was specific for the determination of paracetamol and *p*-aminophenol, its main degradation impurity.

No significant amounts of *p*-aminophenol were found in all the paracetamol formulations.

The developed method could be used for the determination of paracetamol and *p*-amino-phenol as its main degradation product in paracetamol tablets and syrups, and could be applied on other dosage forms.

Proposed method is a convenient and efficient method for simultaneous determination of paracetamol, p-aminophenol and caffeine.

The developed method does not require using gradient or any procedure of extraction and provides determination (qualitative and quantitative) of low levels of the *p*-aminophenol in both paracetamol drug substance and dosage forms.

The results obtained in this study corroborate that the proposed HPLC method is sufficiently precise, rapid and sensitive to be used for routine analyses.

The method is quite suitable for quality assessment of paracetamol in pharmaceutical preparations in the presence of process-related impurities.

4.3 RECOMMENDATIONS

It is recommended that the developed method should be used to analyse paracetamol in combination products of paracetamol and other drugs such as parabru (paracetamol + brufen), fempar (paracetamol + brufen), Zinol (paracetamol + caffeine), etc.

Future work on paracetamol and other degradation products is recommended. This time developing a method that could elute other degradation products of paracetamol.

It is recommended that the developed method should be used to analyse other paracetamol formulations such as paracetamol suppositories, ect.

A method which is capable of detecting process-related impurities, which may be present at trace level in the finished products, should also be developed.

Further stability studies should be done on the paracetamol tablets above six hours but at the same temperature conditions.

A means of subjecting the paracetamol syrups to stability studies should be done.

APPENDICES

Appendix 1

UV-Vis Absorption of Pure Samples using Methanol-Water as solvent

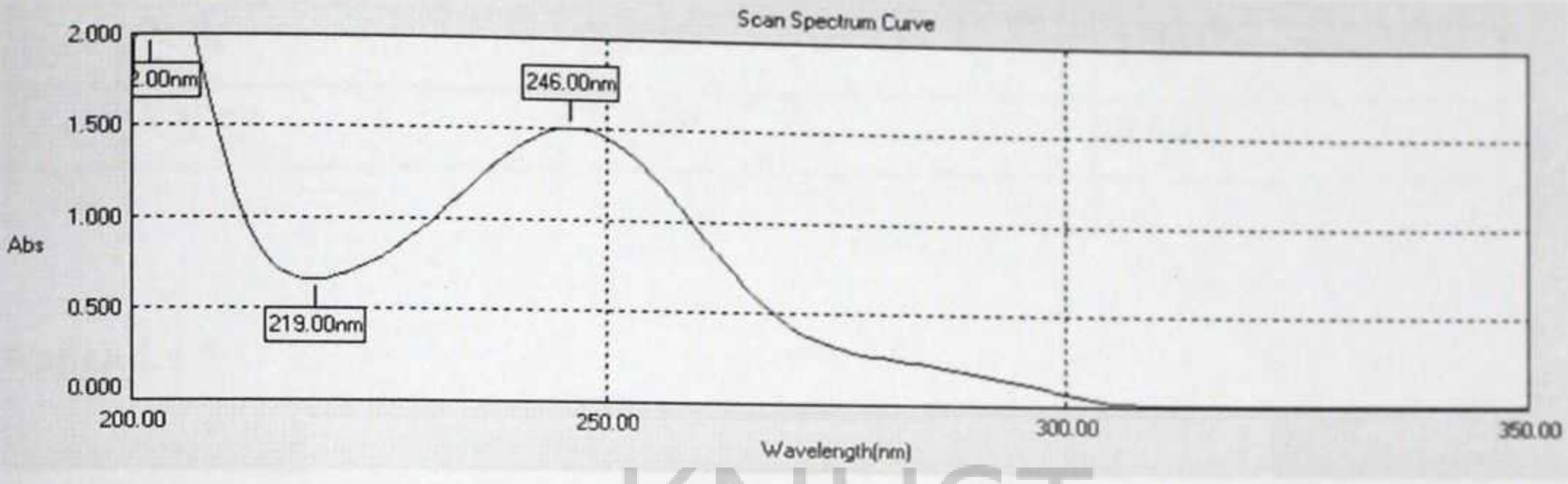


Figure 4.1: UV-Vis Spectrum of Pure Paracetamol powder using Methanol-Water as solvent

Appendix 2

Table 4.1: Data for the UV-visible Spectrum of Pure Paracetamol Powder *using Methanol-Water as solvent*

No.	Peak/Valley	Wavelength(nm)	Absorbance
1	Peak	246.00	1.510
2	Peak	202.00	9.999
1	Valley	219.00	0.660

Appendix 3

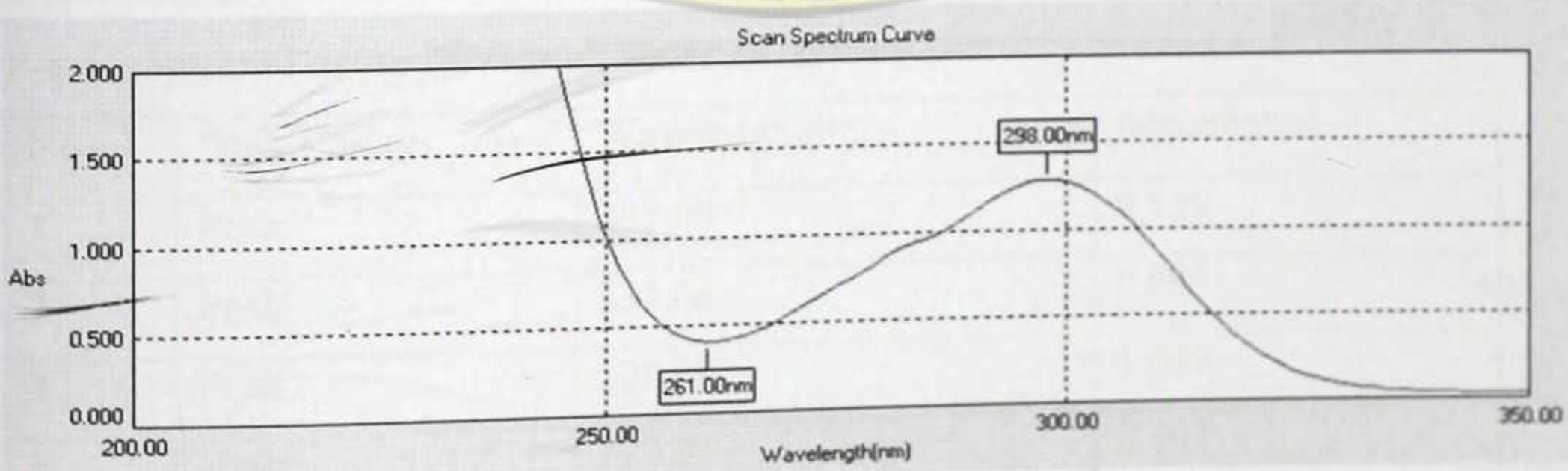


Figure 4.2: UV-Vis Spectrum of Pure P-aminophenol powder using Methanol-Water as solvent

Appendix 4

Table 4.2: Data for the UV-visible Spectrum of Pure P-aminophenol Powder *using Methanol-Water as solvent*

No.	Peak/Valley	Wavelength(nm)	Absorbance
1	Peak	298.00	1.291
1	Valley	261.00	0.404

Appendix 5

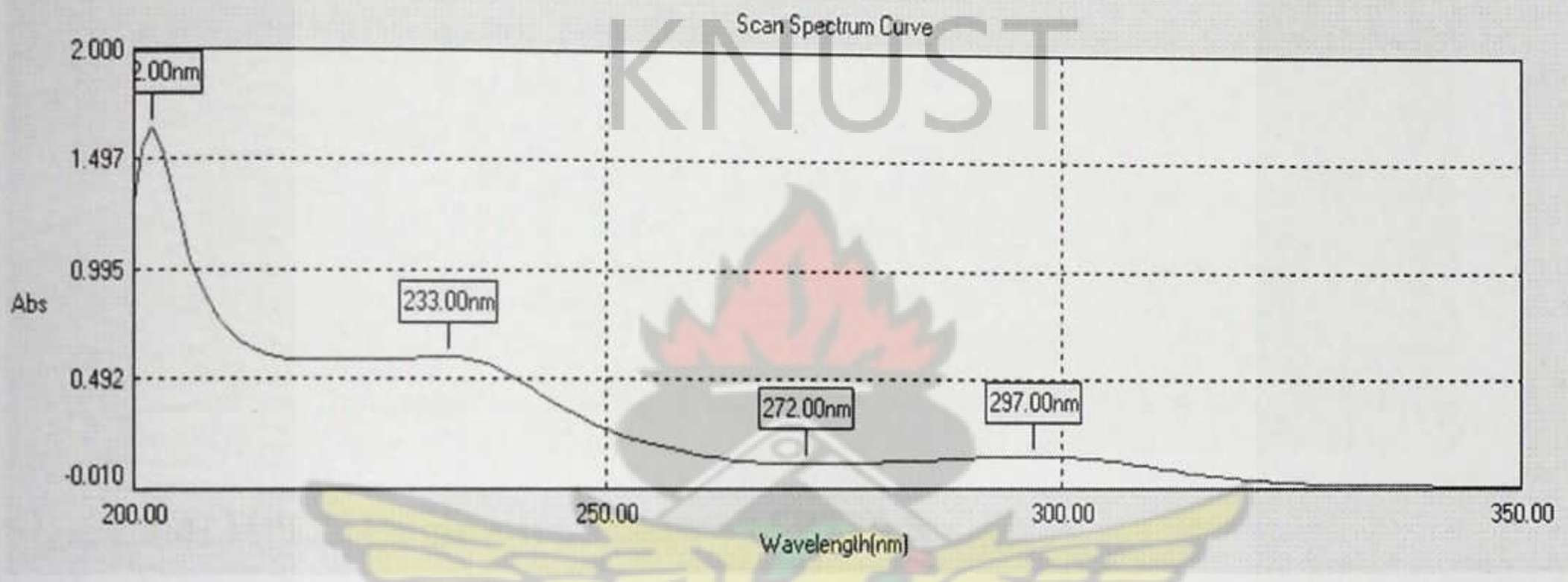


Figure 4.3: UV-Vis Spectrum of a mixture of Pure Paracetamol and Pure P-aminophenol powders using Methanol-Water as solvent

Appendix 5

Table 4.3: UV-visible Data for a mixture of Pure Powders of Paracetamol and P-aminophenol *using Methanol-Water as solvent*

No.	Peak/Valley	Wavelength(nm)	Absorbance
1	Peak	297.00	0.139
2	Peak	233.00	0.595
3	Peak	202.00	1.649
1	Valley	272.00	0.101

Appendix 7

HPLC Qualitative Analysis of Paracetamol formulations

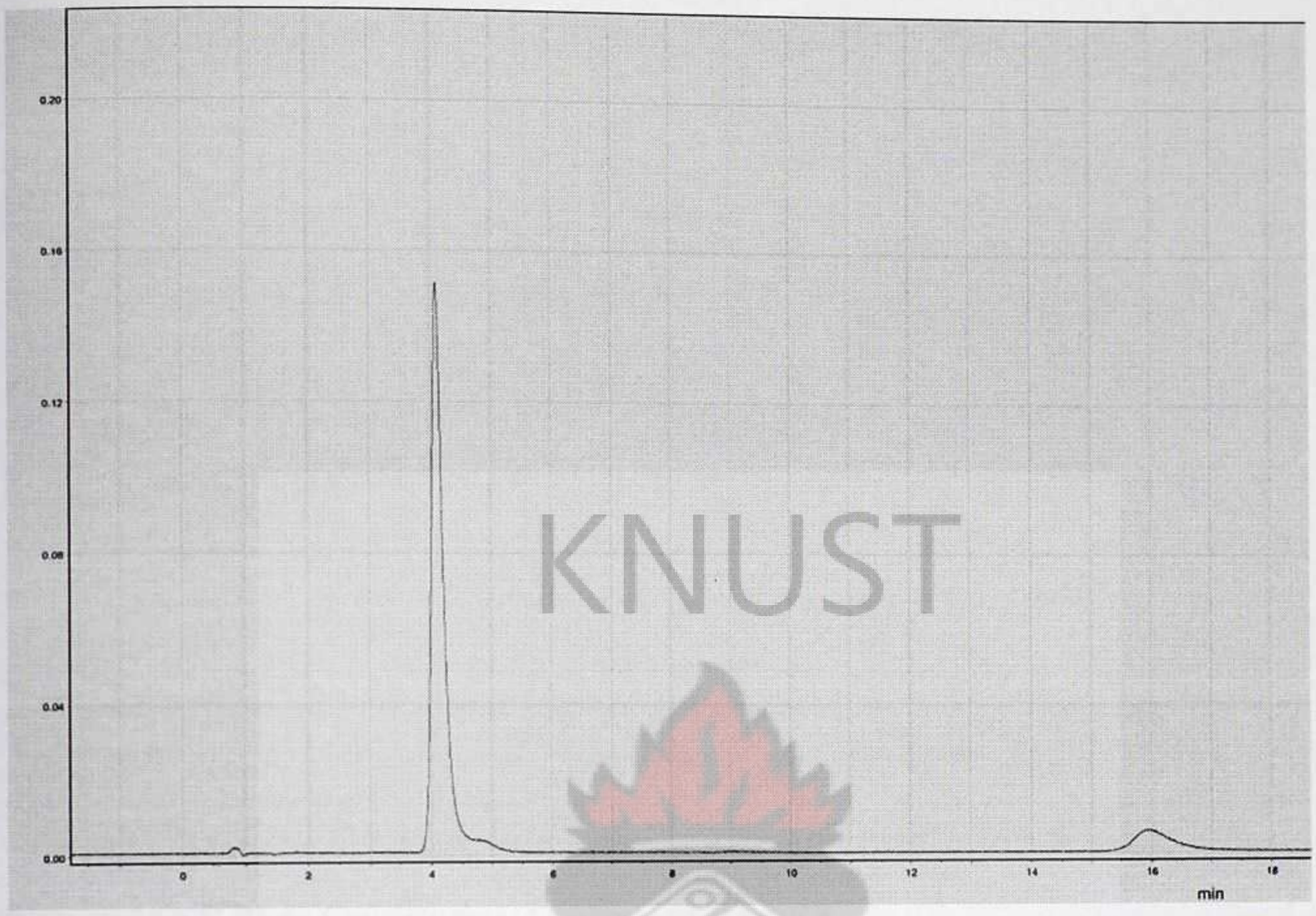


Figure 4.4: HPLC Qualitative Analysis of PTELS

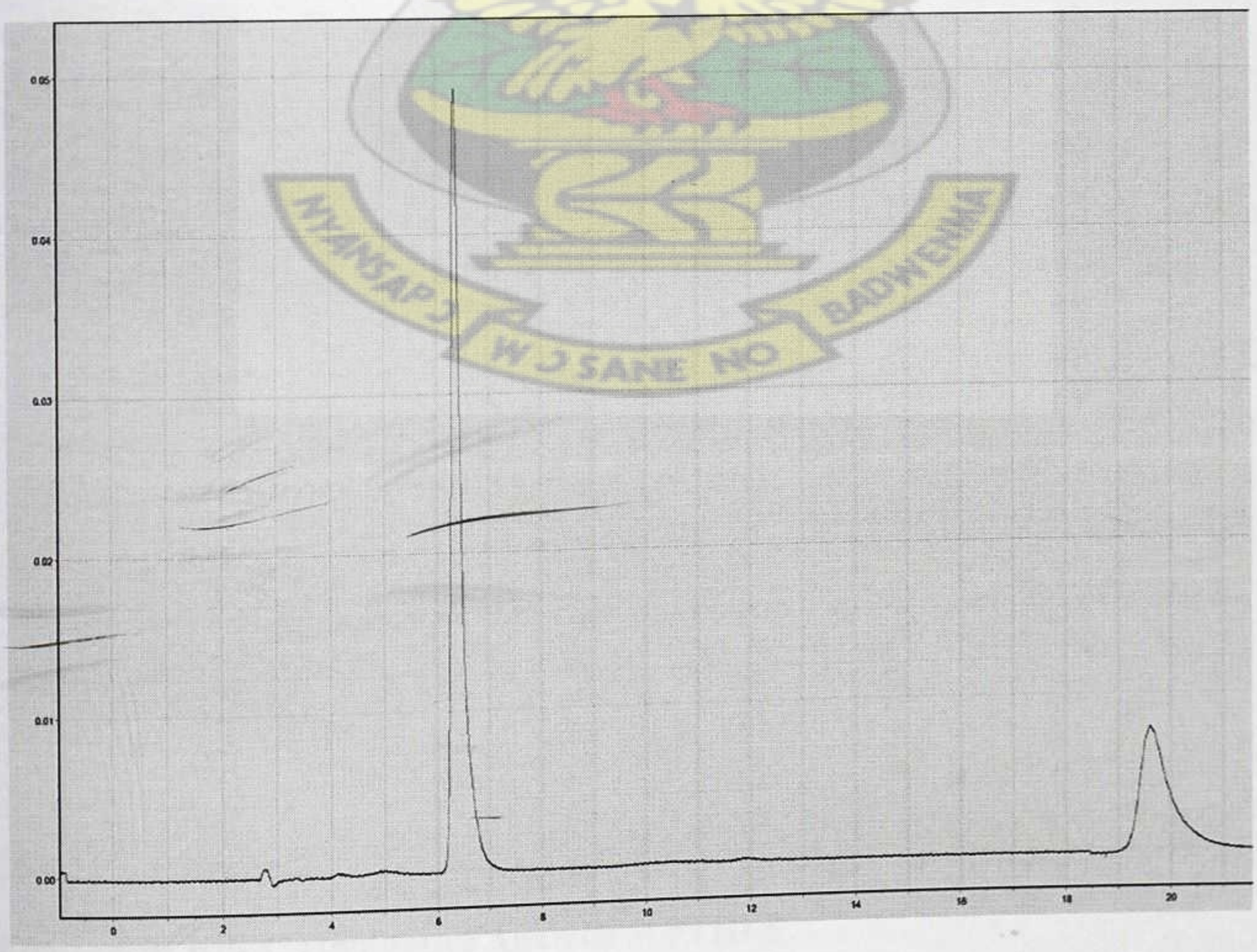


Figure 4.5: HPLC Qualitative Analysis of PTFLS

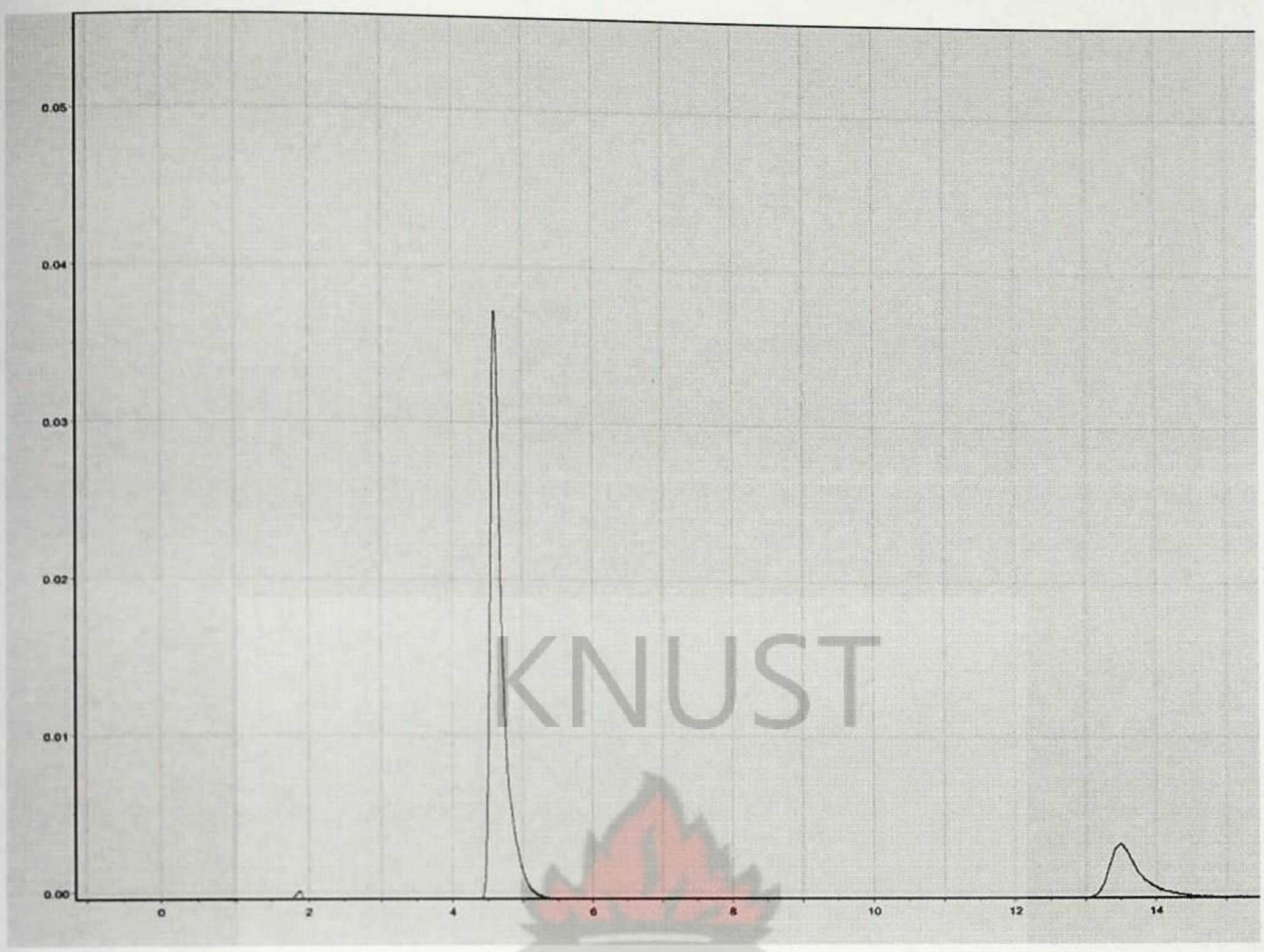


Figure 4.6: HPLC Qualitative Analysis of PTGFS

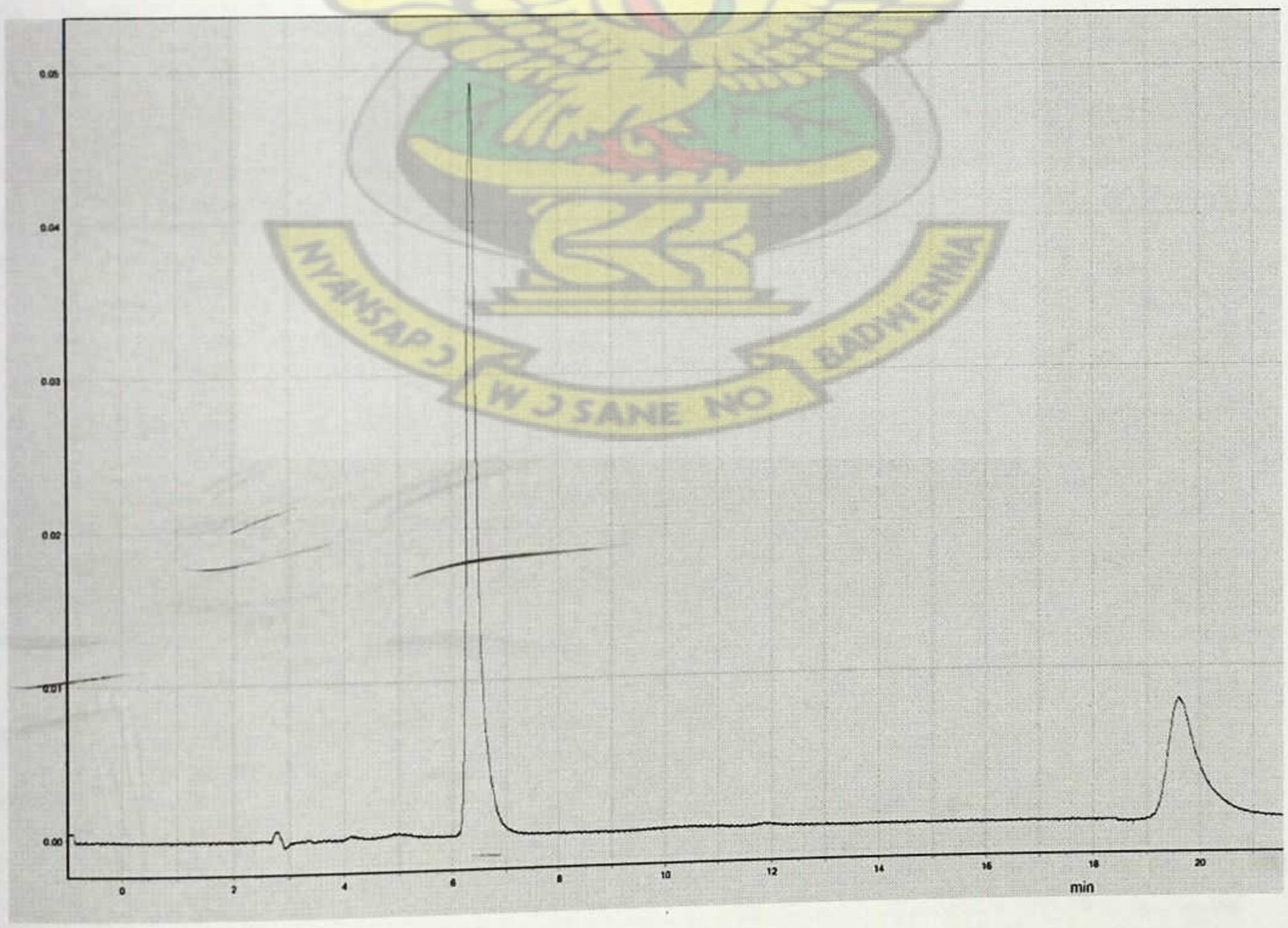


Figure 4.7: HPLC Qualitative Analysis of PTHLS

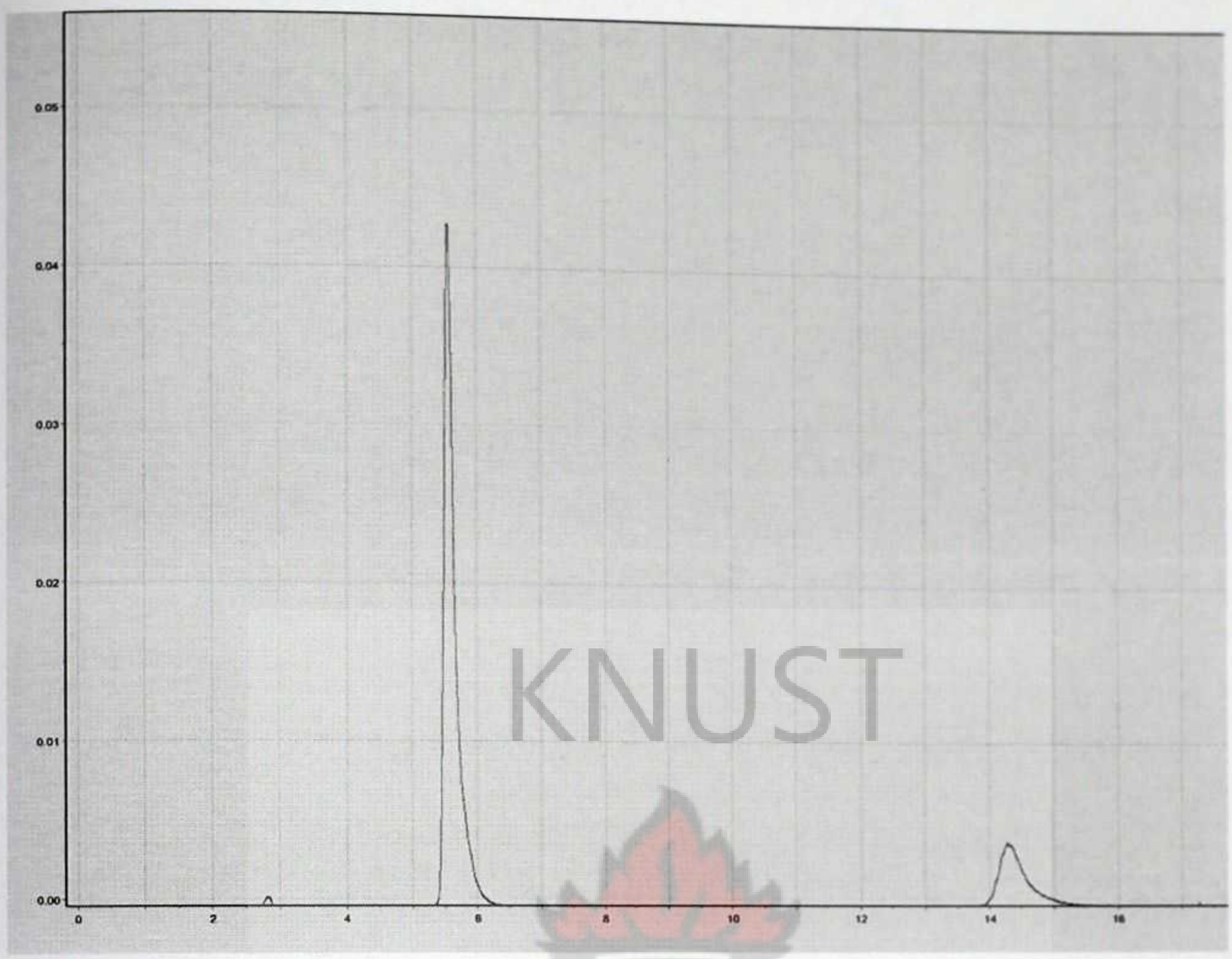


Figure 4.8: HPLC Qualitative Analysis of PTILS

Figure 4.9: HPLC Qualitative Analysis of PTJLS

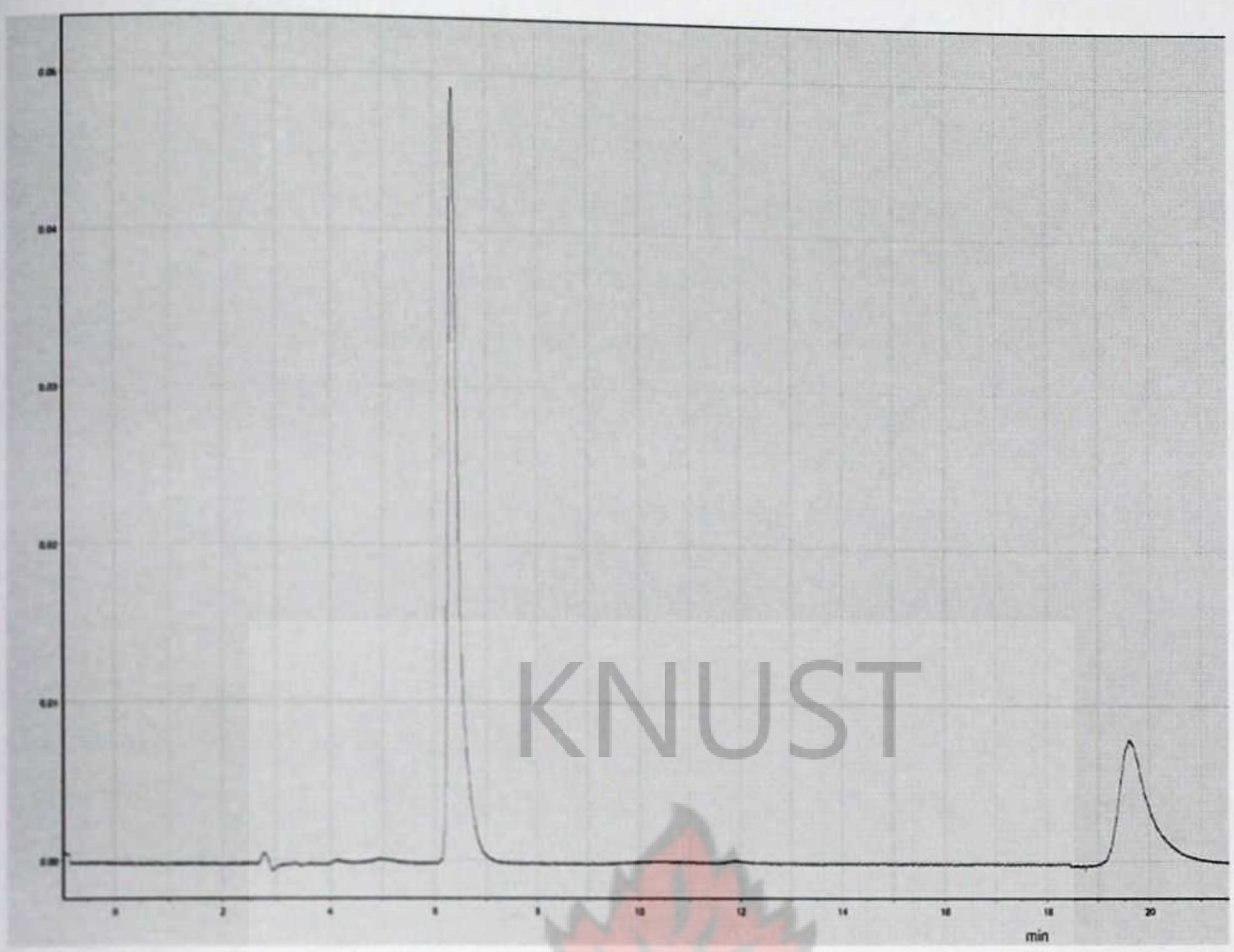


Figure 4.10: HPLC Qualitative Analysis of PTKLS

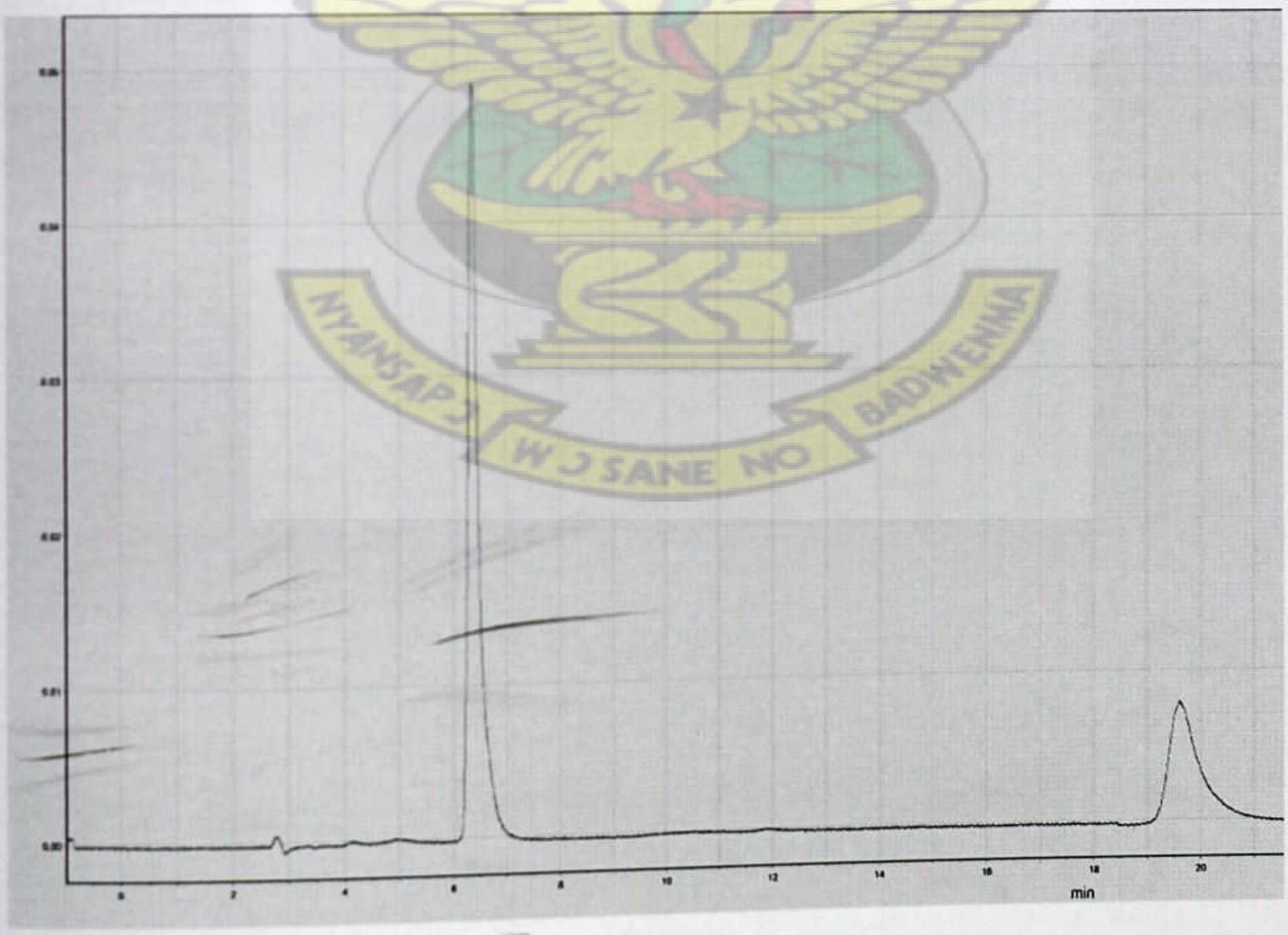


Figure 4.11: HPLC Qualitative Analysis of PTLLS

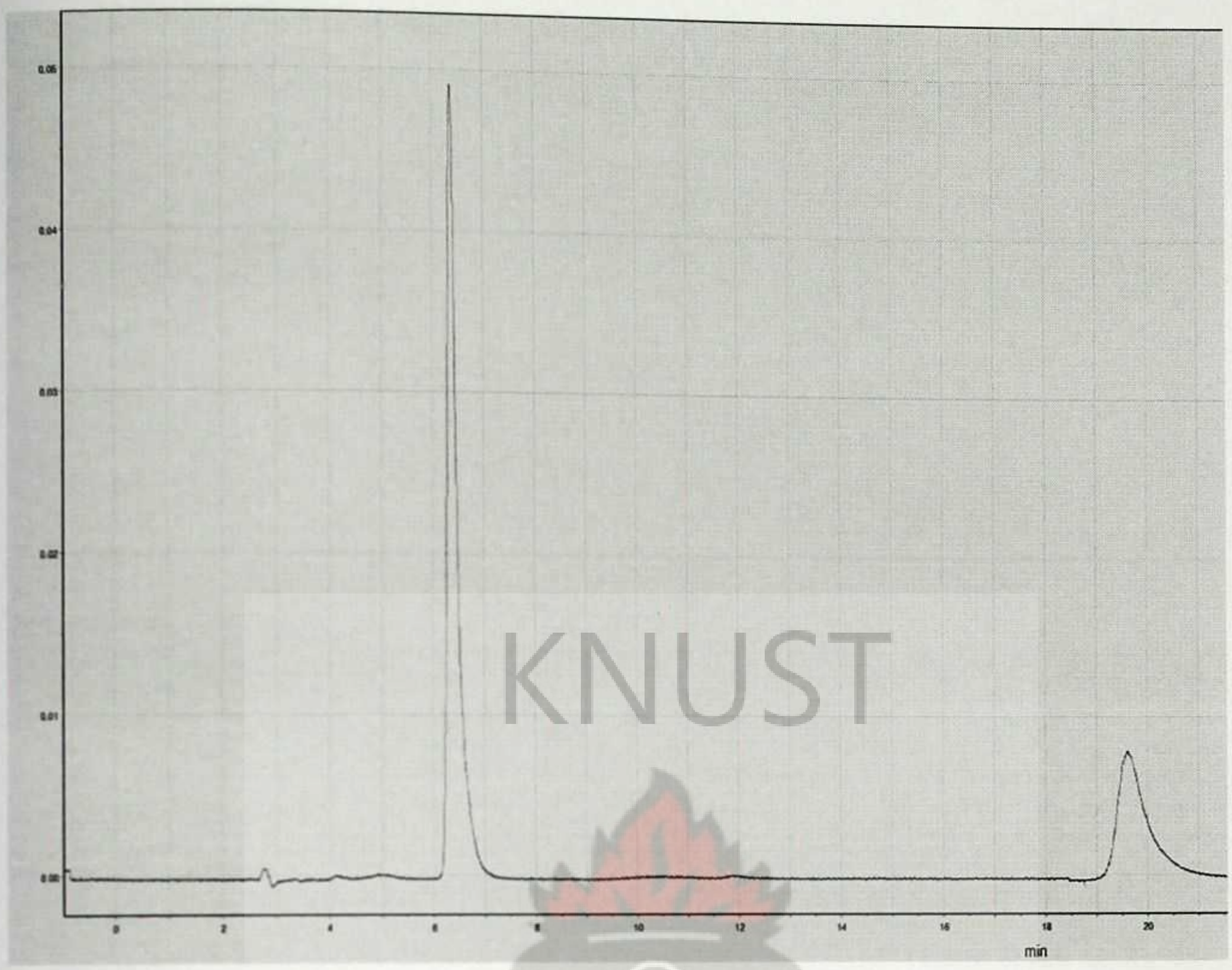


Figure 4.12: HPLC Qualitative Analysis of PTMLS

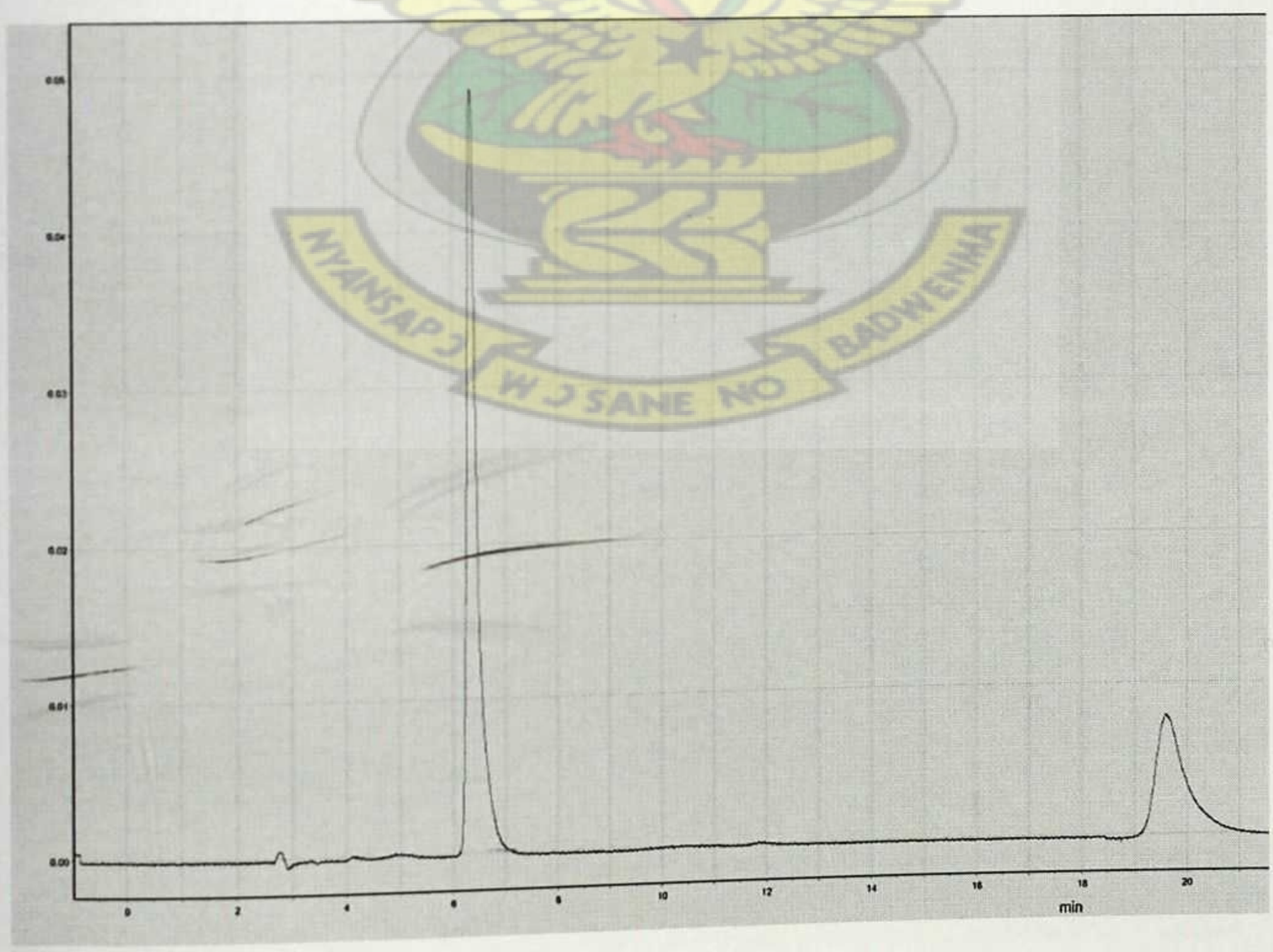


Figure 4.13: HPLC Qualitative Analysis of PTNLS

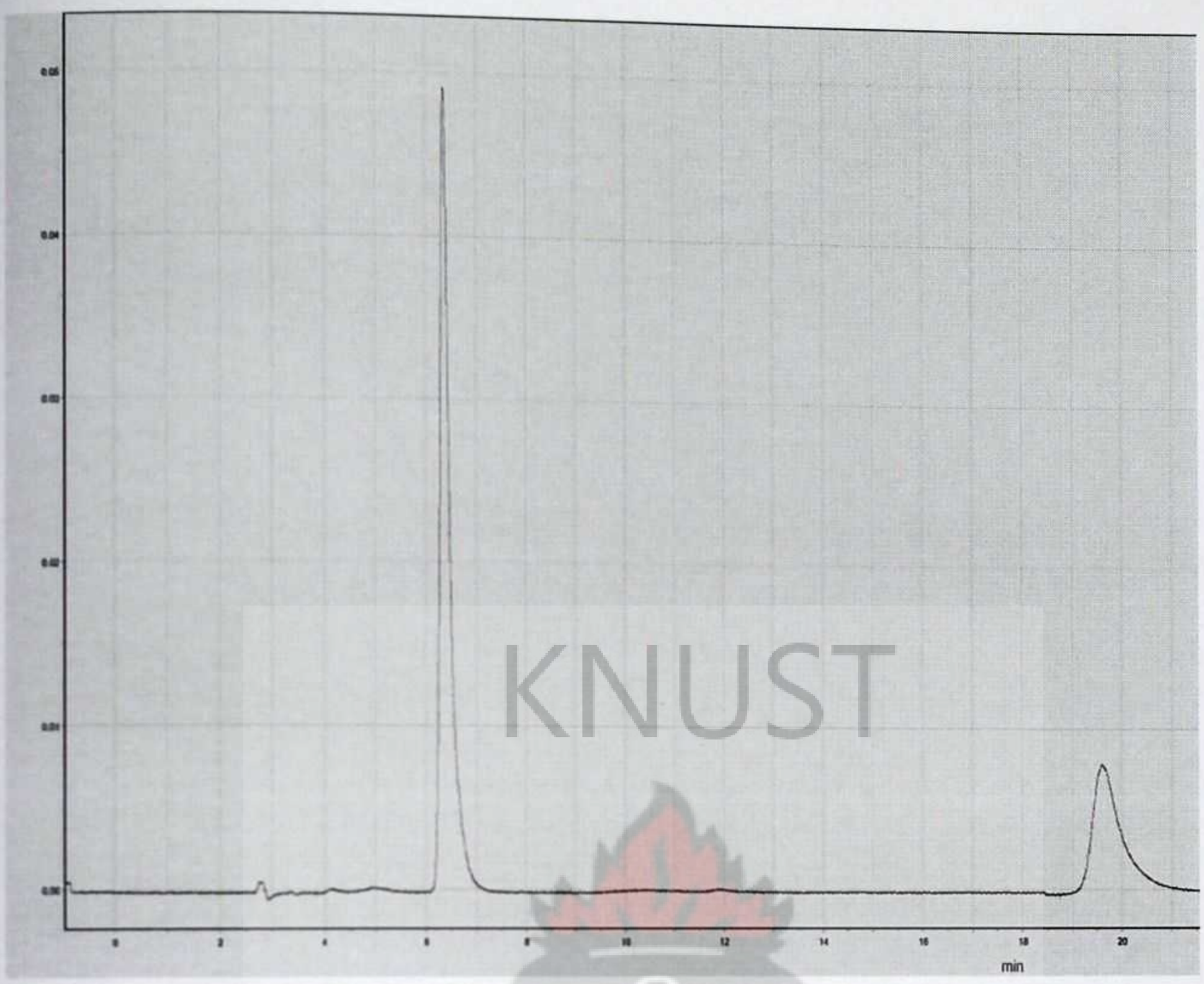


Figure 4.14: HPLC Qualitative Analysis of PTOLS

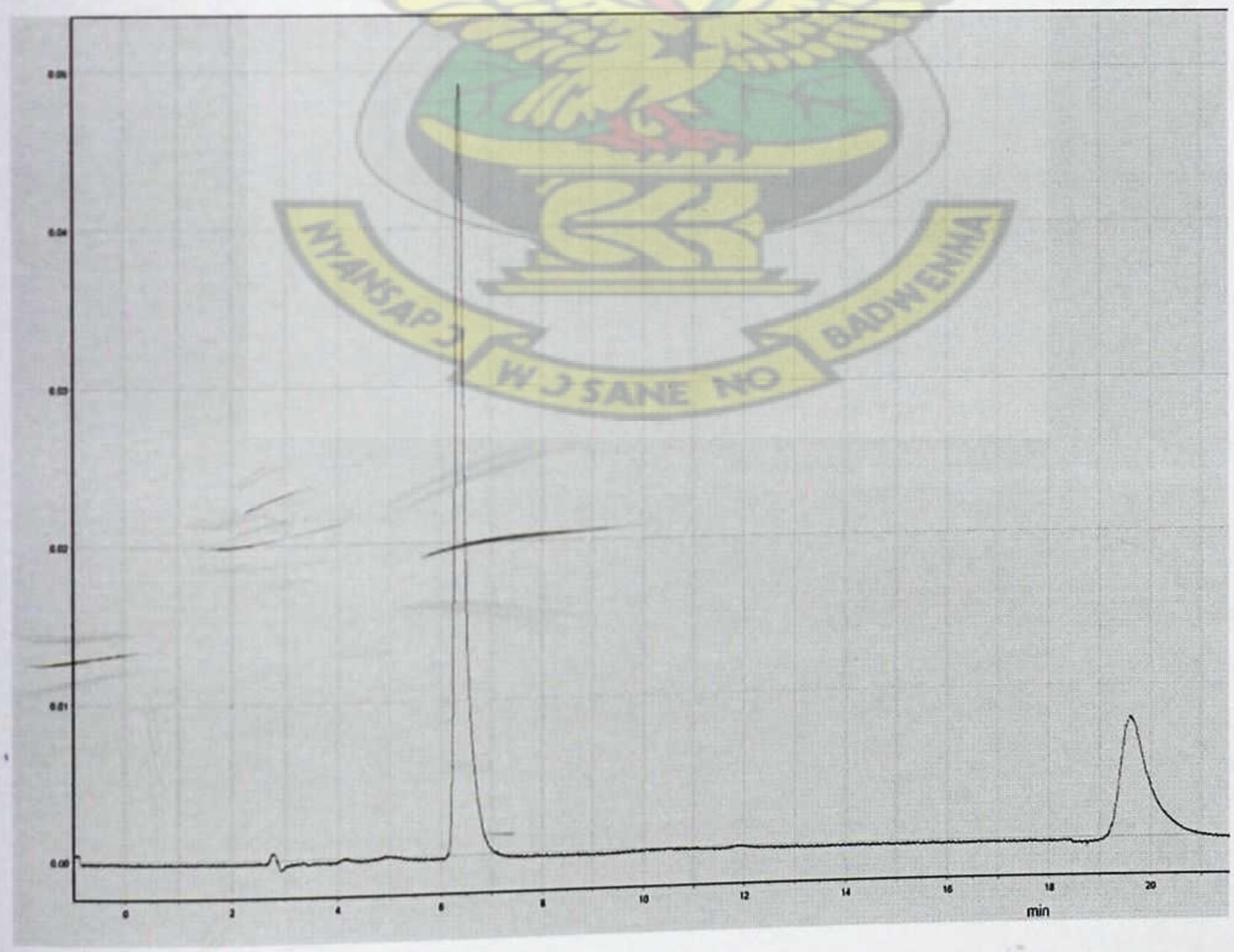


Figure 4.15: HPLC Qualitative Analysis of PTPL

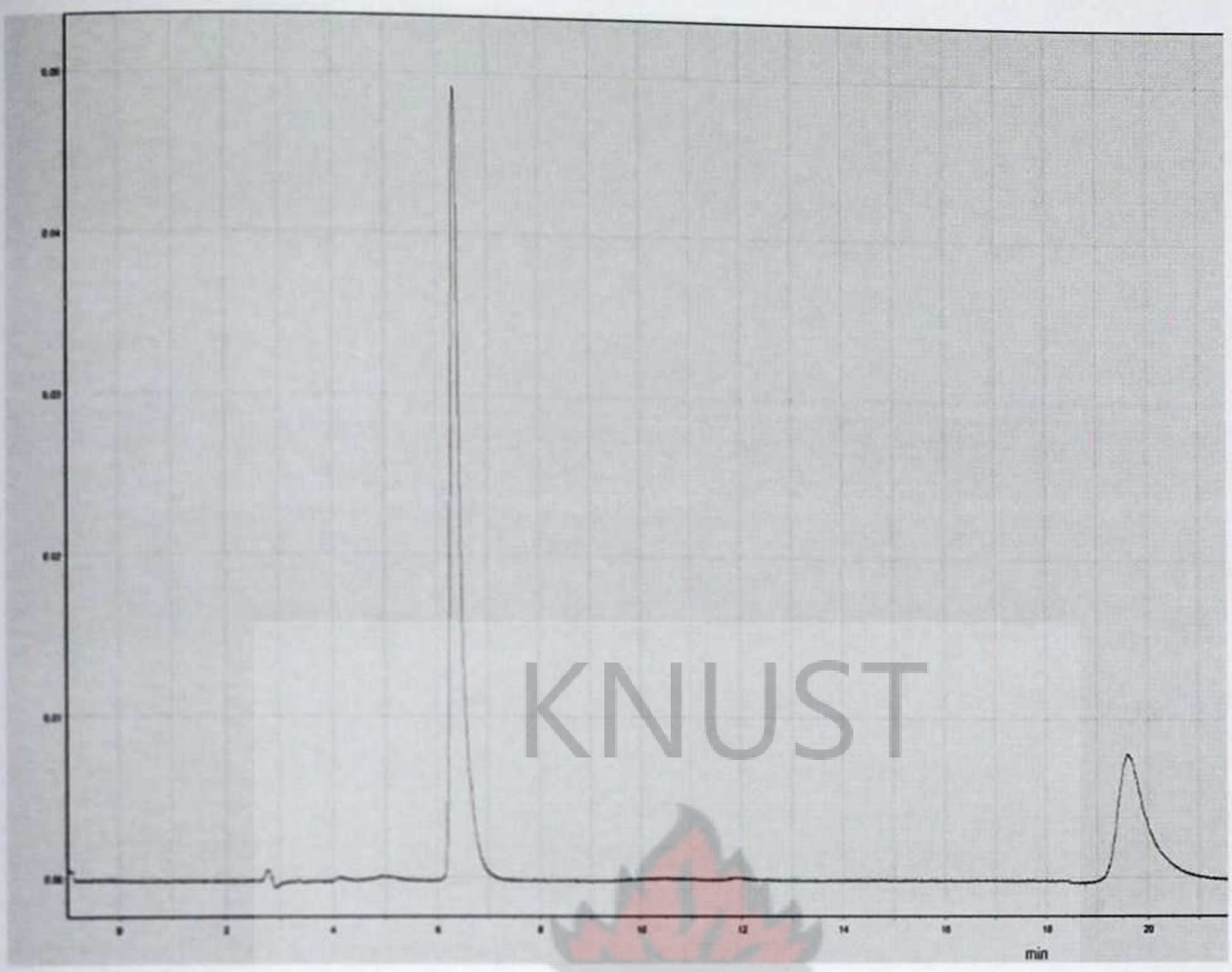


Figure 4.16: HPLC Qualitative Analysis of PSELS

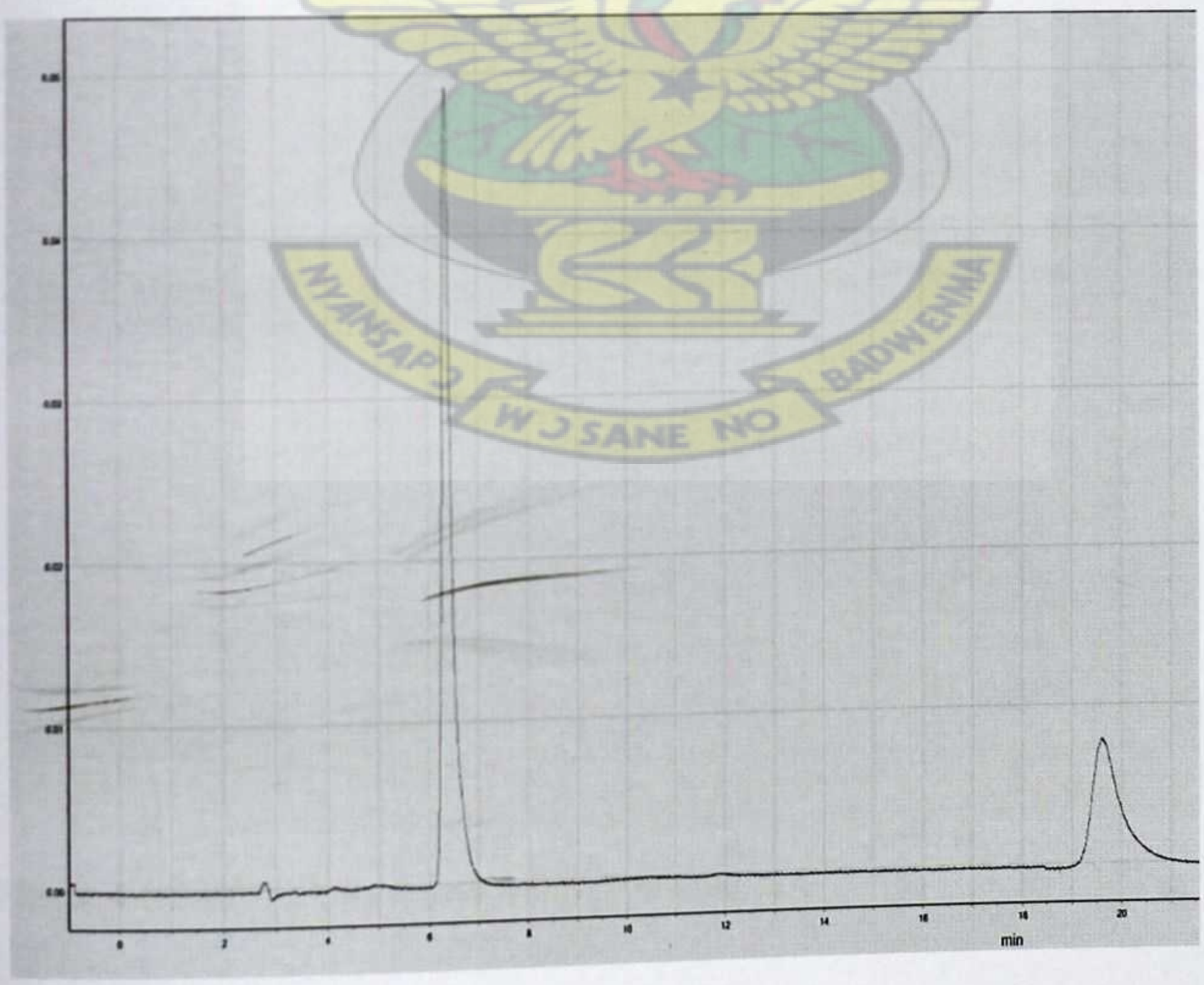


Figure 4.17: HPLC Qualitative Analysis of PSFLS

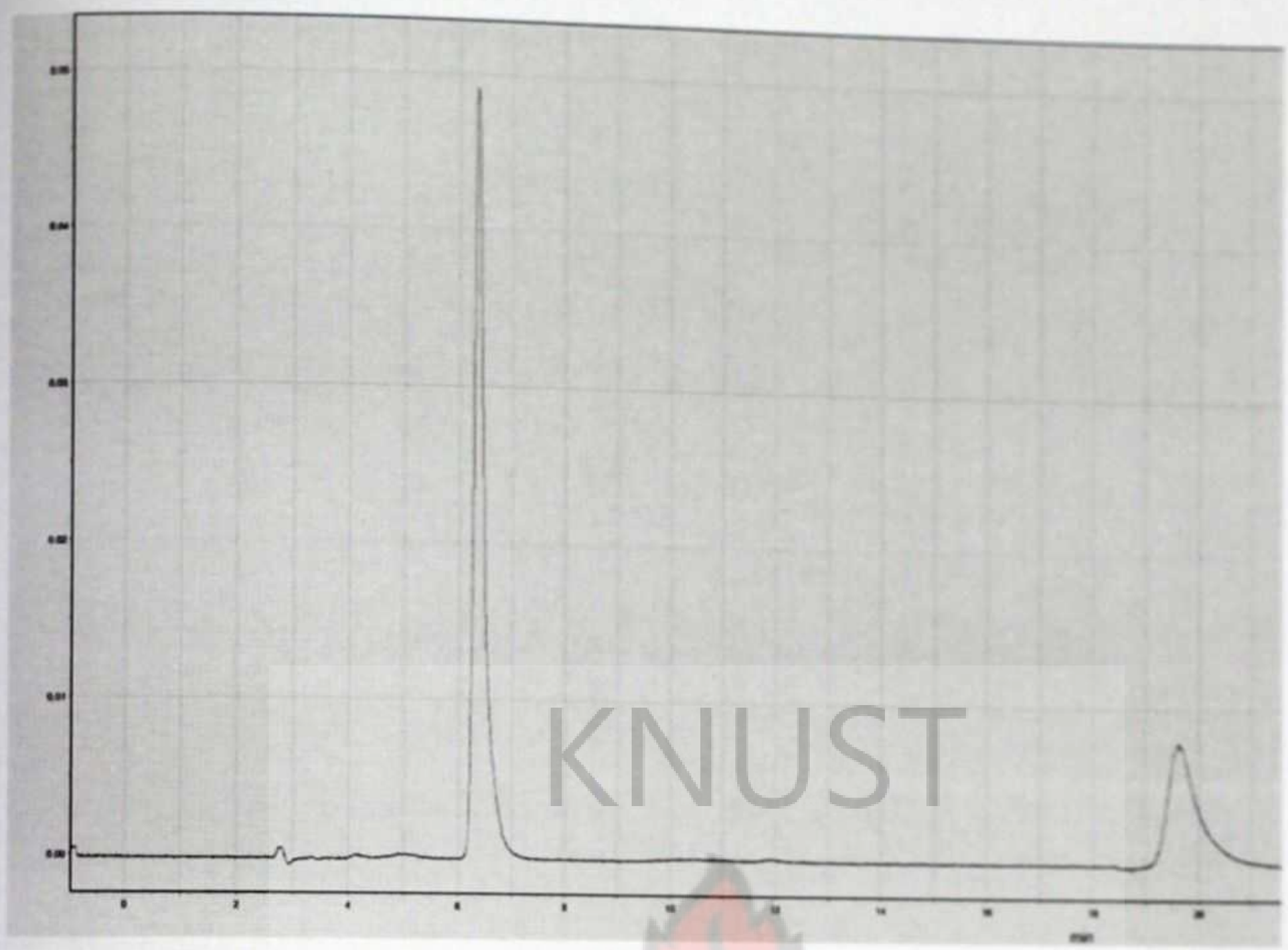


Figure 4.18: HPLC Qualitative Analysis of PSGLS

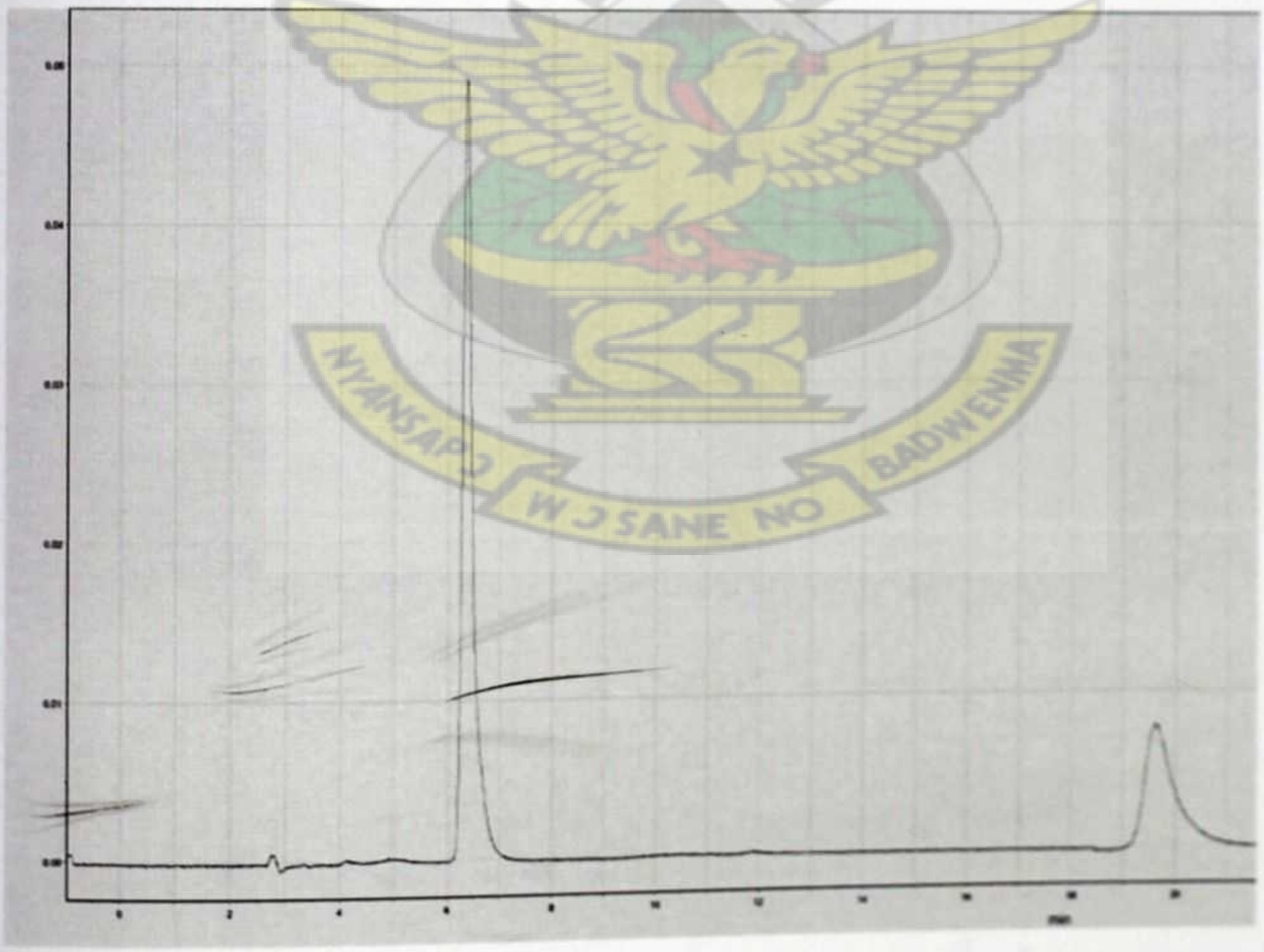


Figure 4.19: HPLC Qualitative Analysis of PSHLS

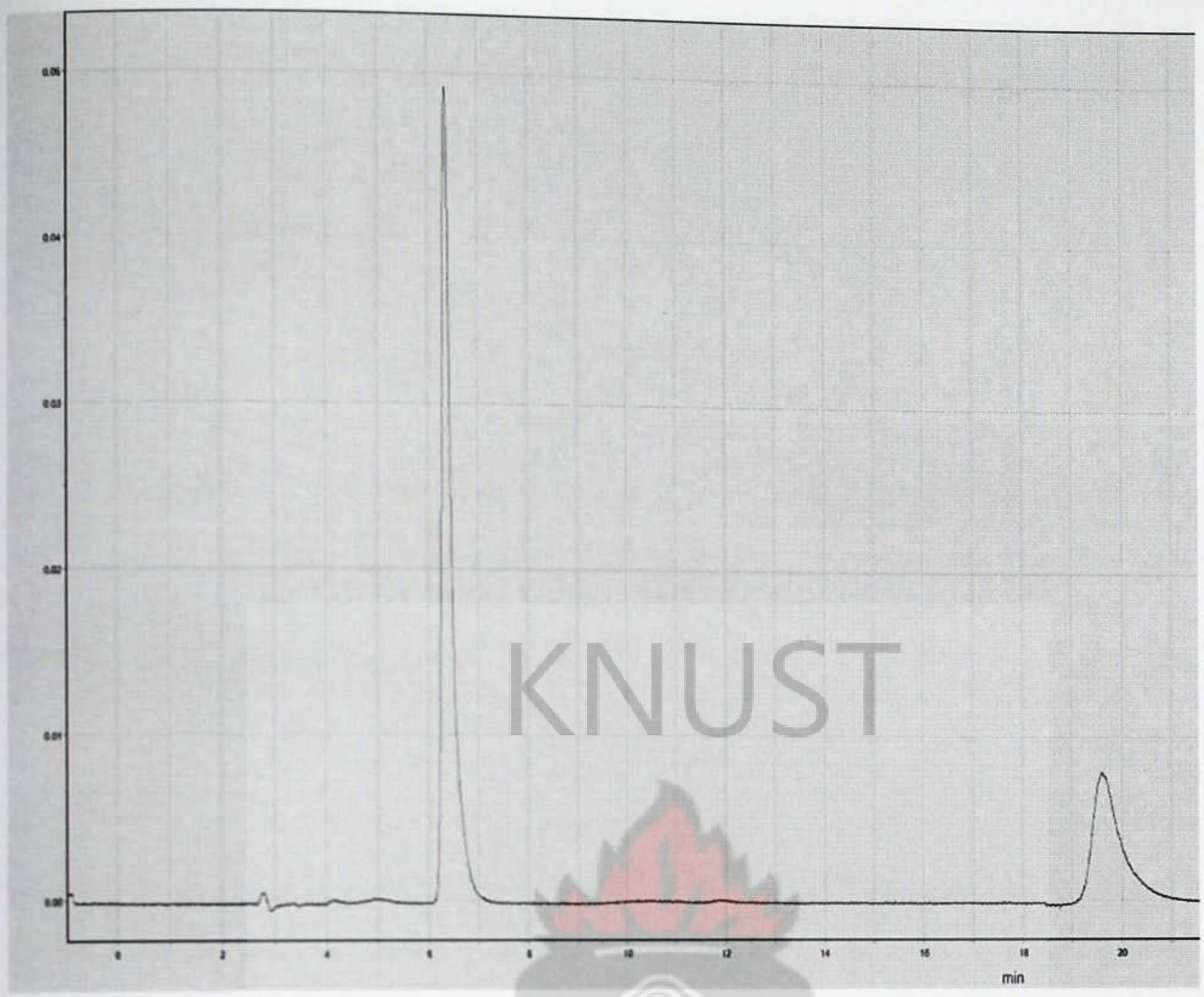


Figure 4.20: HPLC Qualitative Analysis of PSILS

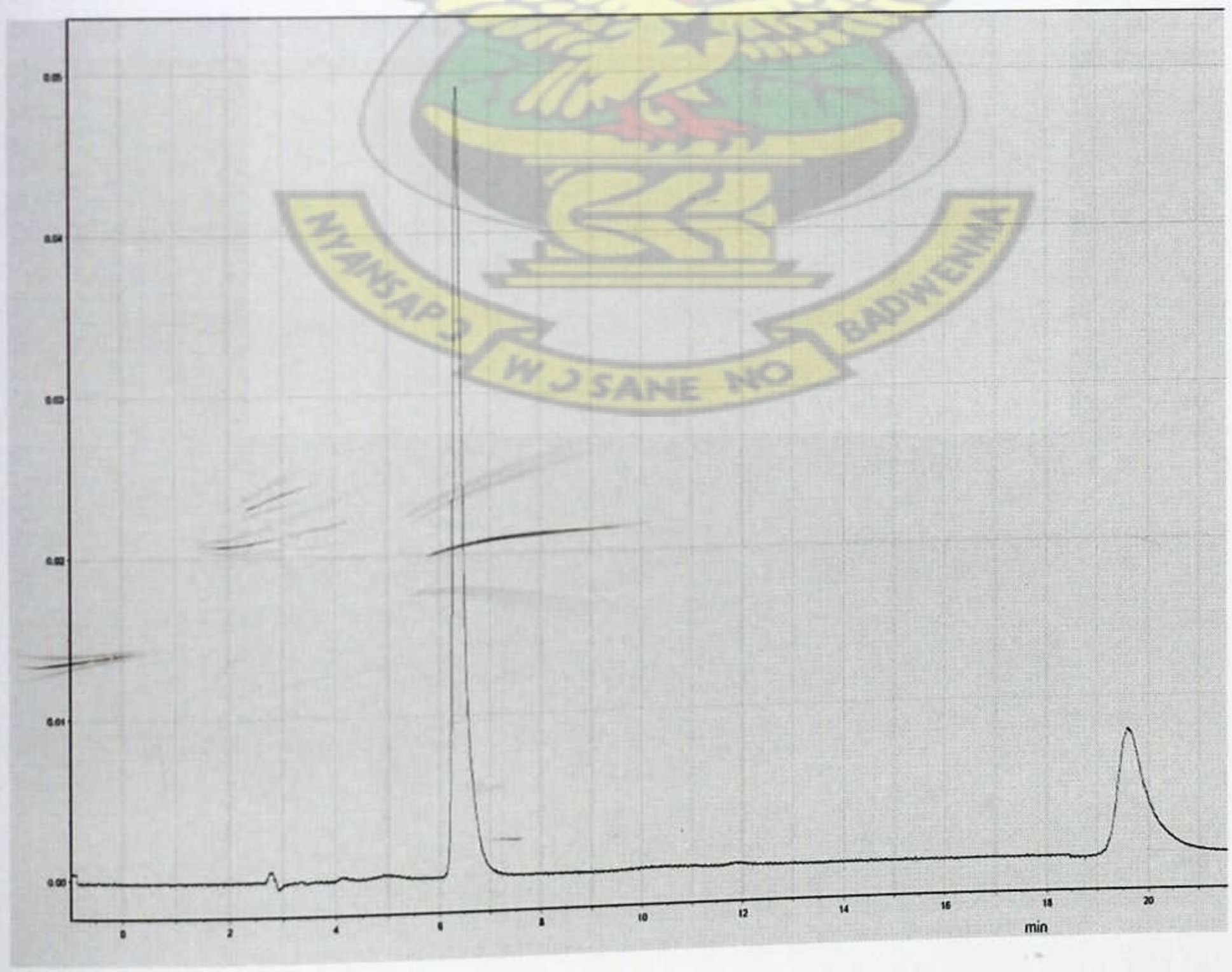


Figure 4.21: HPLC Qualitative Analysis of PSJLS

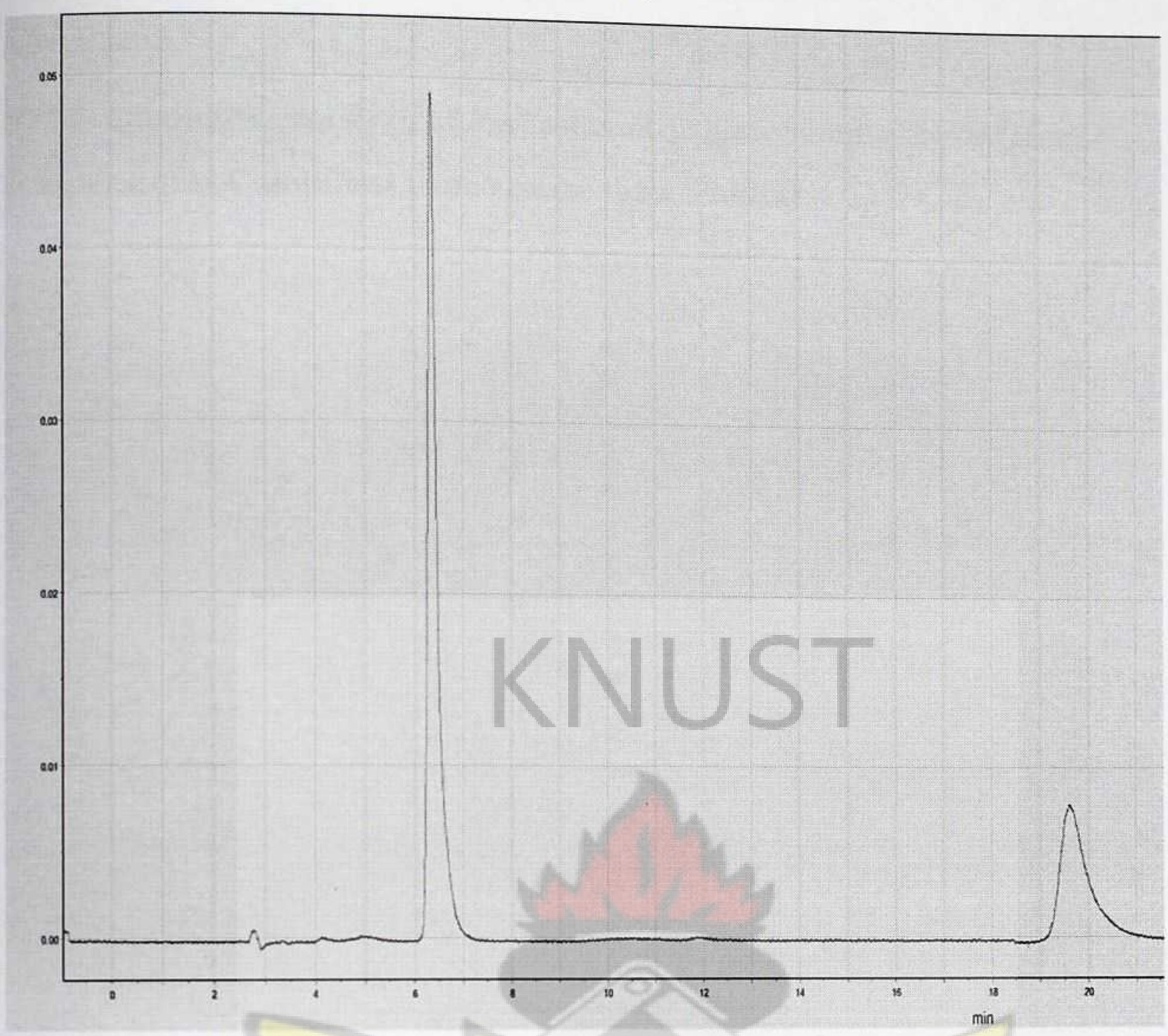


Figure 4.22: HPLC Qualitative Analysis of PSKLS

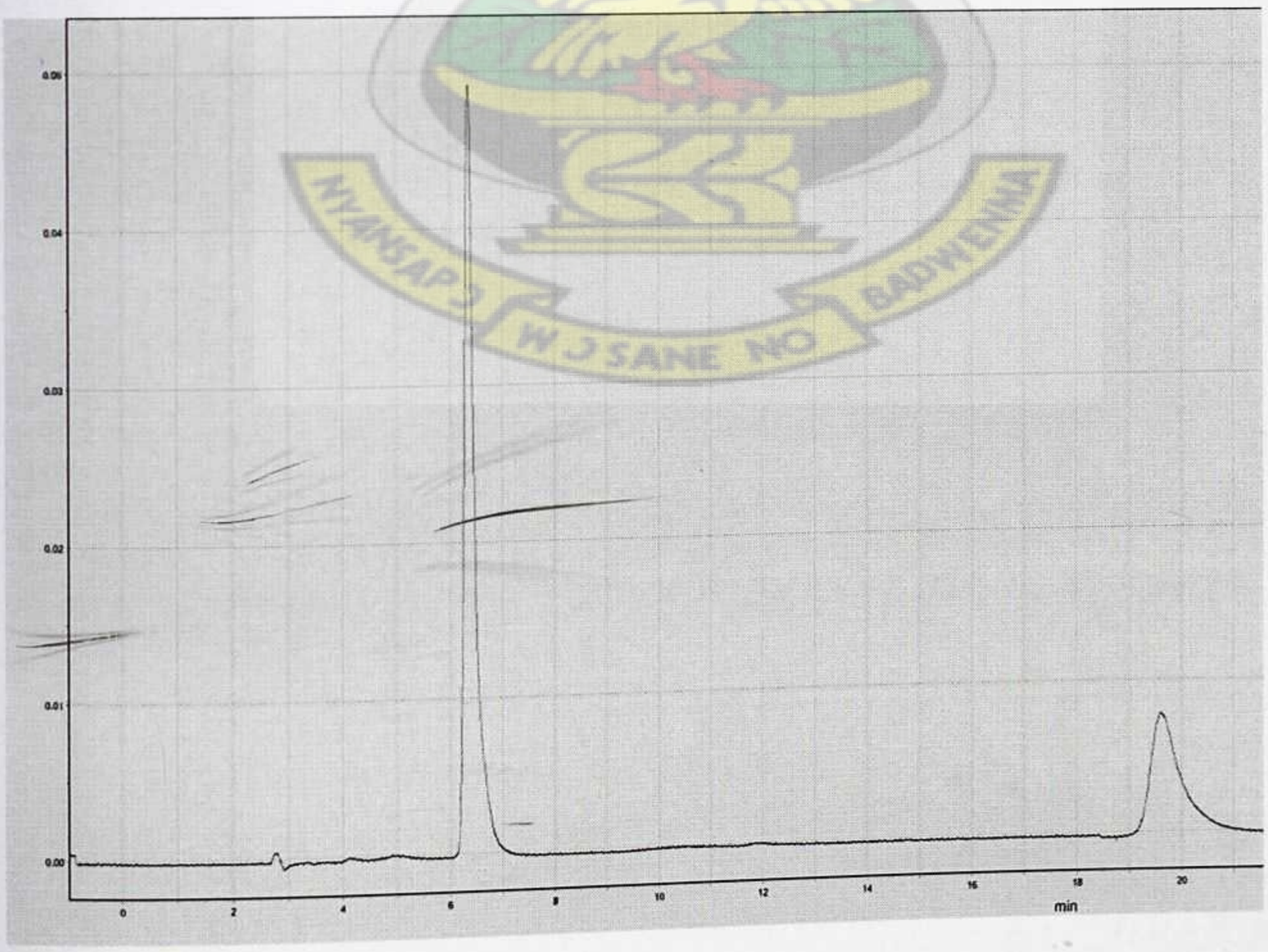


Figure 4.23: HPLC Qualitative Analysis of PSLLS

Appendix 8

HPLC Qualitative Analysis of Paracetamol Tablets Samples at Different Temperatures Conditions to determine their Stability

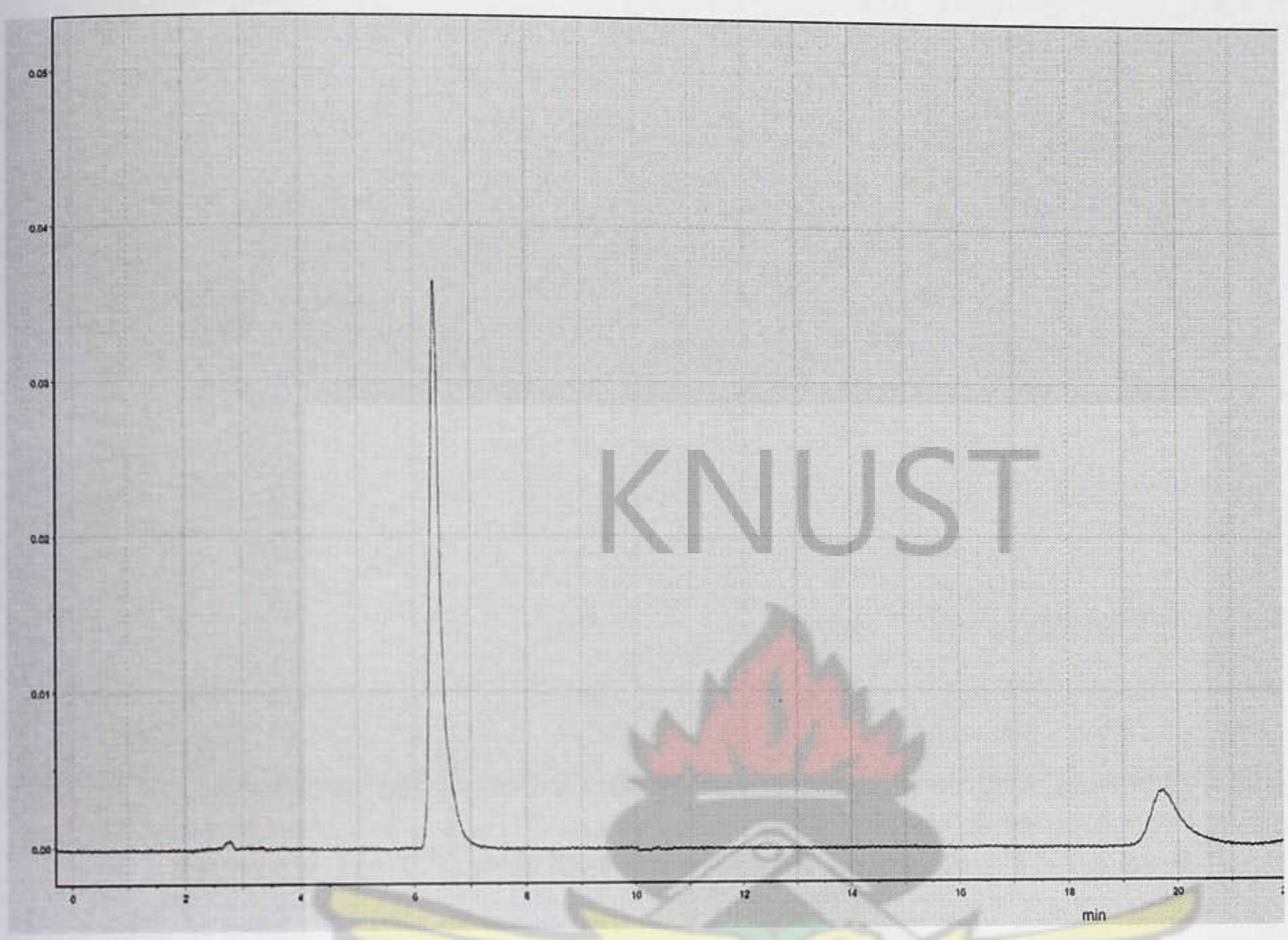


Figure 4.24: Chromatogram of Tablet Sample PTBLS at 28℃

Figure 4.25: Chromatogram of Tablet Sample PTBLS at 35℃

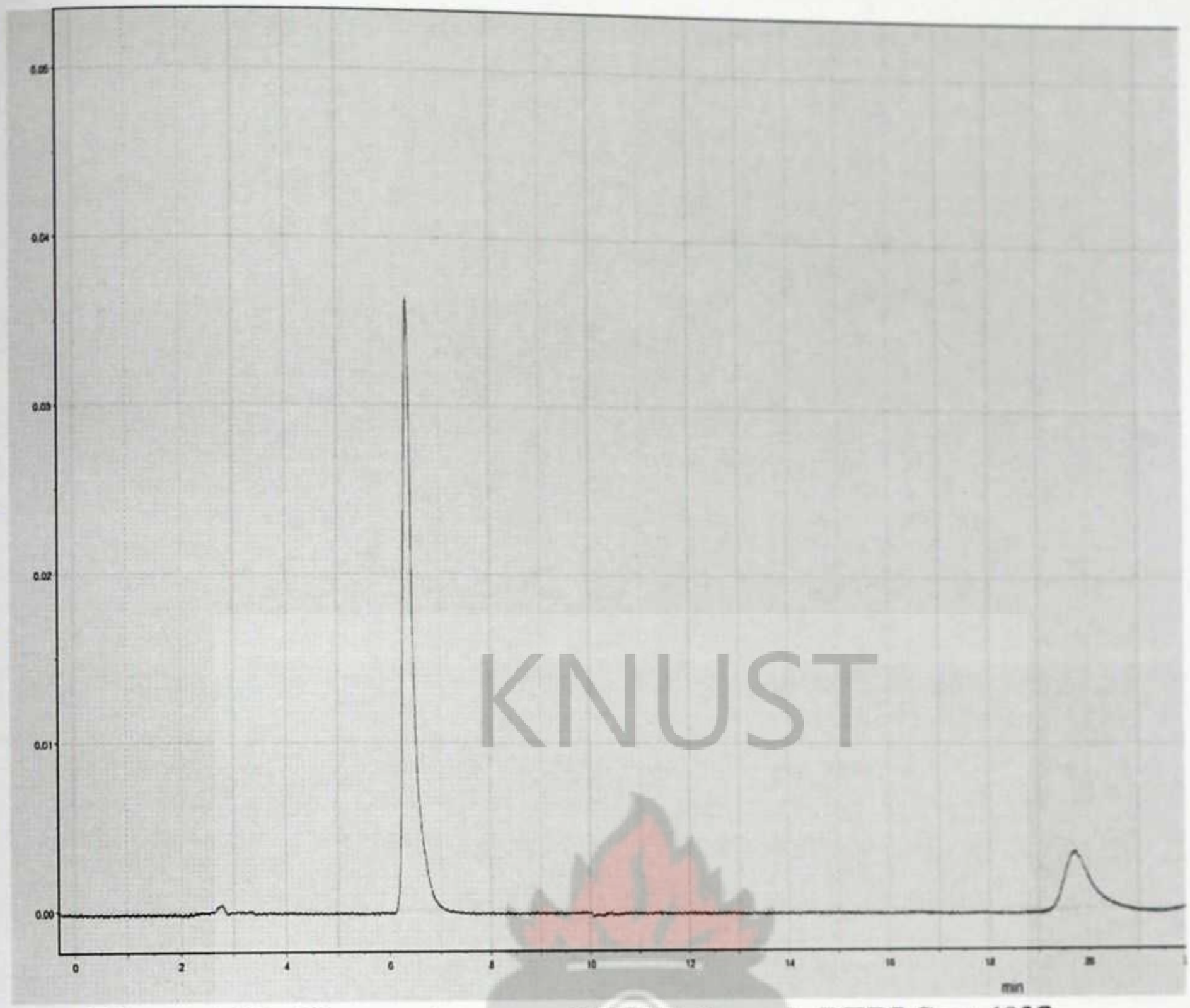


Figure 4.26: Chromatogram of Tablet Sample PTBLS at 40°C

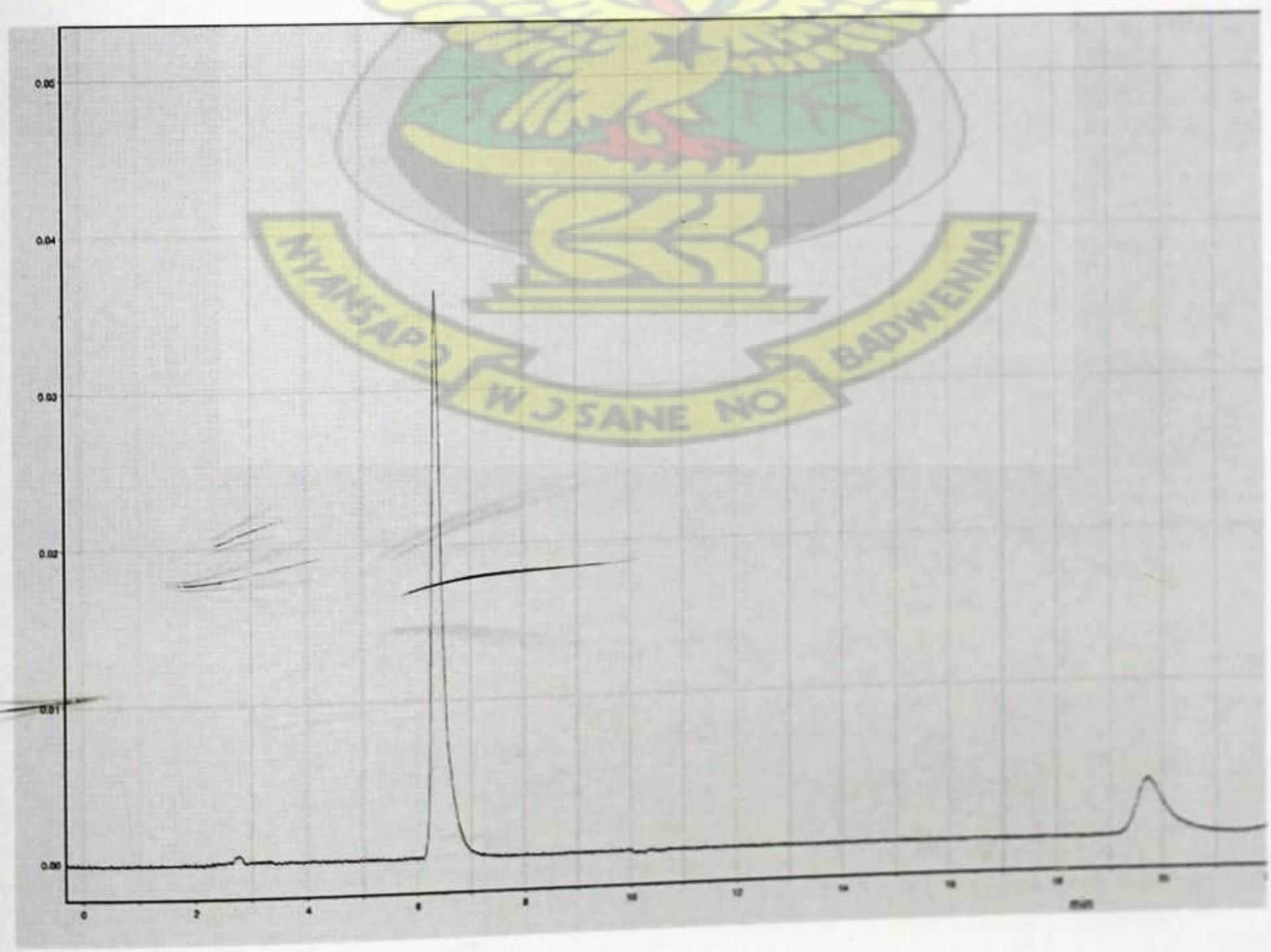


Figure 4.27: Chromatogram of Tablet Sample PTBLS at 50°C

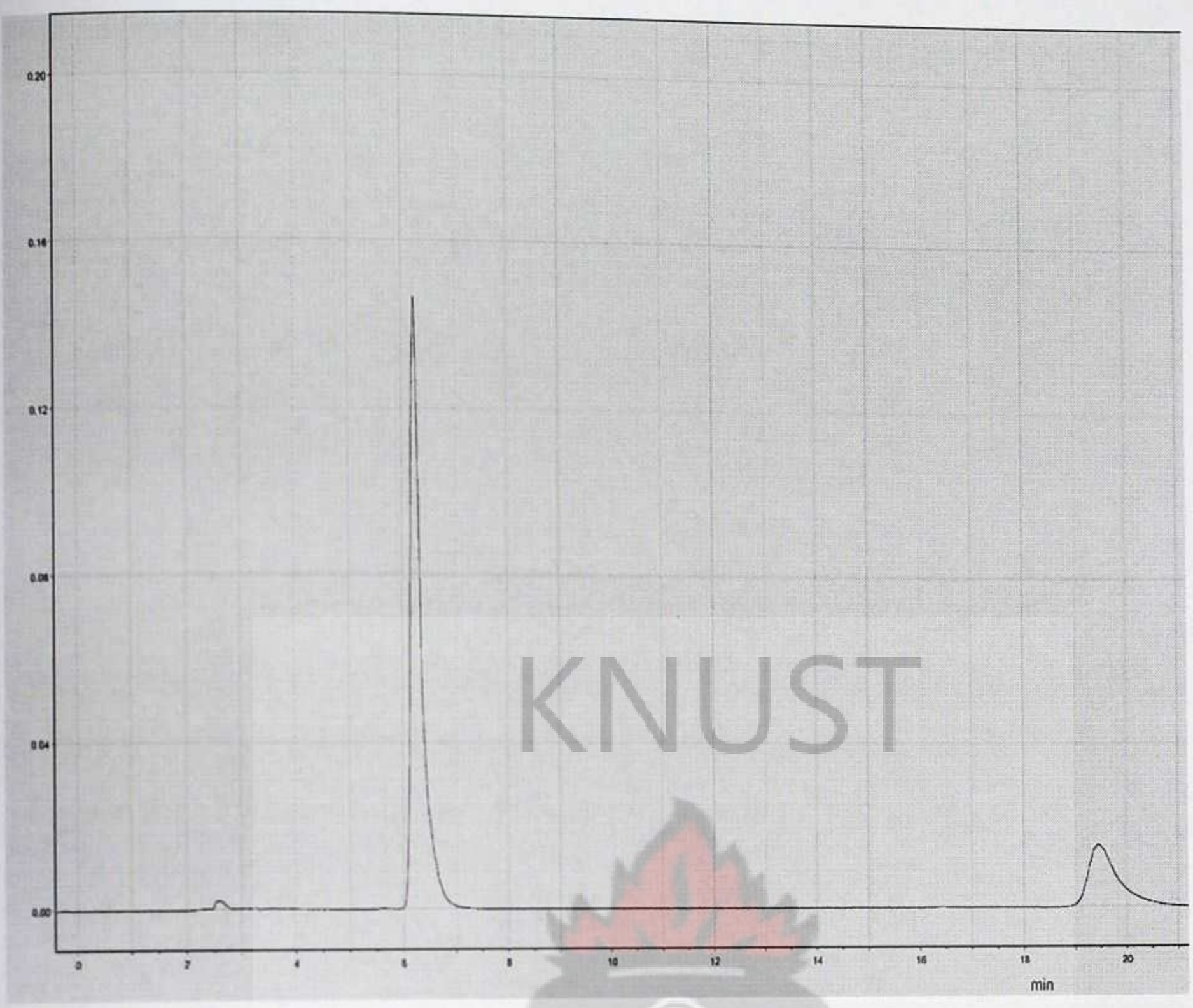


Figure 4.28: Chromatogram of Tablet Sample PTCLS at 28℃

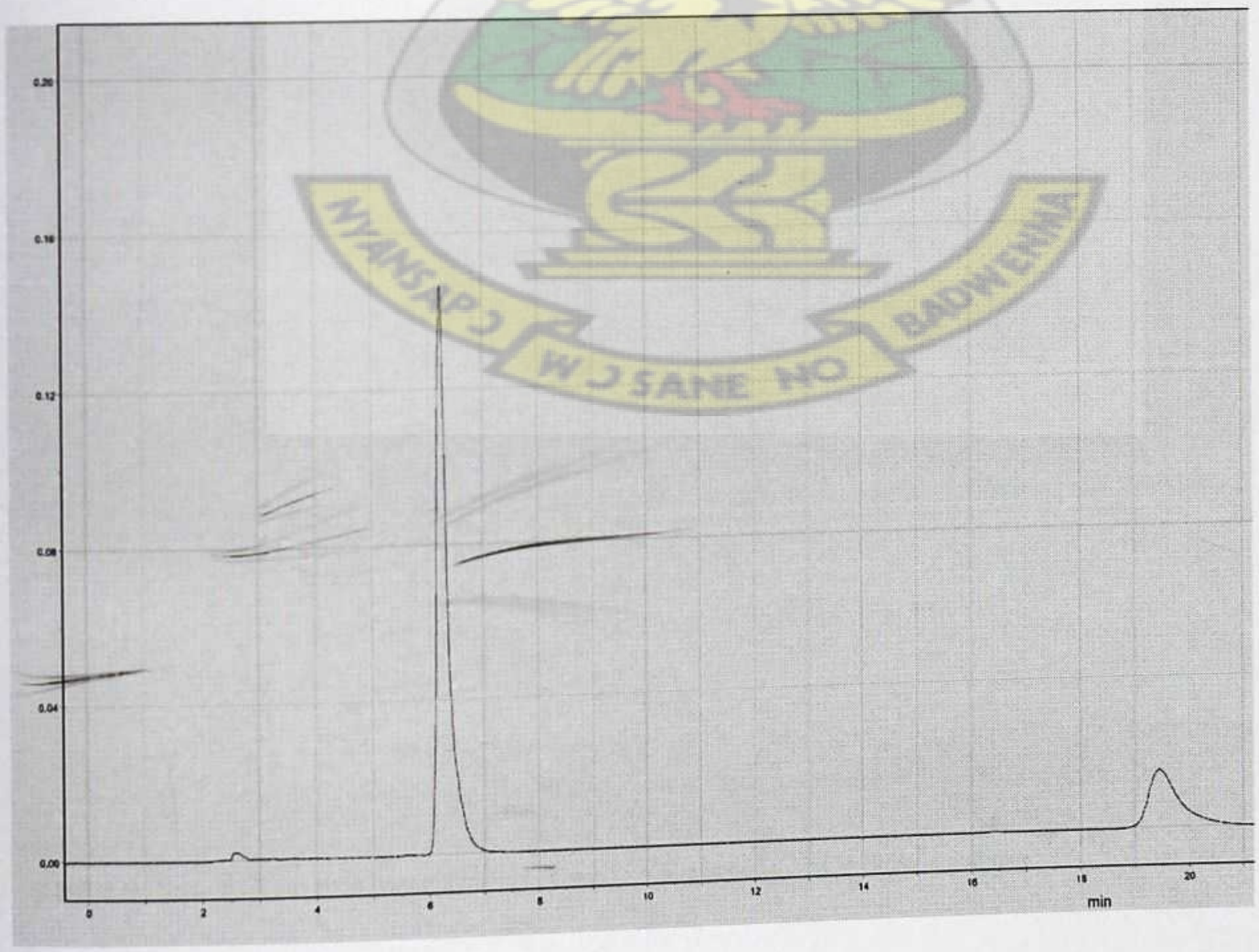


Figure 4.29: Chromatogram of Tablet Sample PTCLS at 35℃

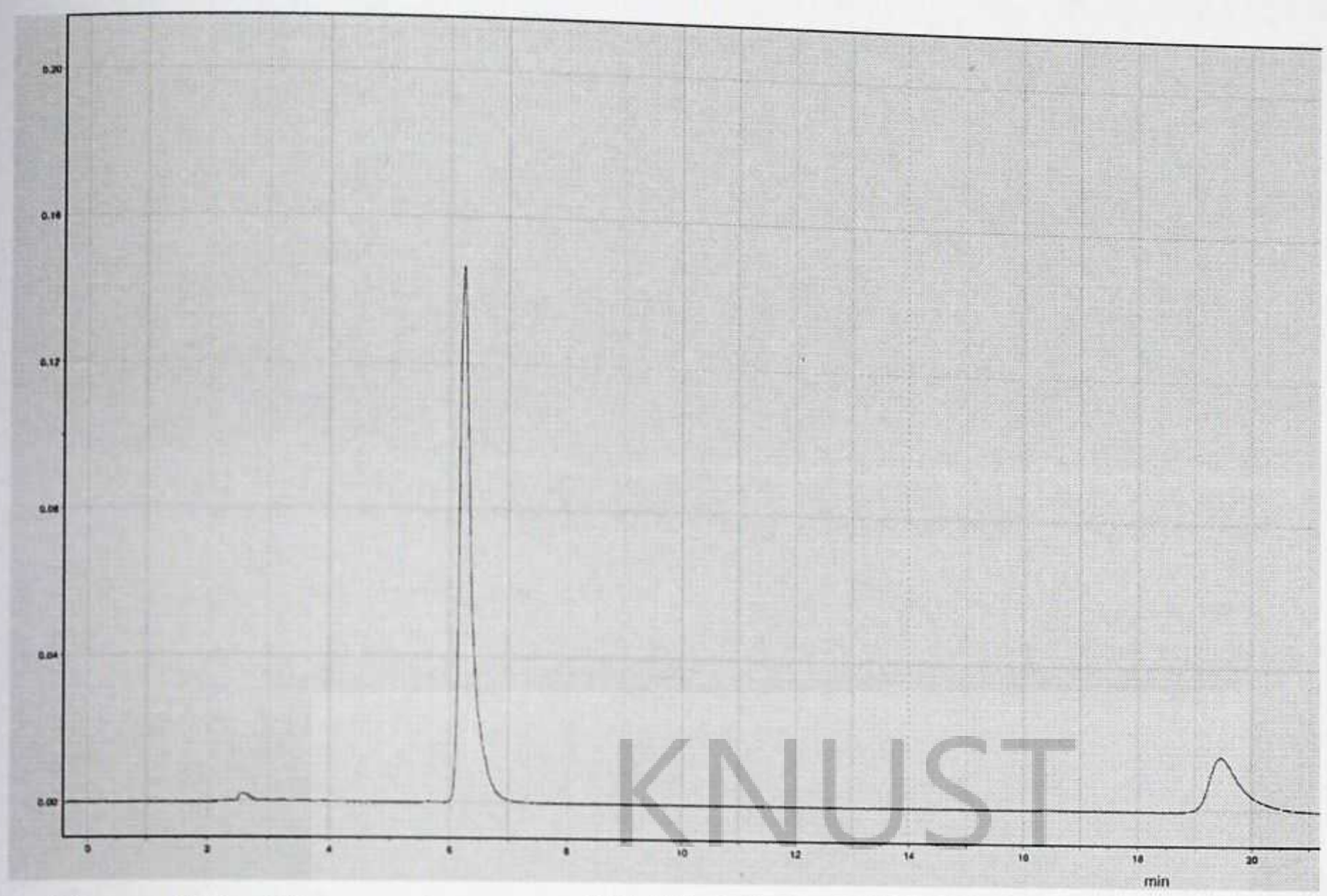


Figure 0.1: Chromatogram of Tablet Sample PTCL S at 40℃

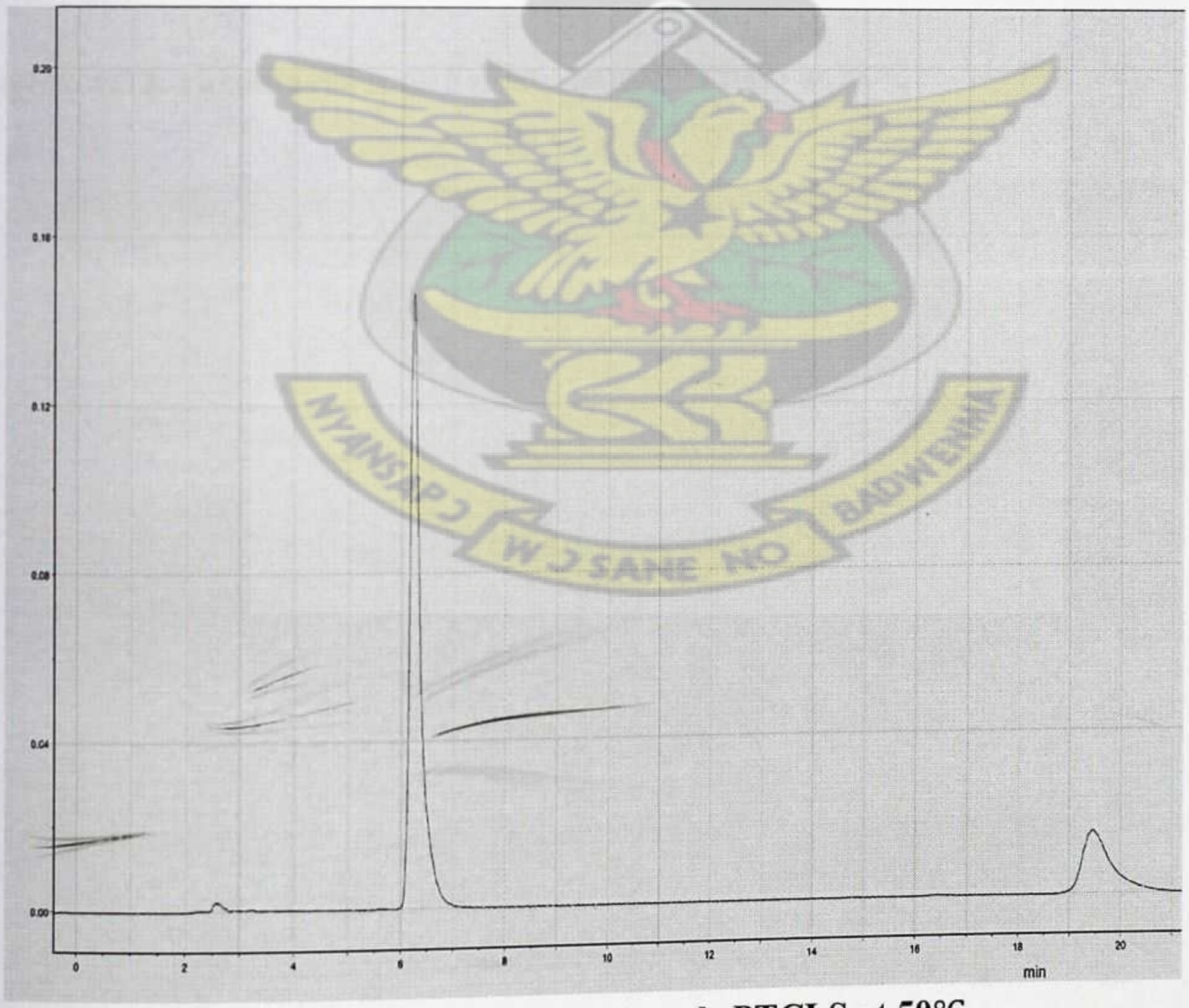


Figure 4.31: Chromatogram of Tablet Sample PTCLS at 50℃

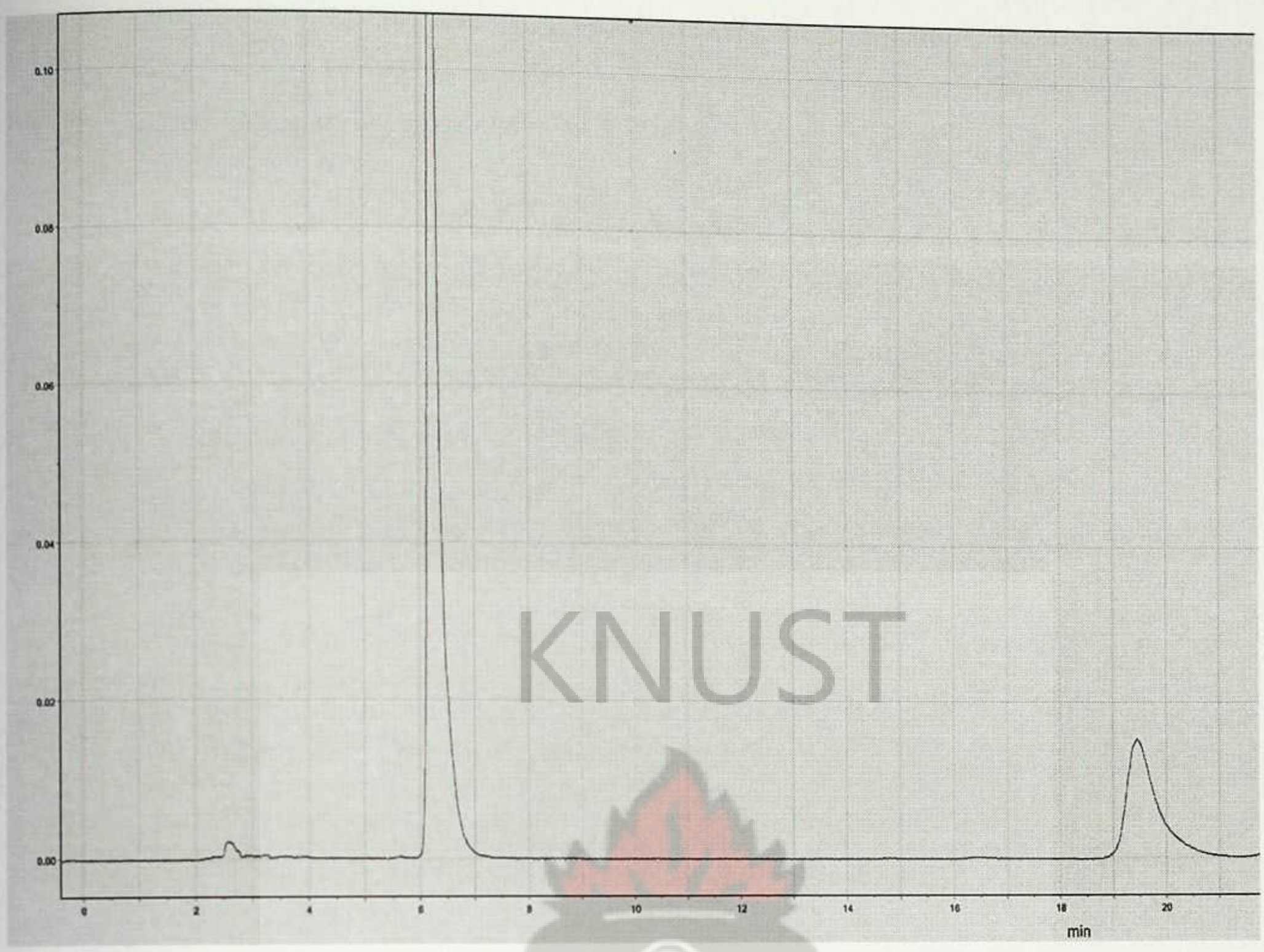


Figure 4.32: chroatogram of Tablet Sample PTDLS at 28℃

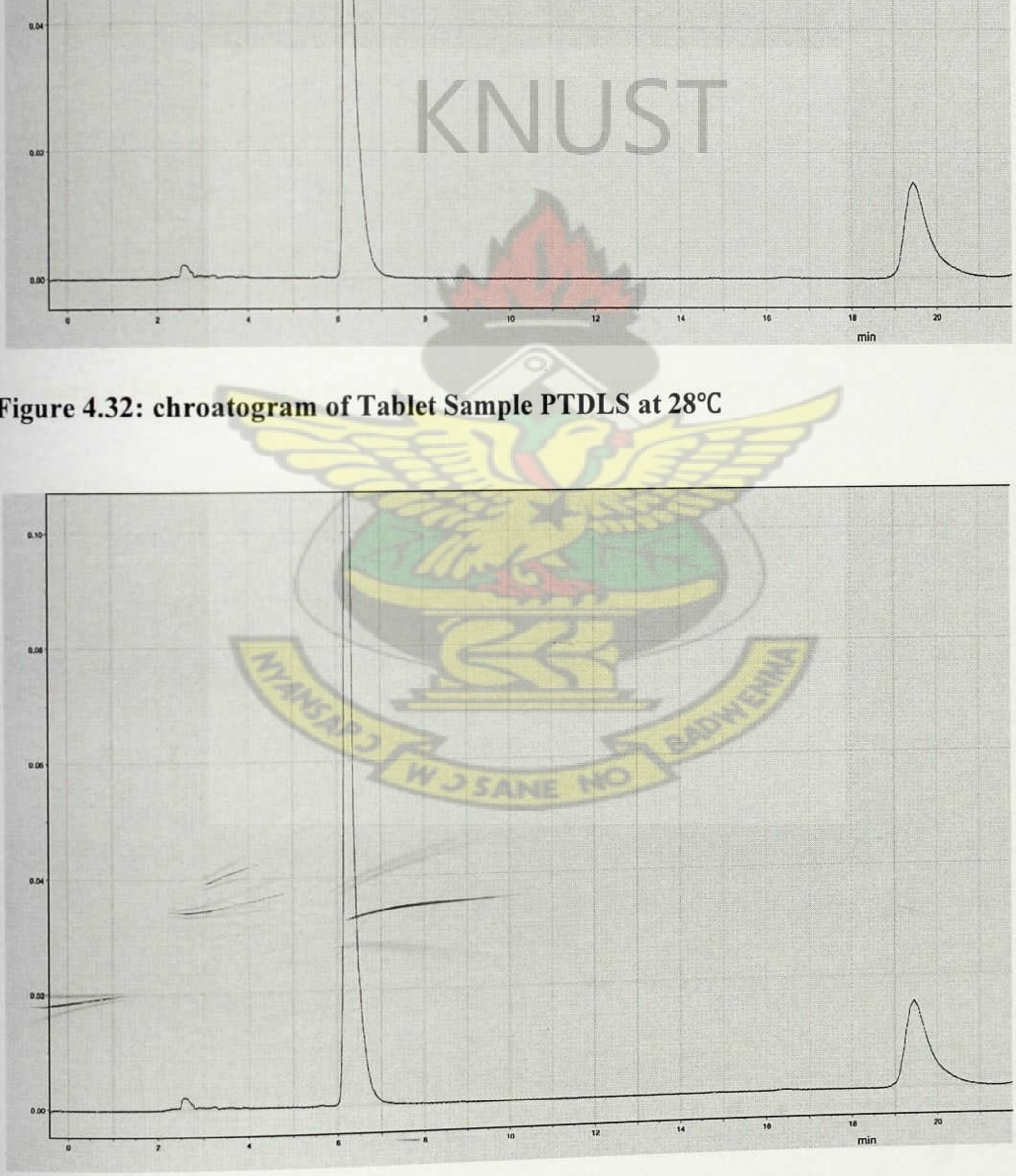


Figure 4.33: Chromatogram of Tablet Sample PTDLS at 35℃

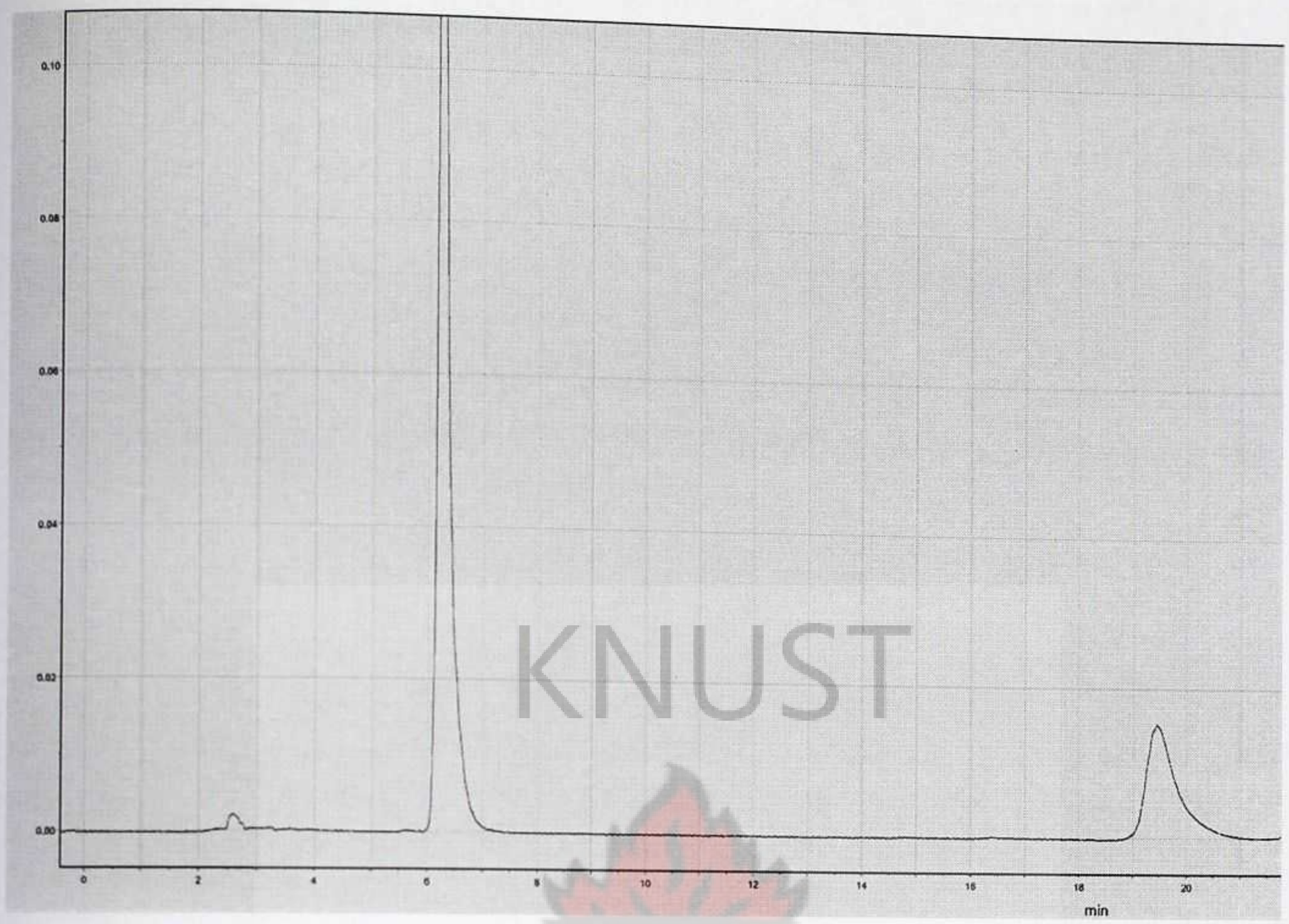


Figure 4.34: Chromatogram of Tablet Sample PTDLS at 40℃

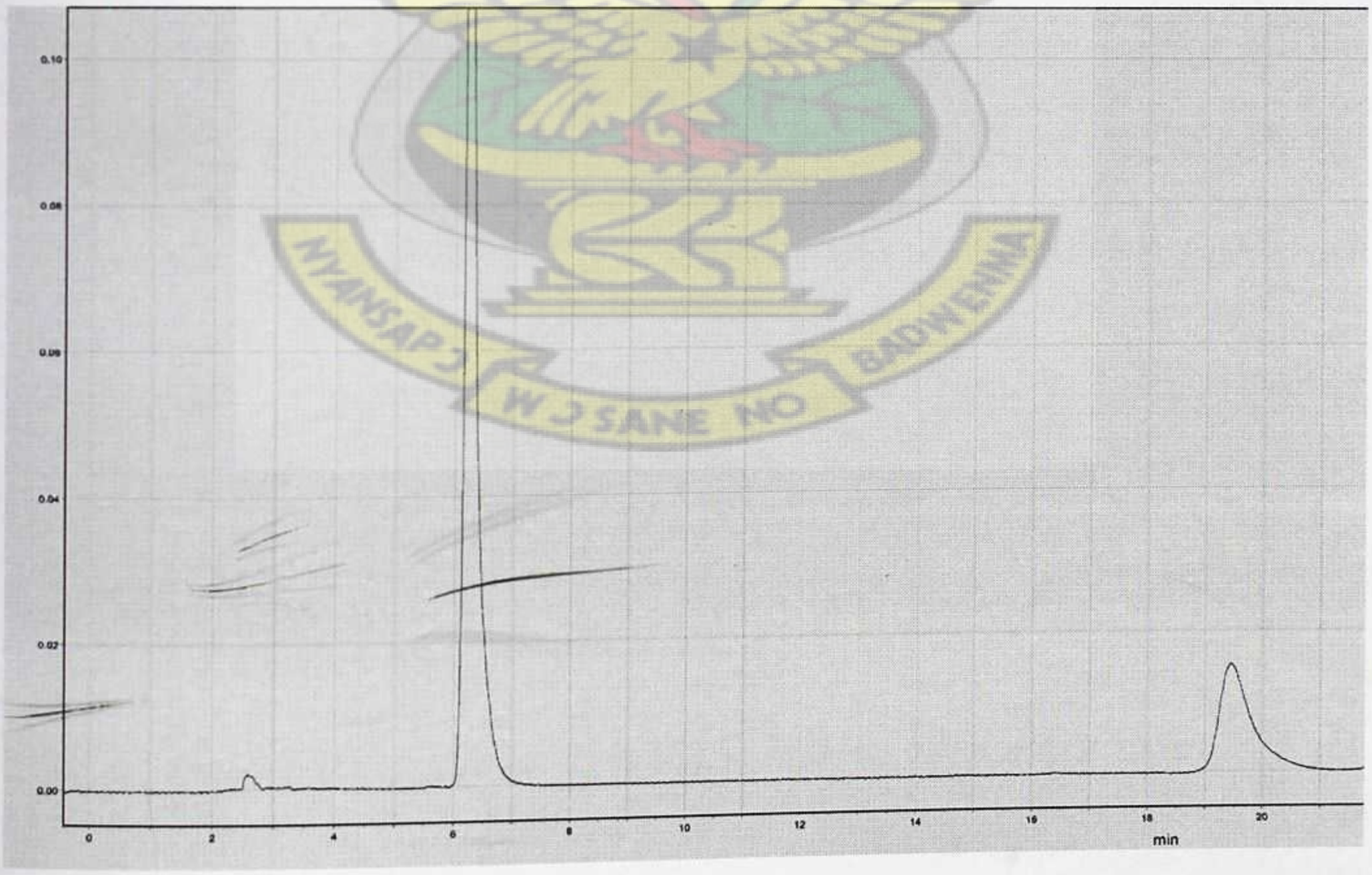


Figure 4.35: Chromatogram of Tablet Sample PTDLS at 50℃

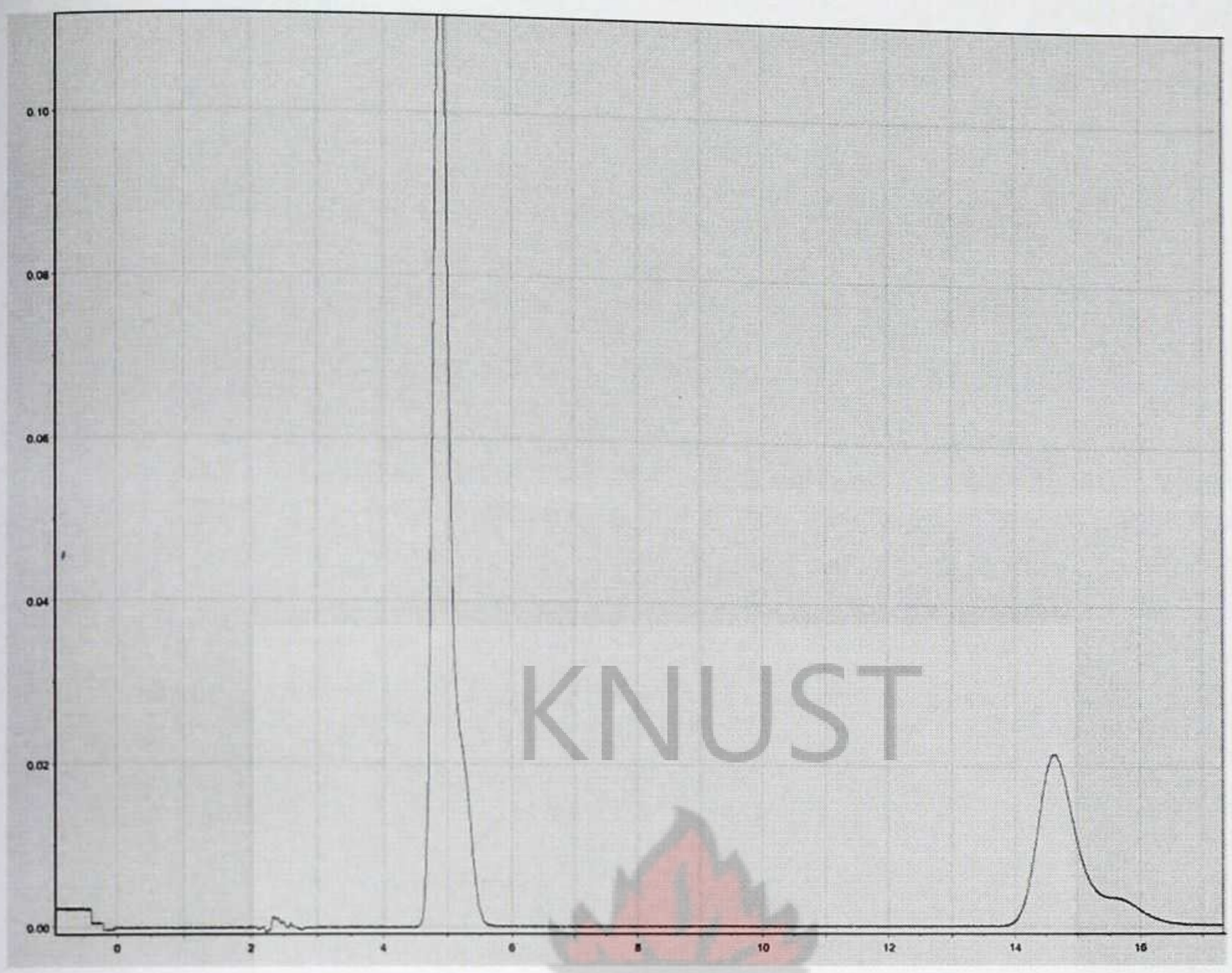


Figure 4.36: Chromatogram of Tablet Sample PTQFS at 28°C

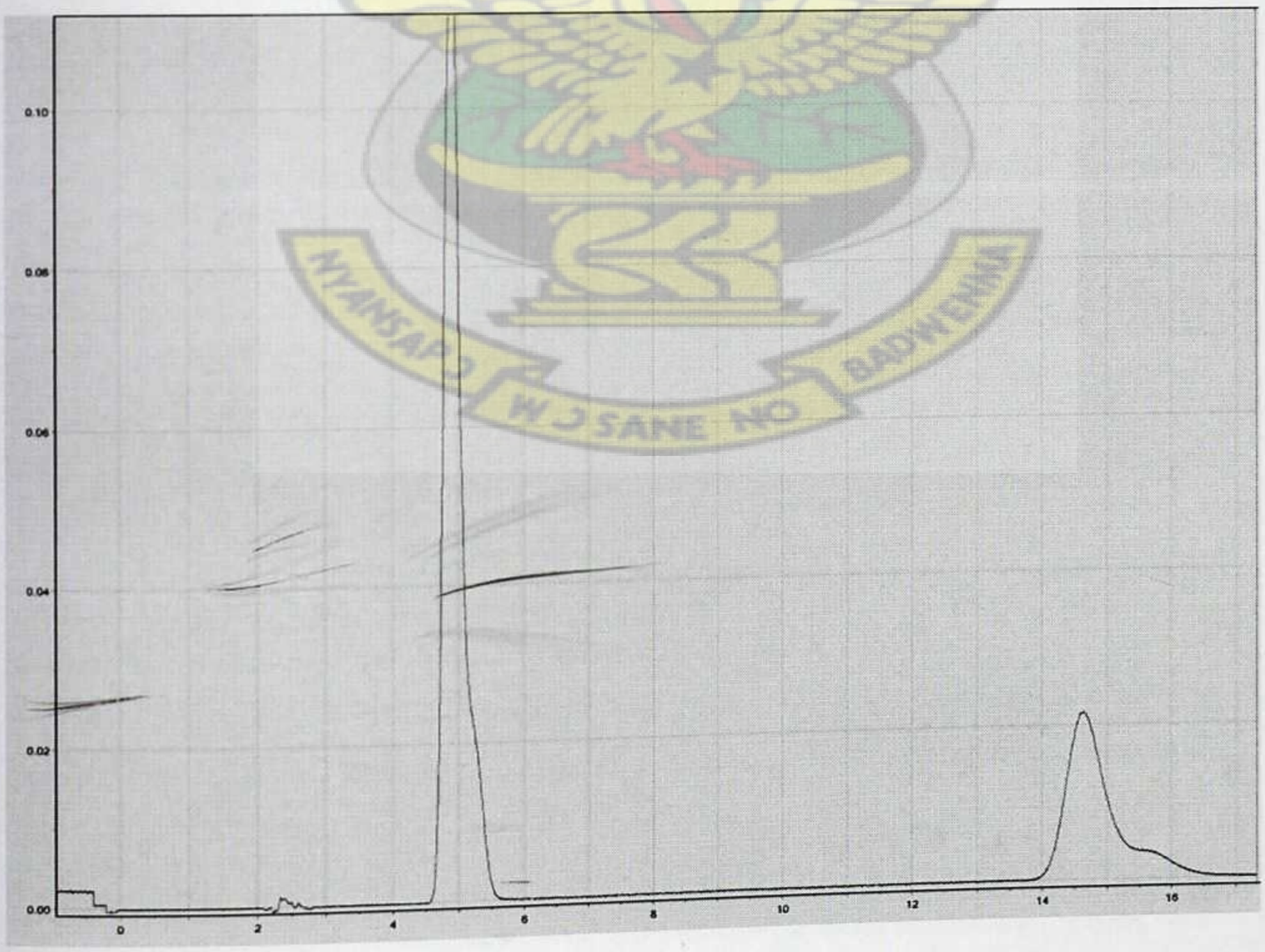


Figure 4.37: Chromatogram of Tablet Sample PTQFS at 35 °C

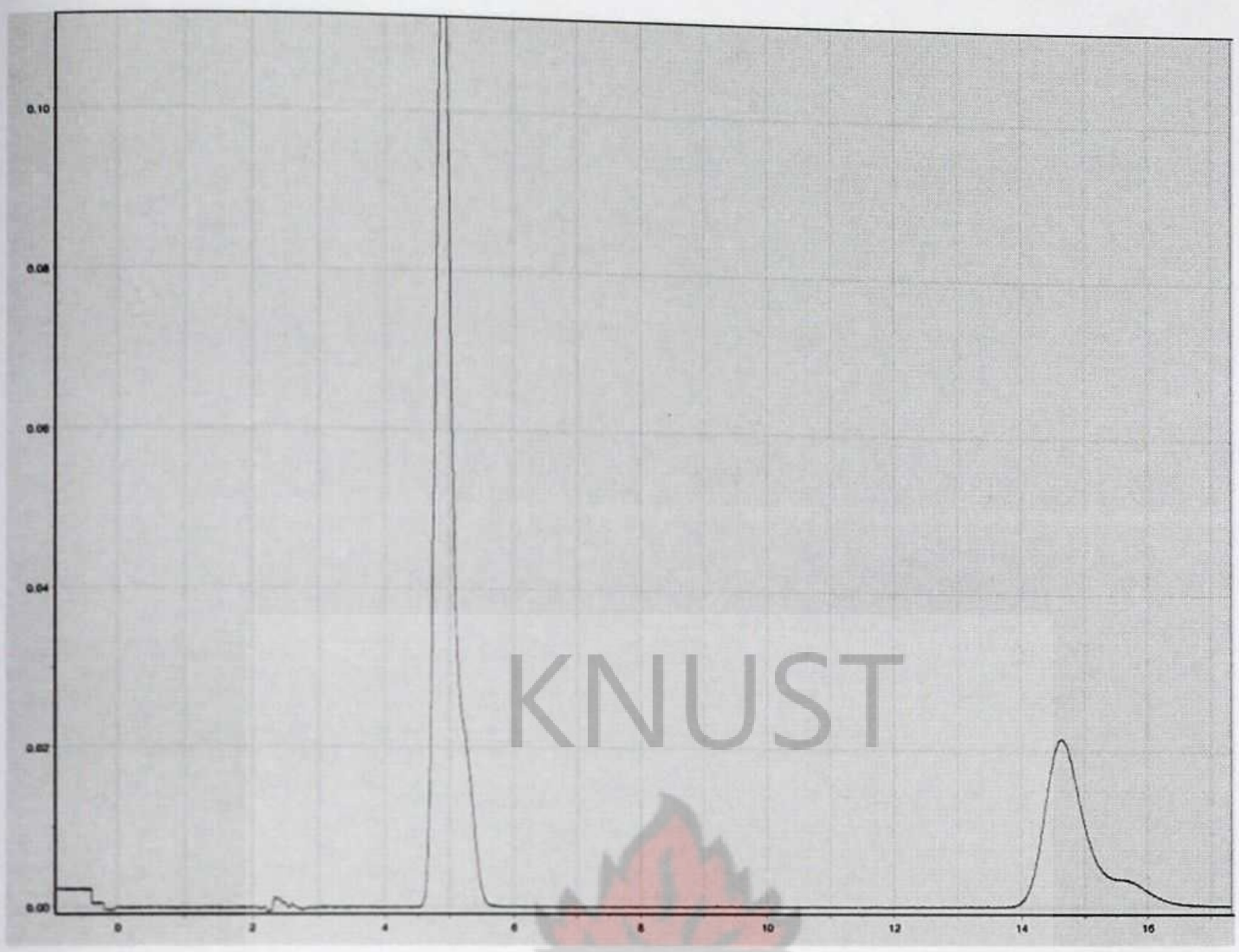


Figure 4.38: Chromatgram of Tablet Sample PTQFS at 40℃

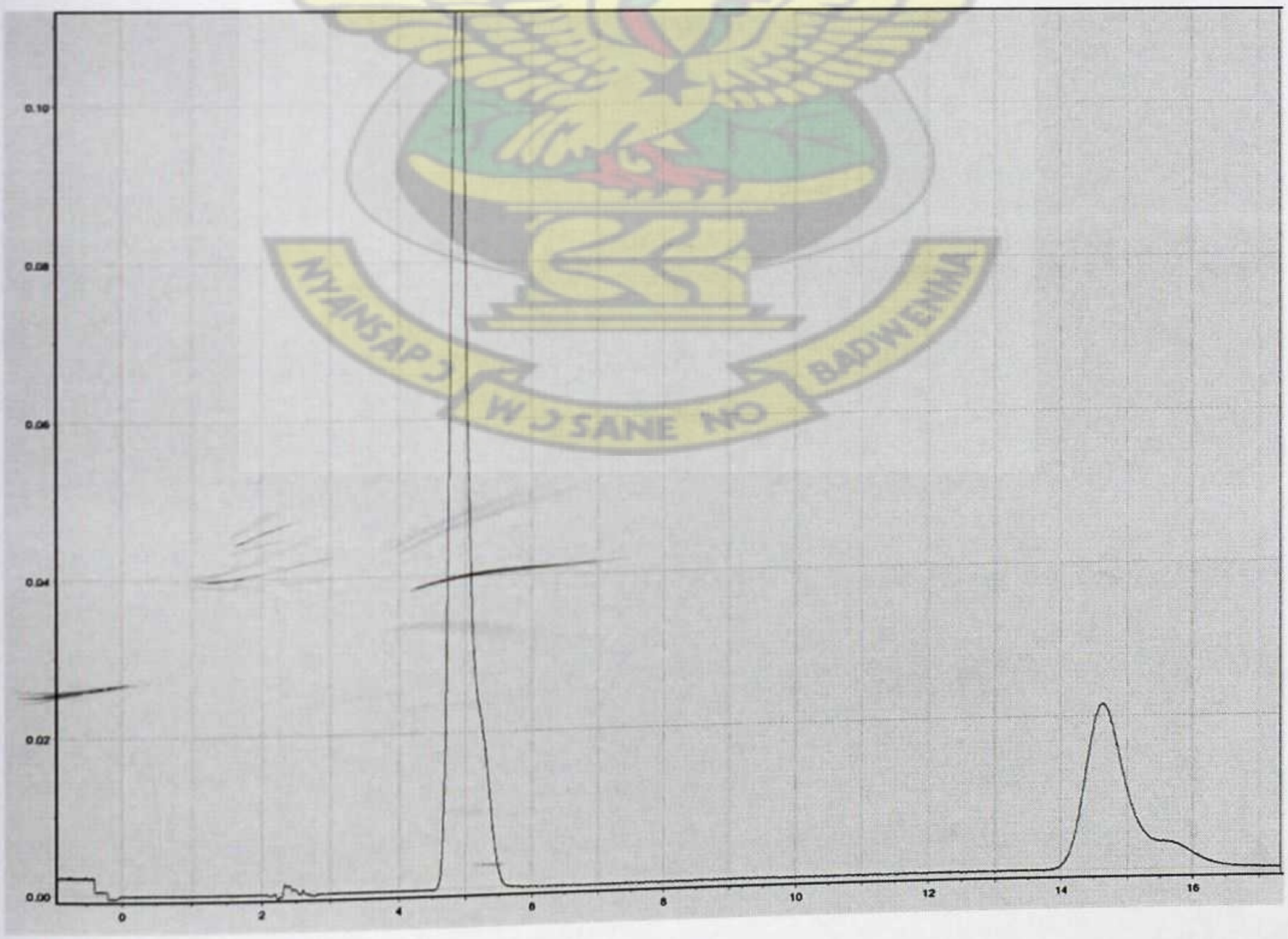


Figure 4.39: Chromatogram of Tablet Sample PTQFS at 50℃

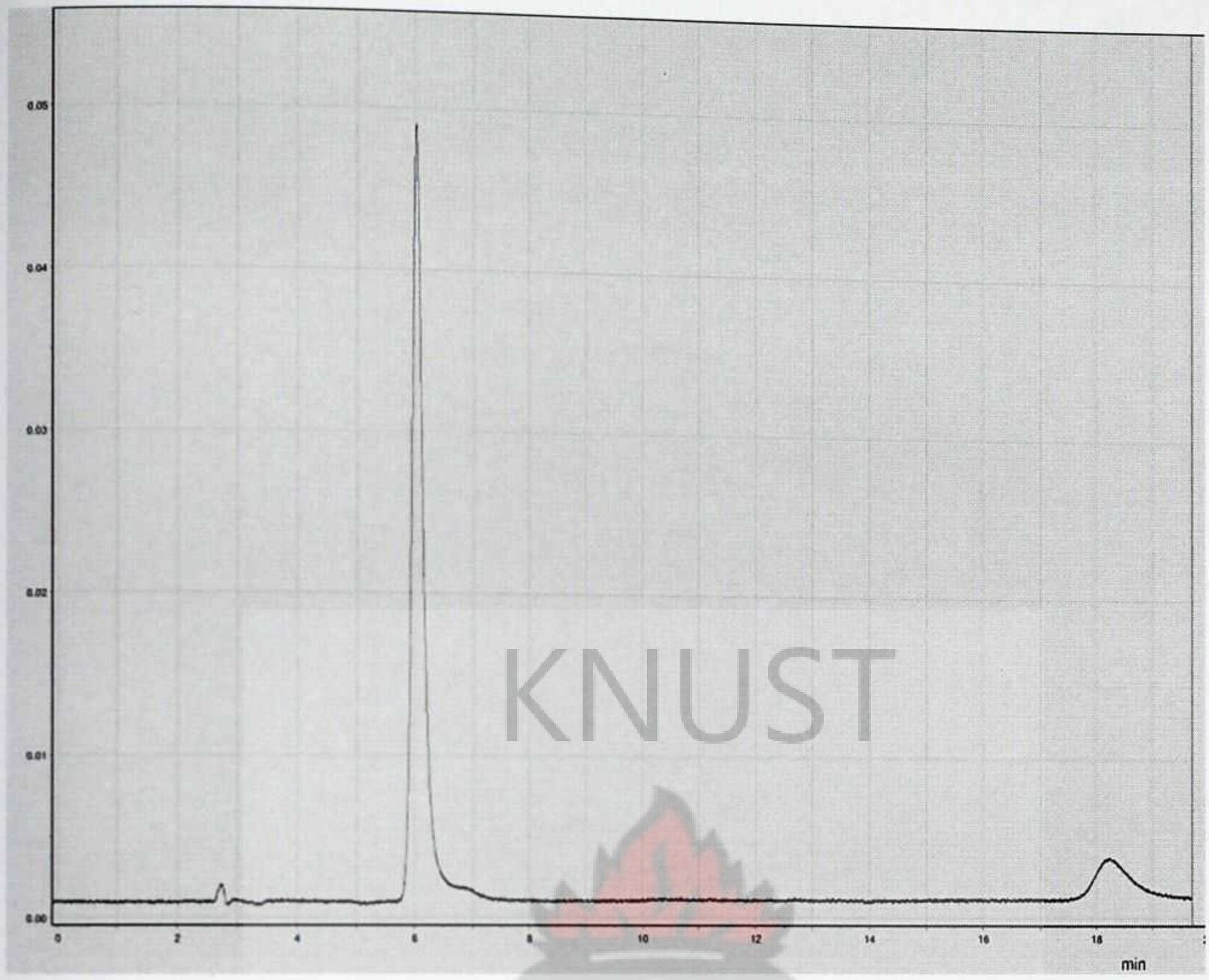


Figure 4.40: Chromatogram of Tablet Sample PTRFS at 28℃

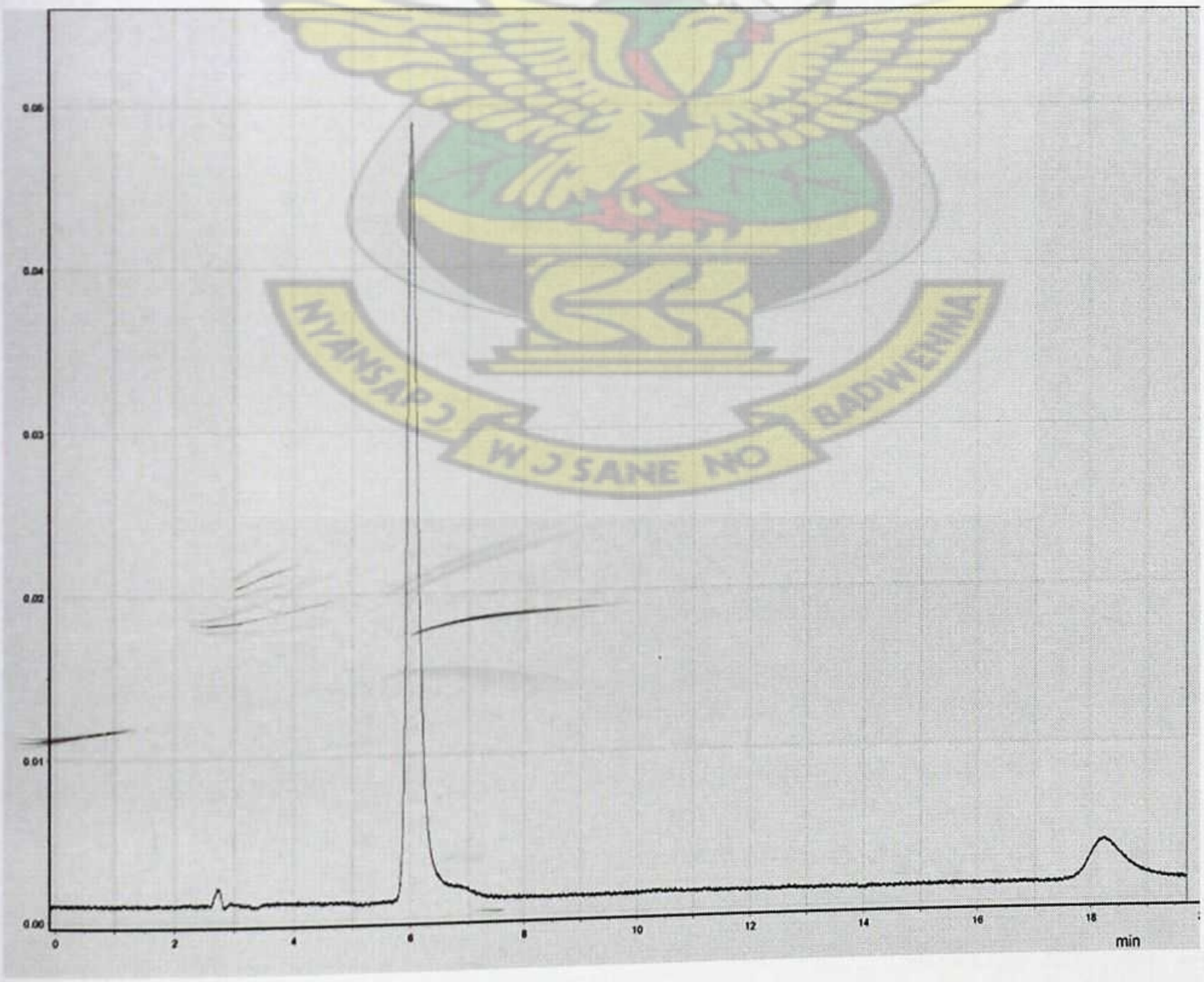


Figure 4.41: Chromatogram of Tablet Sample PTRFS at 35℃

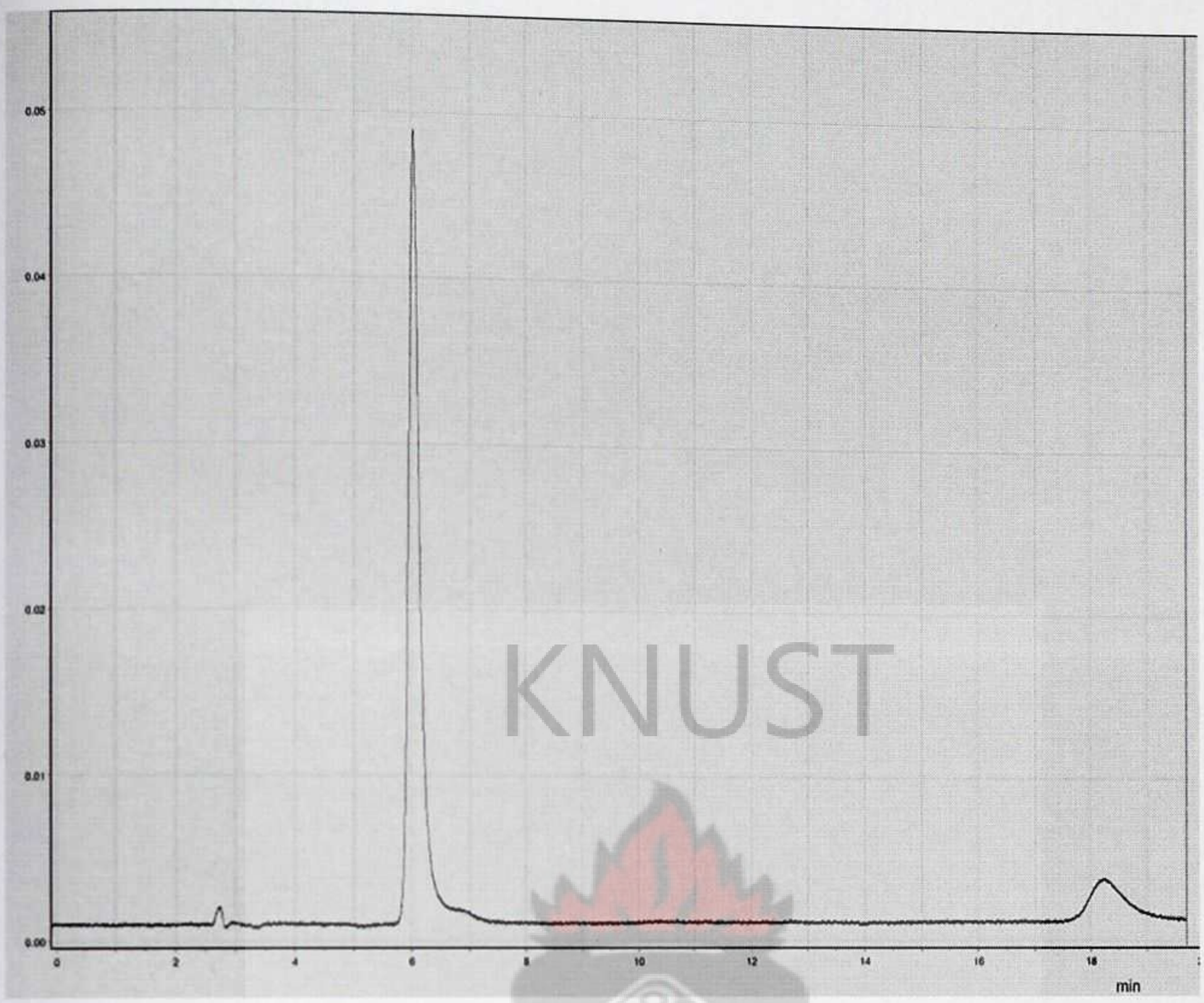


Figure 4.42: Chromatogram of Tablet Sample PTRFS at 40℃

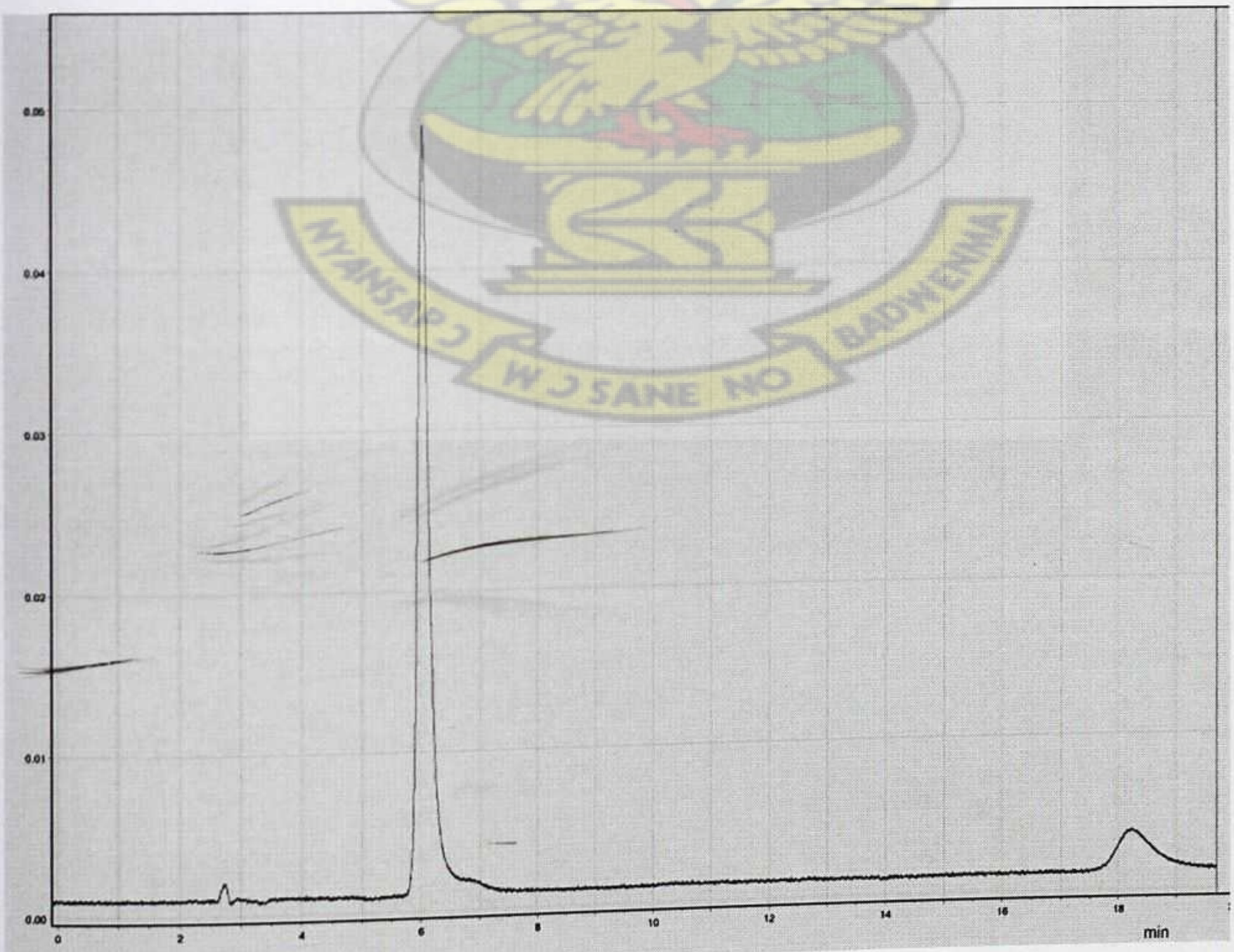


Figure 4.43: Chromatogram of Tablet Sample PTRFS at 50℃

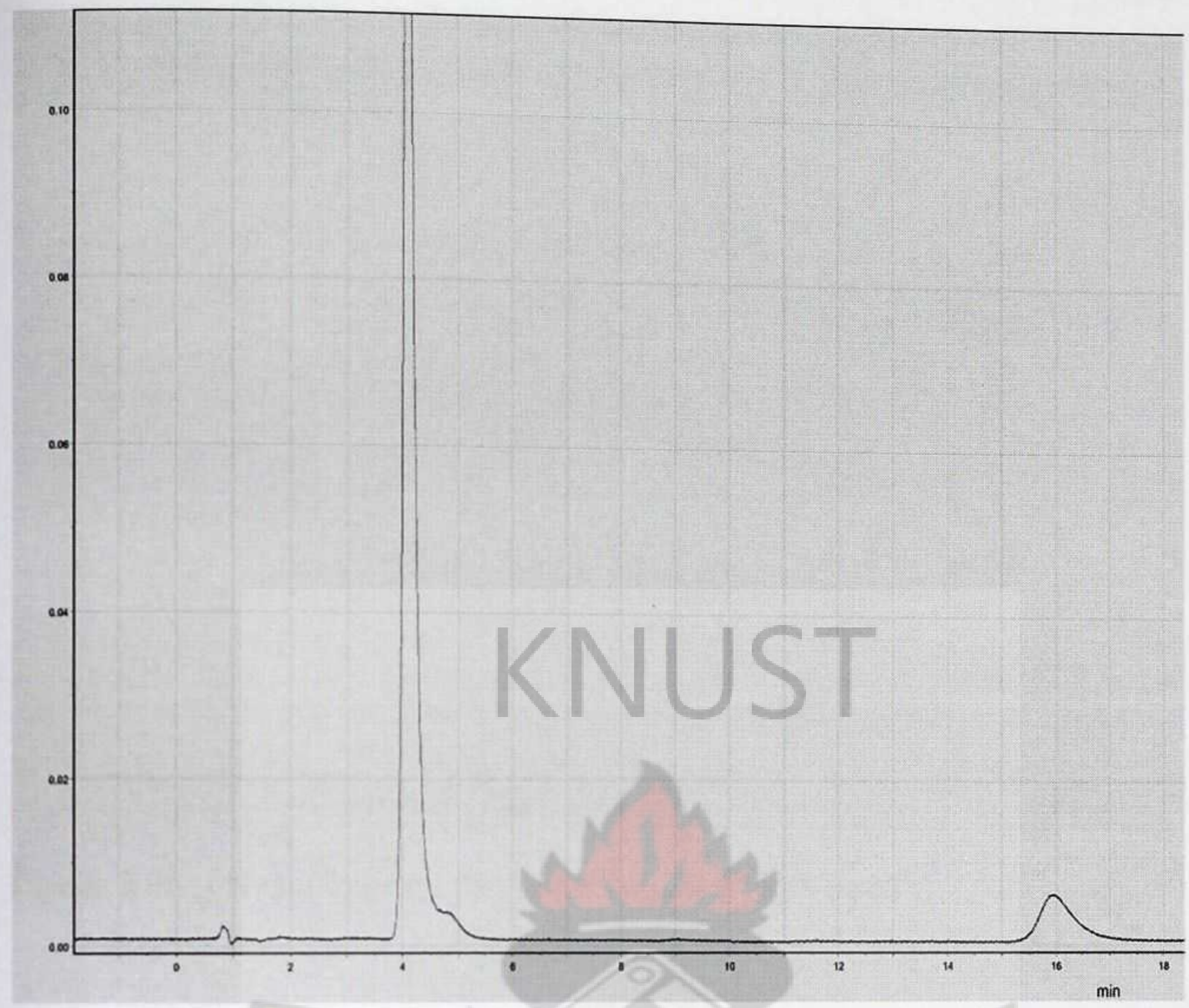


Figure 4.44: Chromatogram of Tablet Sample PTSFS at 28℃

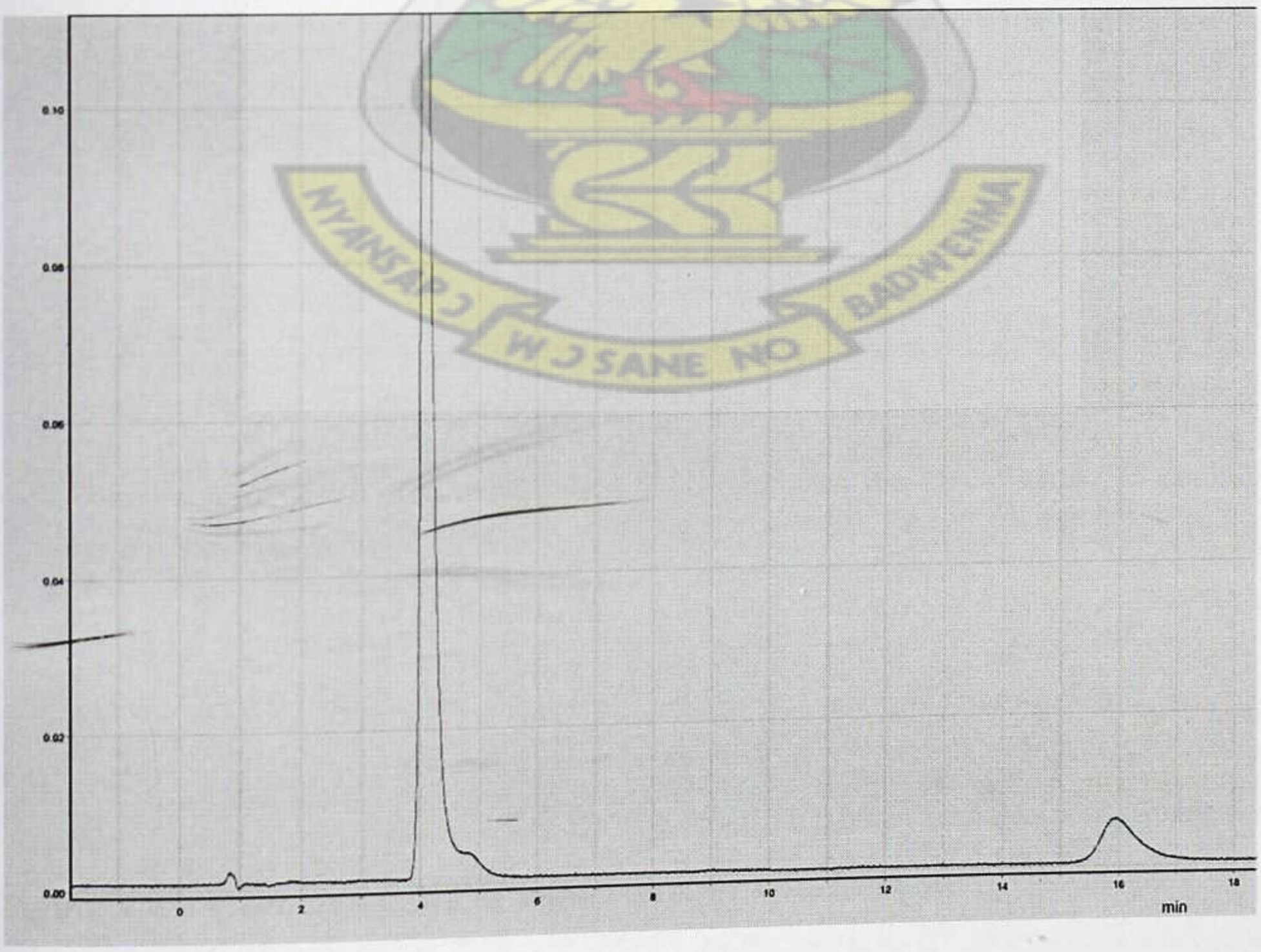


Figure 4.45: Chromatogram of Tablet Sample PTSFS at 35℃

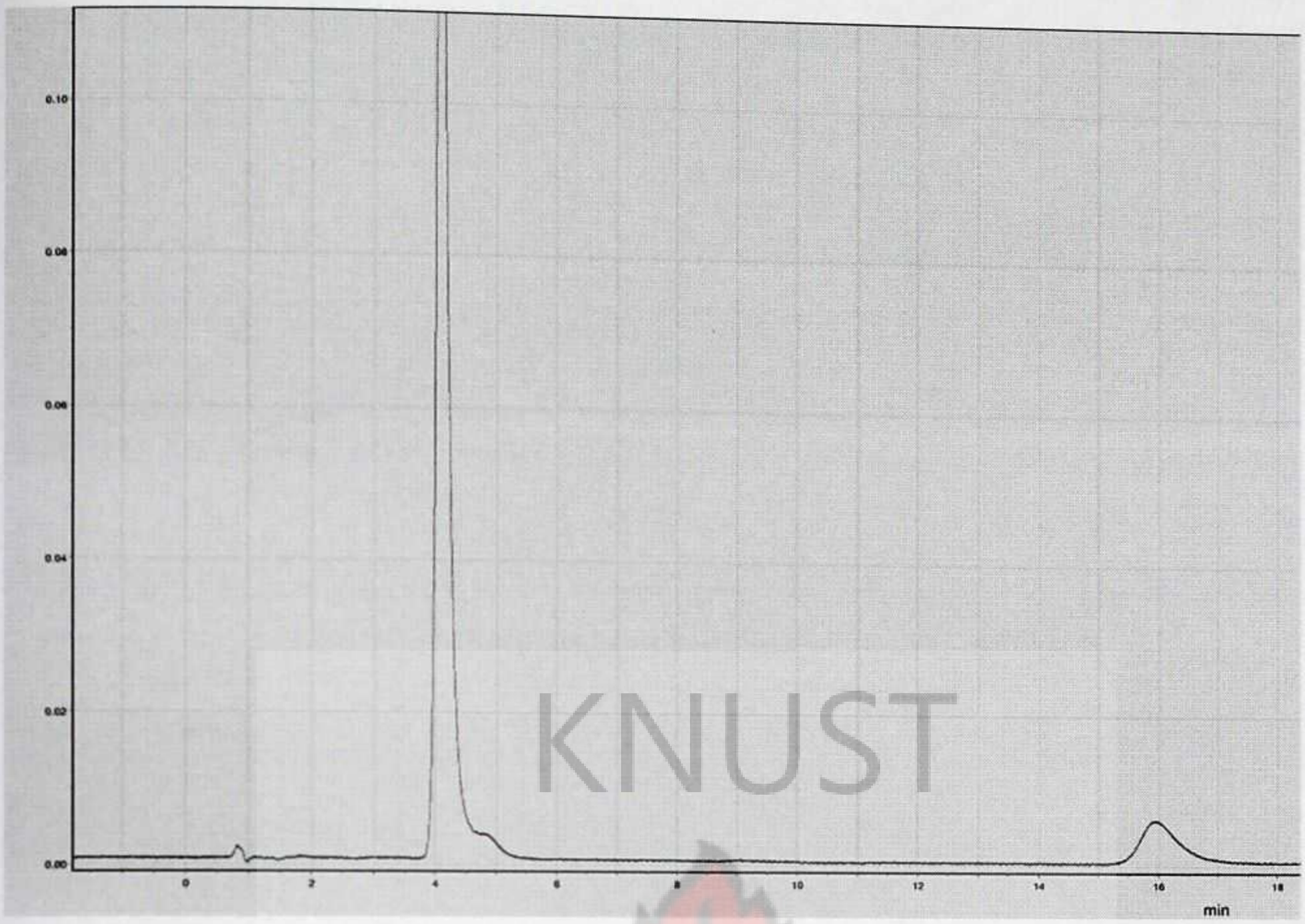


Figure 4.46: Chromatogram of Tablet Sample PTSFS at 40℃

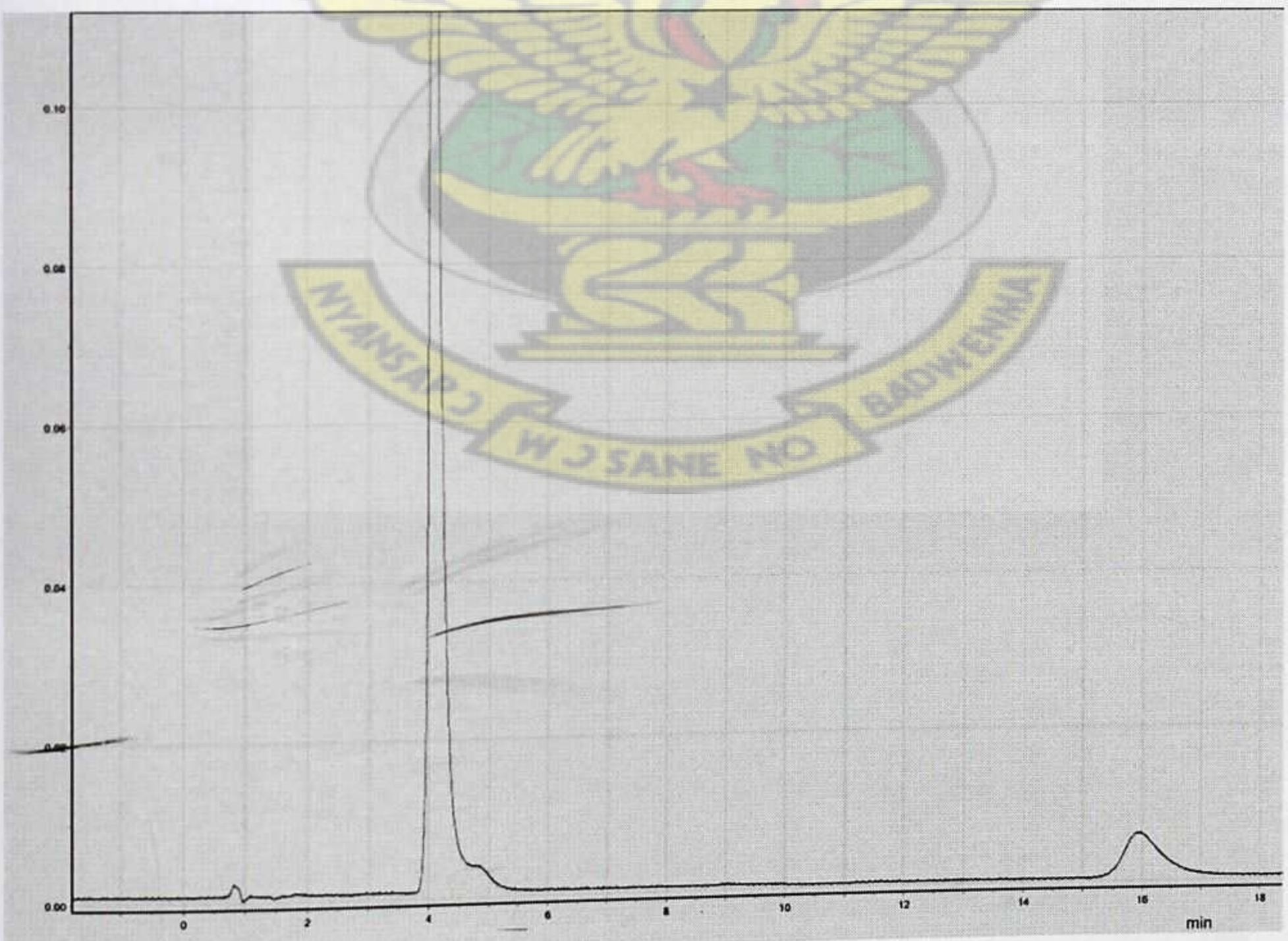


Figure 4.47: Chromatogram of Tablet Sample PTSFS at 50℃

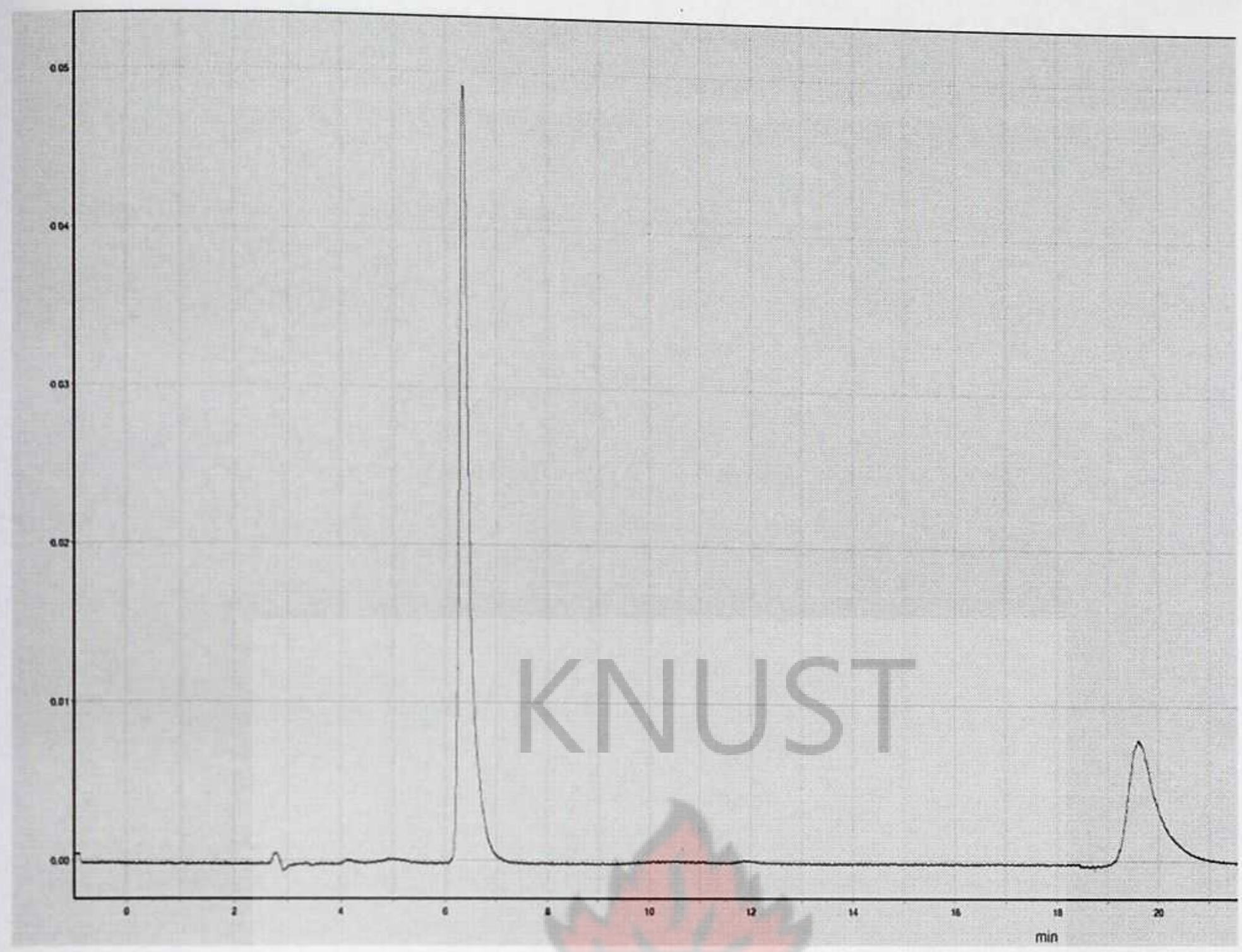


Figure 4.48: Chromatogram of Tablet Sample PTTFS at 28℃

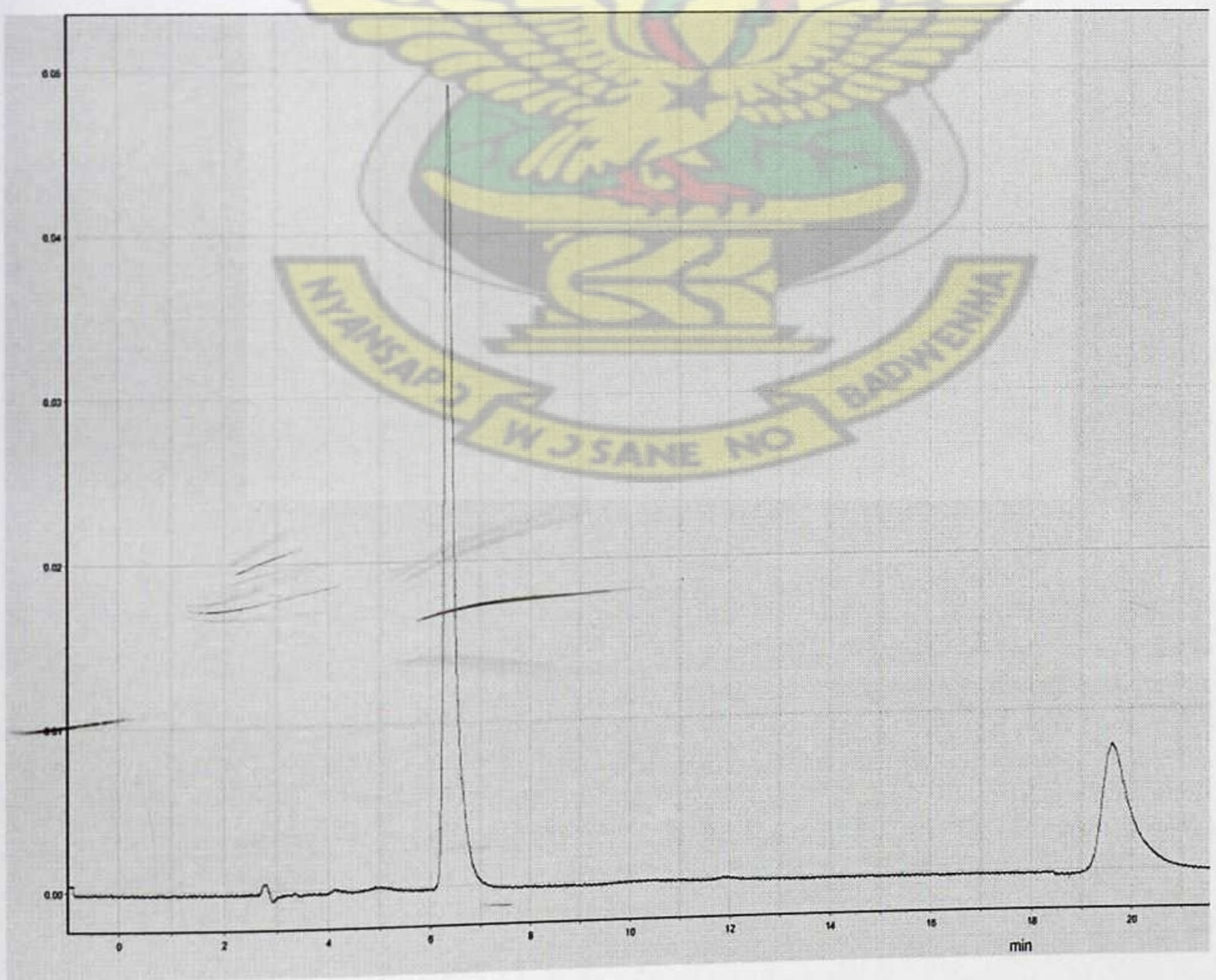


Figure 4.49: Chromatogram of Tablet Sample PTTFS at 35℃

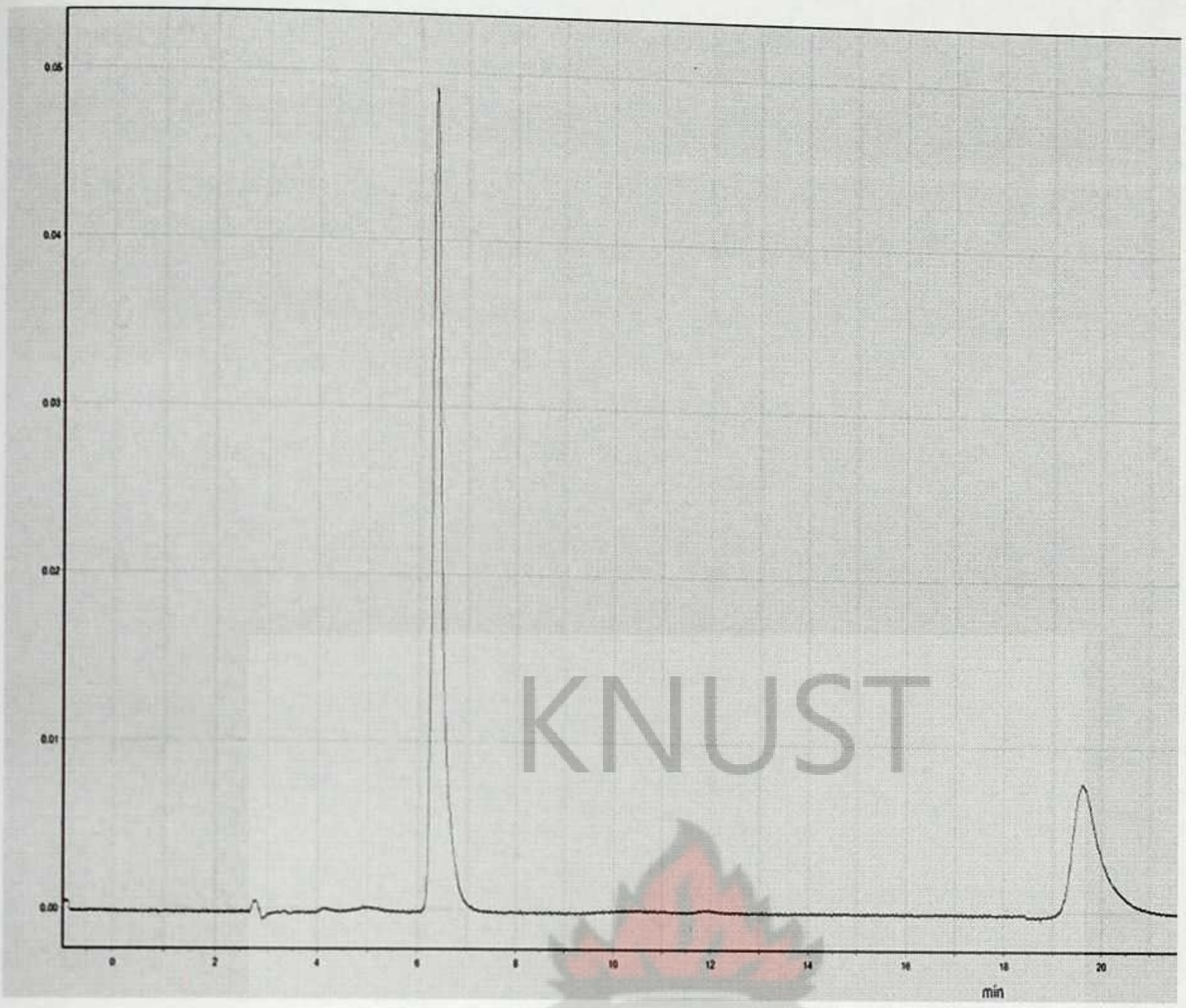


Figure 4.50: Chromatogram of Tablet Sample PTTFS at 40°C

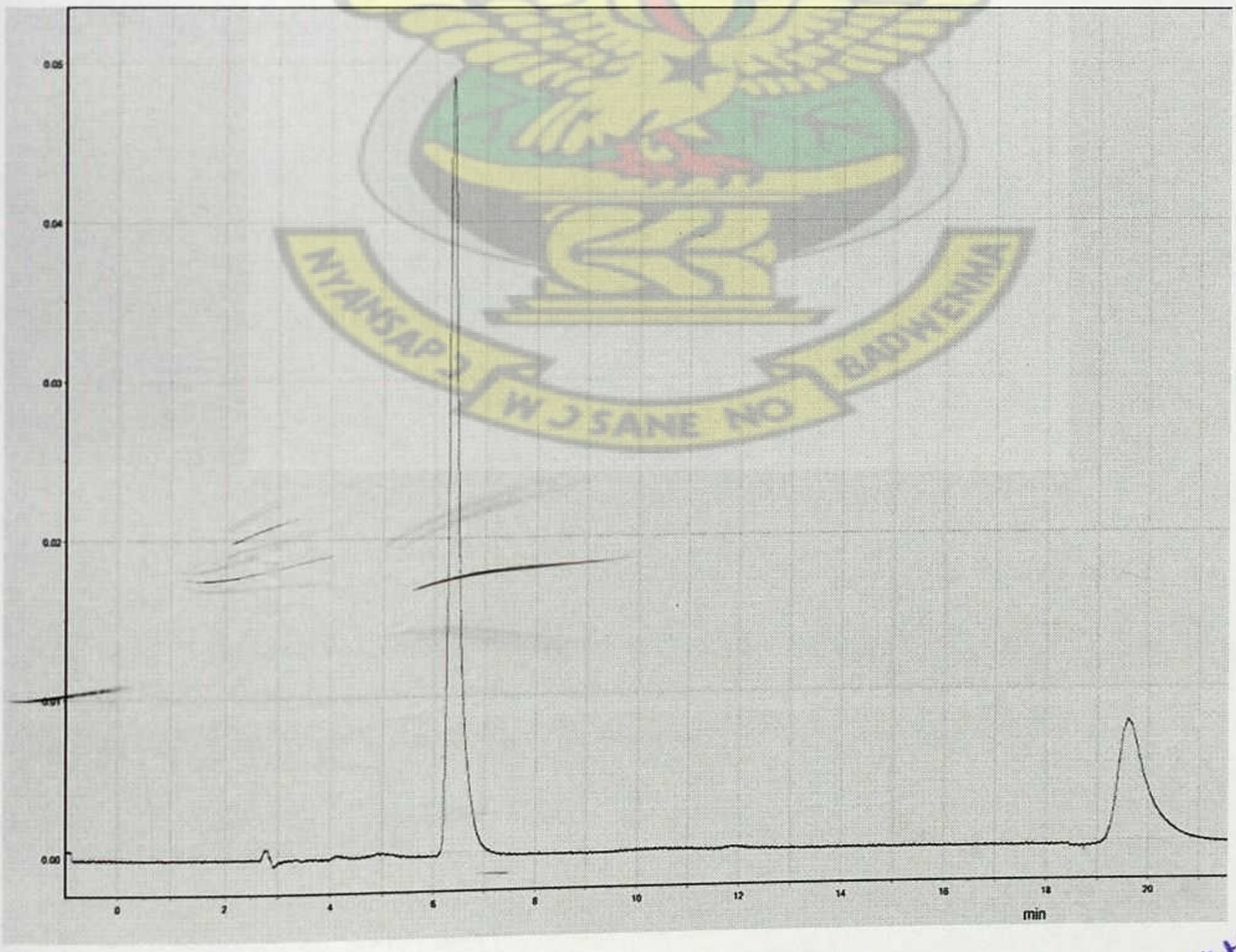


Figure 4.51: Chromatogram of Tablet Sample PTTFS at 50°C

Appendix 9

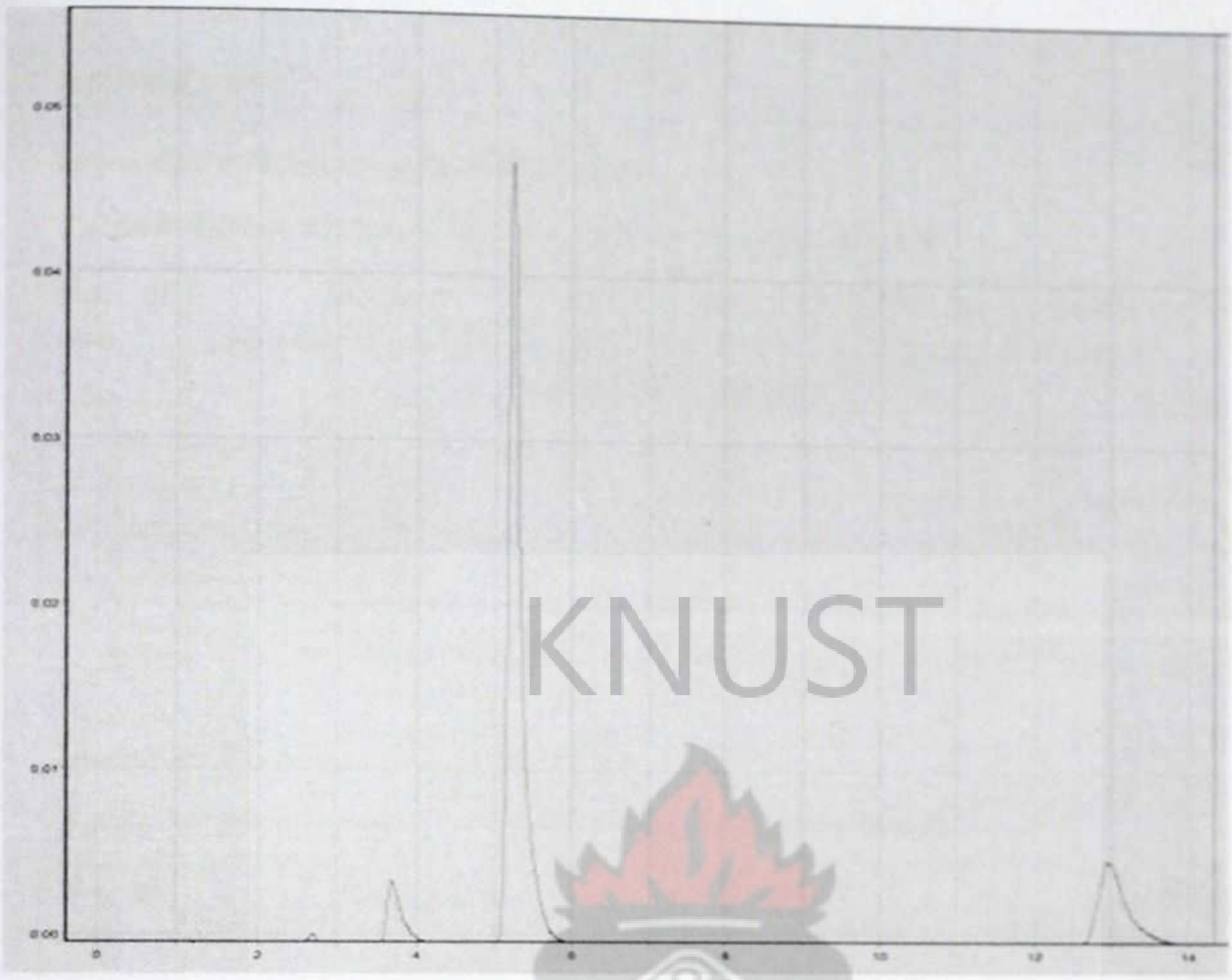


Figure 4.52: Chromatogram of HPLC Investigations

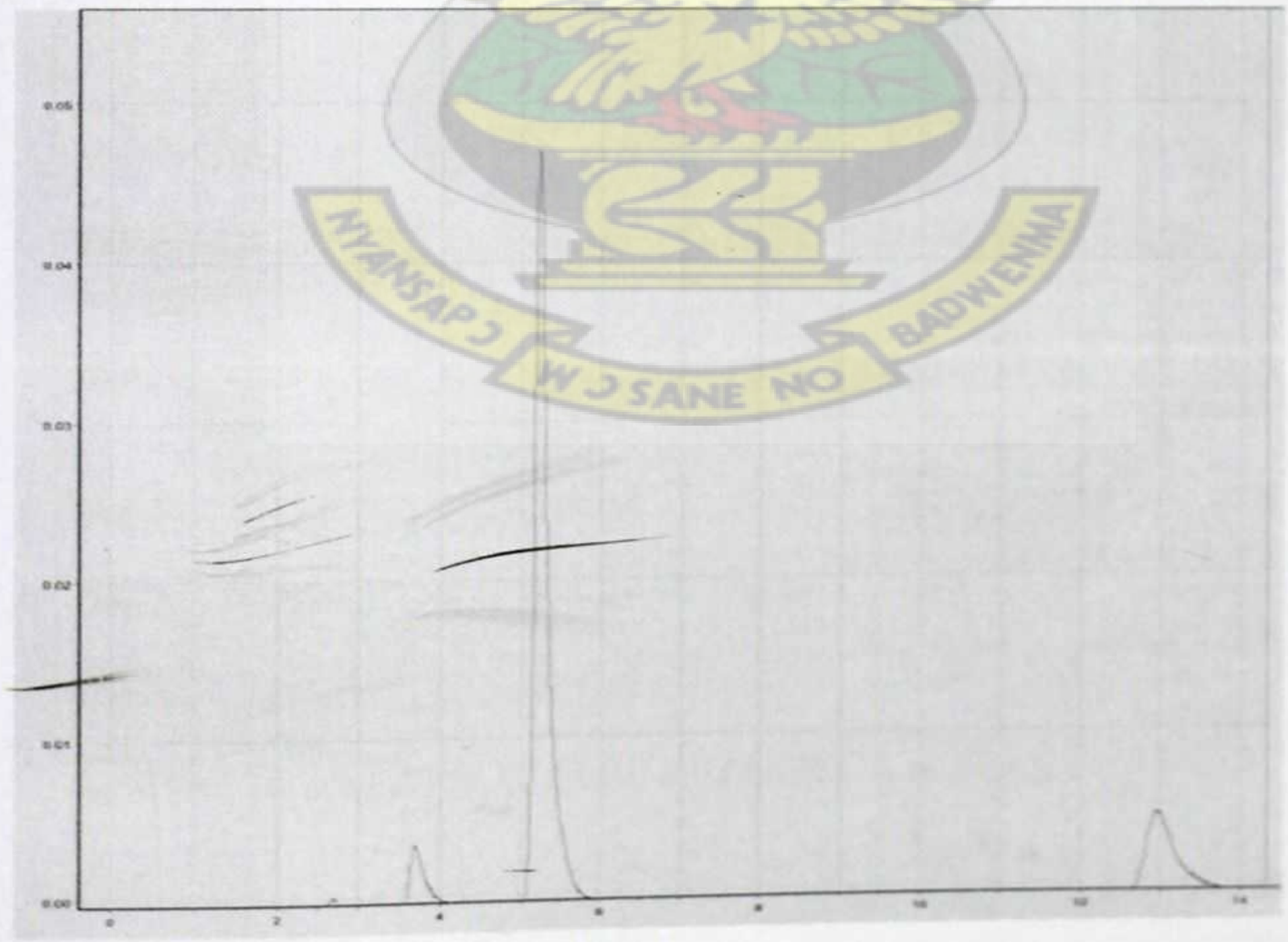


Figure 4.53: Chromatogram of HPLC Investigations

Appendix 10

Intra-day Precision or Repeatability

Table 4.4 Data for Intra-day Precision of p-aminophenol

No. of run	Mean concentration, $\%^{w}/_{v}$	MPA for 2 injections(Pure p-aminophenol)	MPA for 2 injections (Internal Standard)	MPAR
1	0.029931	0.1445	0.1320	1.095
2	0.030010	0.1509	0.1328	1.136
3	0.029922	0.3015	0.1326	2.274
4	0.029881	0.4536	0.1329	3.413
5	0.030009	0.6039	0.1331	4.537
6	0.030033	0.7563	0.1334	5.9669

Inter-day Precision or Repeatability

Table 4.5 Data for Inter-day Precision of p-aminophenol

No. of run	Day 1		Day 2		Day 3	
	MPAR	Mean concentration, $\%^{w}/_{v}$	MPAR	Mean concentration, $\%^{w}/_{v}$	MPAR	Mean concentration,$\%^{w}/_{v}$
1	1.095	0.029931	1.093	0.0448785	1.090	0.0548668
2	1.136	0.030010	1.138	0.0450010	1.135	0.0550010
3	2.274	0.029922	2.272	0.0449055	2.271	0.0548790
4	3.413	0.029881	3.415	0.0448290	3.413	0.0548185
5	4.537	0.030009	4.537	0.04501080	4.536	0.055055
6	5.967	0.030033	5.969	0.0451080	5.968	0.0551705

Table 4.6 Data for test of Precision of the method (student t-test) for p-aminophenol

Days	STATISTICAL PARAMETERS					
	Mean concentration	Standard deviation, S	RSD (%)	t_r	t_0	Remark
Day 1	0.0299643	0.000056	0.187	1.56	2.57	$t_r < t_0$ hence precise
Day 2	0.0449567	0.000101	0.225	1.05	2.57	$t_r < t_0$ hence precise
Day 3	0.0549651	0.000133	0.242	0.64	2.57	$t_r < t_0$ hence precise

μ_0 for day 1 = $0.0200\%^{w}/_{v}$, μ_0 for day 2 = $0.0250\%^{w}/_{v}$, μ_0 for day 3 = $0.0300\%^{w}/_{v}$

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