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Forensic examination of Castor seeds (Ricin): Analytical method development using ALS microscopy, Planar Chromatography and UV-spectroscopy

Bitasta Sarkar¹, Punya V¹, Rahul Das^{2*}

¹Department of Forensic Science, School of Sciences, JAIN (Deemed-to-be University), Bengaluru, India

²Department of Forensic Science, School of Basic and Applied Sciences, Adamas University, Kolkata, India

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ABSTRACT

Ricin, a highly toxic protein found in castor bean seeds, is a potential agent for biological warfare and a major concern for national authorities worldwide. The assassination of Georgi Markov using ricin highlighted the forensic importance of detecting this poison. The present study aims to develop an analytical method, to facilitate detection and forensic analysis of ricin. The study employs visual characterization to examine the physical features of the seeds, and thin-layer chromatography (TLC), and UV-visible spectrometry for chemical examination to confirm the presence of ricin. The research design involves conducting solvent extraction and TLC, followed by optical characterization by the use of UV-Vis spectrometry, for quick and unambiguous identification of ricin for forensic purposes. This study reports, for the first time, extensive morphological analysis of castor seeds, green solvent extraction and optimization of TLC. By developing an easy and effective method for the examination of castor seeds and ricin, this study provides a reliable additional test for detecting ricin in castor seeds, which is crucial for forensic and security purposes.

Introduction

Ricin is a highly toxic protein found naturally in castor bean seeds (*Ricinus communis L.*)⁽¹⁾. Ricin is one of the most potent toxins. It is a potential agent for biological warfare⁽²⁾. Castor seed is the source of castor oil, which has various uses. The seeds contain between 40% and 60% oil rich in triglycerides, mainly ricinolein⁽³⁾. The seed also contains ricin, a highly potent water-soluble toxin, and is also present in low concentrations throughout the plant. Although the lethal dose in adults is considered to be four to eight seeds, reports of actual poisoning are relatively rare. Brazil is the fourth world producer of castor bean oil, just behind India, China and Mozambique^(4,5).

Ricin is highly toxic through a variety of exposure routes.

In humans, the LD50 is estimated to be 5–10 µg/kg by inhalation and 1–20 mg/kg by oral ingestion (6). The Centre for Disease Control and Prevention (CDC) has identified ricin as a Category B biological threat agent due to its toxicity and widespread availability (7). Ricin is a dangerous substance that shares toxicity similar to sarin, a neurotoxic agent (8). It can be easily extracted from castor bean seeds as a fine white powder after removing the endosperm. This powder is water-soluble and can remain stable at a wide range of pH levels. Due to its potential as a chemical and biological warfare agent, it is classified as such by both the Chemical Weapons Convention and the Biological Weapons Convention.

Ricin can be dispersed through the air as small particles or used to contaminate water and agricultural products.

Corresponding author

Email Address: dasrahulfs@gmail.com

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This makes it a prime candidate for terrorist attacks and a major concern for national authorities worldwide⁽⁹⁾. In the case which dates back to September 7, 1978, in London, outspoken Bulgarian dissident Georgi Markov was stabbed in the leg with an umbrella by an unknown foreign man. Within three days he was dead. It was found that the tip of the umbrella had held a minuscule metal sphere containing a pellet of ricin that remained in the wound and killed him⁽¹⁰⁻¹²⁾.

The analysis is usually based on the oral toxicity or penetration of ricin directly to the bloodstream and other experiments with an animal that can be influenced by several factors such as species, age and feeding time. Spectrometric techniques for identification, quantification and analysis of morphological features of the products formed have yet to be used for these studies. The most common methods used to detect ricin are based on enzyme-linked immunosorbent assay, or by performing bioassays where the inactivation of an RNA substrate is measured. Other techniques also used are sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), real-time quantitative-polymerase chain reaction (RTQ-PCR), and toxicological analyses in cell culture and guinea pigs (13-15). In this work, we report, for the first time, an extensive morphological analysis of castor seeds, green solvent extraction and optimization of TLC.

Materials and Methods

The samples, i.e. castor seeds, were collected from Bengaluru, Karnataka. Different equipment and chemicals were used for the extraction and examination of ricin from castor seeds. Video Spectral Comparator (VSC 80) from Foster+Freeman, Acetone (Nice Chemicals Pvt Ltd, Indi), TLC Silica gel 60 F254 plates (Merck KGaA Germany), UV-Vis Spectrophotometer (UV 1900, Shimadzu) and Digital weighing balance, were used.

Physical examination

i) Weight and dimension measurement

A total of 110 seeds were measured on a digital weighing balance and their average was obtained. The "Tare" button was pressed to reset the scale to zero in the weighing machine. The samples were then placed on the weighing machine and the weight was measured. The dimensions (length and diameter) of the seeds were measured for 20 random samples, in order to achieve a generalised result.

ii) Microscopic Examination

A Video Spectral Comparator (VSC) is a specialised instrument used in forensic analysis. It has multiple light sources, including UV (ultraviolet), IR (infrared), and visible light. These different light sources and filters help

in revealing details that might not be visible to the naked eye. Filters isolate and enhance specific characteristics or elements within the sample.

The samples i.e. 10 castor seeds, were observed under Flood Light (reflected light) and Violet filter (410 nm). The examination was carried out based on the colour and pattern under reflected light and luminescence under Violet filter, are considered and observed.

Parameters used in VSC:

Field View-14.0x7.9 m

B-50

Iris- 62%

Zoom-24.35

Light and filters- Flood light (reflected light);

Fluorescence light with Violet Filter

(410 nm)

A comparative study was conducted on 10 sets of samples, containing two seeds each (castor seeds) based on colour, pattern on the front side of the seed, radicle and embryo axis on the rear side. To determine the morphological changes encountered in cases of crushed seeds, used as poison, two castor seeds were partially and fully crushed respectively using a mortar and pestle.

Solvent extraction:

The samples (2-3 seeds) were taken into a mortar. The seeds were crushed and grounded in a circular motion with a pestle and the paste was transferred into a beaker, followed by the addition of 20 ml of acetone. The beaker was then completely covered with aluminium foil and kept idle for 24 hours for extraction.

Preliminary test

Agglutination Test

A lancet was used to collect blood from one of the volunteering authors, and two drops of blood were collected onto microscopic glass slides. The first slide contained only blood and the second slide contained 1 drop of extracted sample along with the blood sample. The results were then observed.

Confirmatory test

Thin Layer Chromatography and its optimization

TLC is a chromatographic technique used to separate the components of a mixture using a thin stationary phase supported by an inert planar surface⁽¹⁶⁾. It can be performed on the analytical scale as a means of monitoring the progress of a reaction, or on the preparative scale to purify small amounts of a compound. TLC is an analytical tool widely used because of its simplicity, relatively low cost, high sensitivity, and separation speed⁽¹⁷⁾.

For optimization and obtaining the best result, a total of nine solvent chambers were developed by using Hexane:

Methanol in the following ratios - 1:9,2:8,3:7,4:6,5:5,6:4,7:3,8:2,9:1. Out of all the 9 solvent systems, only 3:7 (Hexane: Methanol) showed proper spot, while the rest showed an inverted 'w' shape. Hence, Hexane: Methanol in the ratio 3:7 was selected for further analysis.

Selection of TLC Plate - Commercialised TLC plates (60 F254 plates (Merck KGaA Germany) were used as stationary phases and they have different polarities suitable for separating specific types of compounds.

Sample Preparation - The sample i.e. crushed castor seeds were dissolved in acetone for 24 hours, followed by spotting on the TLC plate. The spotting was carried out three times on the same region.

Developing Solvent: The solvent system was selected based on the polarity of the compounds to be separated. As for our sample is concerned, we chose hexane which is nonpolar (0.1) and methanol which is moderately polar (5.1)

Chamber Saturation: The solvent chamber was pre-saturated with the developing solvent to ensure a consistent vapour phase within the chamber. This helps in preventing uneven migration of compounds on the TLC plate.

Developing Process: The solvent was allowed to ascend the plate until it reached an appropriate height (3/4th of the total plate length).

Visualisation: After development, the TLC plate was then visualised for separated compounds under UV light source (using VSC and UV Chamber).

Rf Value Calculation: The Rf values were determined using the standard formula below

$$R_f = \frac{\text{Distance travelled by the compound}}{\text{Distance travelled by the solvent front}}$$

UV Spectrometry.

The sample extract was centrifuged to obtain a clear sample using a centrifuge at 4000-5000 RPM for 5-10 minutes. The process was repeated 4-5 times and the supernatant was collected in each of them.

A UV-1900 Series spectrometer from Shimadzu, was used for the optical characterization of ricin, using LabSolutions UV-Vis (1.12 version) software and appropriate steps were followed according to lab protocol. Acetone was considered for the blank sample. The samples were run three times to ensure that the λ_{\max} values were of a specific range.

Results

Weight and dimension measurement

The average weight of 10 individual seeds was found to be 0.1336 g. Hence, the weight of a single castor seed would range from 0.13 to 0.14 grams. The average length and diameter of castor seeds were found to be 1.06 cm and 0.68 cm respectively.

Microscopic Examination under VSC

The results of few seeds are reported in Table 1, based on the colour and pattern under reflected light and luminescence under Violet filter. The majority of the seeds had brown and yellow colour with linear fibrous patterns and most of the radicles showed luminescence under the violet filter (410 nm).

From the comparative study, it was found that the majority of the seeds had dark brown and yellow colour and most of the seeds showed visible embryo axis on the back or rear side, as given in Table 2.

The findings from the crushed samples are given in Figure 1. It is observed that, under flood light, the colour of the seed coat is either brown or dark brown or pale brown while the colour of the endosperm is pale yellow. This endosperm contains oil content which is luminescent under long UV light (365nm). It also contains fibre material which is fluorescent under UV light.

Agglutination Test

The slide containing blood samples and the extract started to agglutinate. which shows the presence of ricin. Ricin agglutinates red blood cells (RBCs) by binding to certain sugar molecules (glycoproteins), present on their surface, causing them to clump together or agglutinate.

Thin Layer Chromatography

The Rf value obtained for the optimized solvent system i.e. Methanol:Hexane (7:3), using the formula mentioned above, was found to be 0.75

UV Spectrometry

From UV Spectrometry, the λ_{\max} value was found to be 335 nm. The same sample was analysed three times to determine specific λ_{\max} values.

Discussion

This study presents a comprehensive analysis of castor seeds using various techniques. Weight and dimension measurements revealed consistent seed weight and dimensions, which can be used for physical examination in various cases. Microscopic examination under VSC have shown distinctive coloration, patterns, and luminescence characteristics aiding seed identification. Thin Layer Chromatography provided essential data on compound separation and identification, using the Rf value. UV Spectrometry identified λ_{\max} values, which is crucial for assessing chemical composition. There is a scope for further research, by increasing the sample size and utilization of high-end instrumental techniques for characterization. Together, these methodologies offer a holistic understanding of castor seeds, significant for forensic, pharmaceutical, and

industrial applications. The findings contribute significantly to forensic identification of castor (*ricin*).

Conclusion

Ricin is a highly potent toxic protein that occurs naturally in castor bean plant seeds. It is known to be among the most powerful toxins and is produced during the production of castor oil. Ricin is soluble in water and is found in lower concentrations throughout the plant. Guinness World Records recognizes castor beans as the most toxic common plant due to the presence of ricin. A reliable, sustainable, cost-effective, and easily reproducible method for detecting ricin through tests and assays has been reported in this paper. The average weight of a castor seed is found to be 0.13 to 0.14 grams. The average length and diameter are found to be 1.0675 cm and 0.68 cm respectively. Majority of the seeds had brown and yellow colour with linear fibrous patterns and most of the radicles showed luminescence. Also, the crushed seeds exhibit fluorescence under long UV light. The Rf value for the system used is 0.75 and the λ_{\max} value for UV Spectroscopy ranges between 334 nm to 335 nm. The findings from this research study can be used for rapid physical and chemical examination of castor seeds (*ricin*) for forensic and security purposes.

Author contributions:

All authors contributed to the study design and conception, manuscript drafting, and approved the final draft of the manuscript.

Conflict of interest:

The authors have no conflict of interest to declare.

Source of funding:

None.

Ethical Clearance-

Not applicable

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Table legends:

Table 1: Microscopic examination of the samples using VSC

Table 2: Table showing the results from comparative microscopic examination



Figure legends:

Figure 1: (a). Partially crushed seed under flood light (b). Partially crushed seed under long UV (365 nm) (c). Completely crushed seed under flood light (d). Completely crushed seed under long UV (365 nm)

Figure 2: (a) Agglutination test for Ricin - Blood sample (b) Agglutination test for Ricin - Blood+Extract (c) Agglutination test (Under 40x of a Compound microscope for Ricin - Blood sample (d) Agglutination test (Under 40x of a Compound microscope for Ricin - Blood+Extract

Figure 3: Spectrum obtained in the UV range of UV-Spectrometry

Table 1: Microscopic examination of the samples using V

| SL.No | SEED FIGURE | OBSERVATION |
|-------|---|--|
| 1. |  | <p>i. Flood Light</p> <ul style="list-style-type: none"> • Colour-Brown and yellow • Pattern-linear fibrous stripes <p>ii. Violet Filter</p> <ul style="list-style-type: none"> • Luminescence-The linear pattern is radiating |
| 2. |  | <p>i. Flood Light</p> <ul style="list-style-type: none"> • Colour-brown and dirty gold • Pattern-linear fibrous stripes <p>ii. Violet Filter</p> <ul style="list-style-type: none"> • Luminescence- the radicle is showing Luminescence |

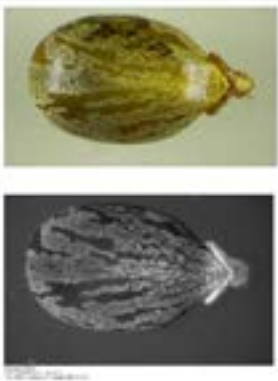


| | | |
|----|---|--|
| 4. |  | <p>i. Flood Light</p> <ul style="list-style-type: none"> • Colour-pale brown and off-white pattern • The fibrous strips became wider at the bottom <p>ii. Violet Filter</p> <ul style="list-style-type: none"> • Luminescence- a line below the radicle is luminescence |
|----|---|--|

Table 2: Table showing the results from comparative microscopic examination

| SL. NO. | SEED FIGURE | OBSERVATION |
|---------|---|---|
| 1. | <p>FRONT</p> <p style="text-align: center;">A B</p>  <p>BACK</p>  | <p style="text-align: center;">A</p> <p>FRONT</p> <ul style="list-style-type: none"> • Colour- Dark brown with dirty gold • Pattern- Ruptured pattern at the center <p>BACK</p> <ul style="list-style-type: none"> • Radicle - present • Embryo axis - present <p style="text-align: center;">B</p> <p>FRONT</p> <ul style="list-style-type: none"> • Colour- Dark brown with dirty gold • Pattern- linear fibrous <p>BACK</p> <ul style="list-style-type: none"> • Radicle - present • Embryo axis - present |
| 2. | <p>FRONT</p>  <p>BACK</p>  | <p style="text-align: center;">A</p> <p>FRONT</p> <ul style="list-style-type: none"> • Colour- dark brown • Pattern- linear fibrous pattern <p>BACK</p> <ul style="list-style-type: none"> • Radicle - present • Embryo axis - present <p style="text-align: center;">B</p> <p>FRONT</p> <ul style="list-style-type: none"> • Colour- light brown with pale yellow • Pattern- Thick linear fibrous pattern <p>BACK</p> <ul style="list-style-type: none"> • Radicle - present • Embryo axis - present |
| 10. | <p>FRONT</p>  <p>BACK</p>  | <p style="text-align: center;">A</p> <p>FRONT</p> <ul style="list-style-type: none"> • Colour- light brown and dirty green colour • Pattern- linear fibrous strip <p>BACK</p> <ul style="list-style-type: none"> • Radicle- Absent <p style="text-align: center;">B</p> <p>FRONT</p> <ul style="list-style-type: none"> • Colour- brown and yellow colour • Pattern- Ruptured fibrous strip <p>BACK</p> <ul style="list-style-type: none"> • Radicle- present |

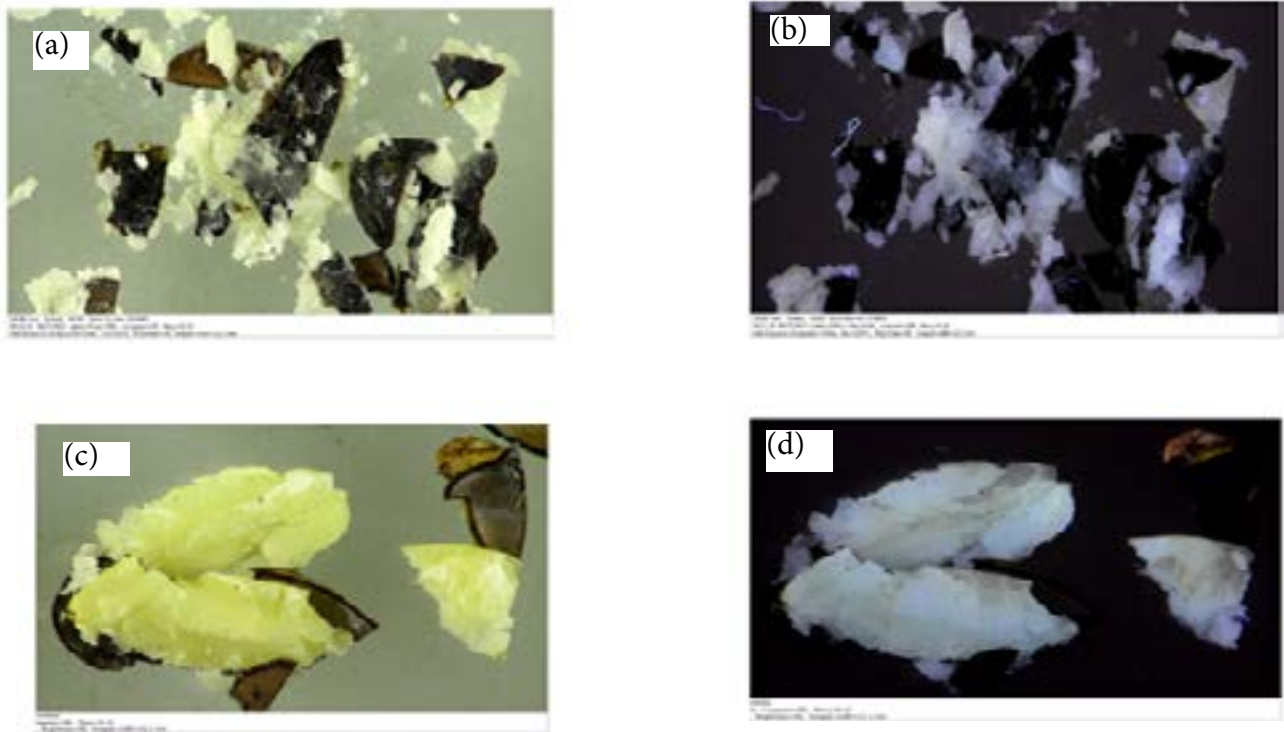


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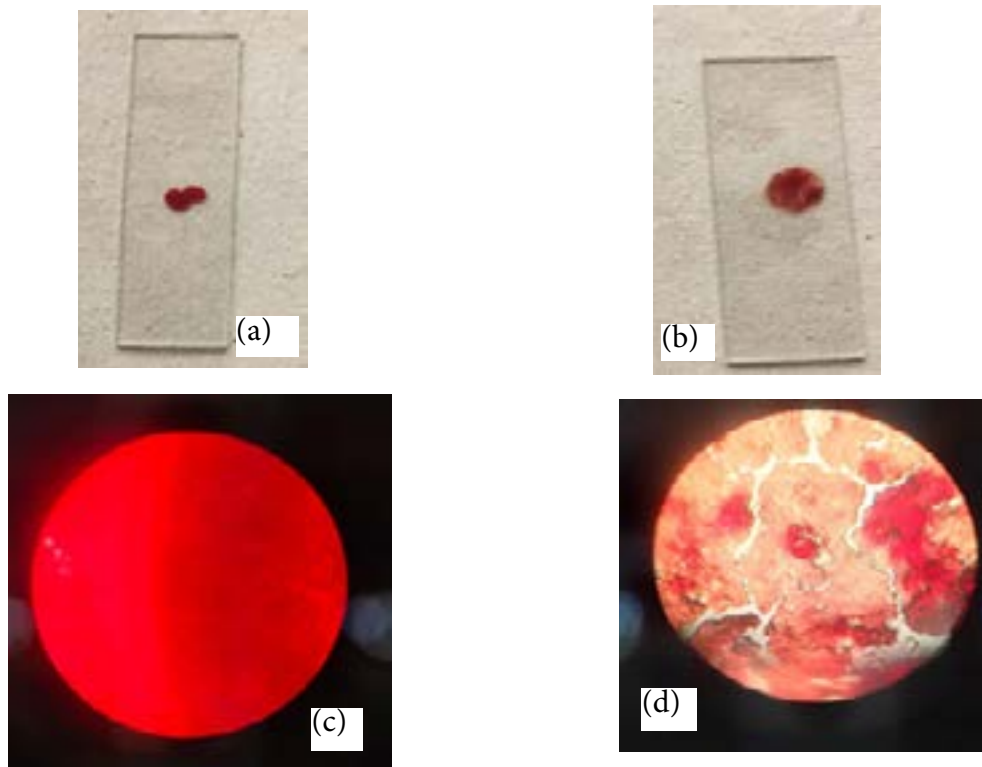


Fig 2: (a) Agglutination test for Ricin - Blood sample (b) Agglutination test for Ricin - Blood+Extract (c) Agglutination test (Under 40x of a Compound microscope for Ricin - Blood sample (d) Agglutination test (Under 40x of a Compound microscope for Ricin - Blood+Extract

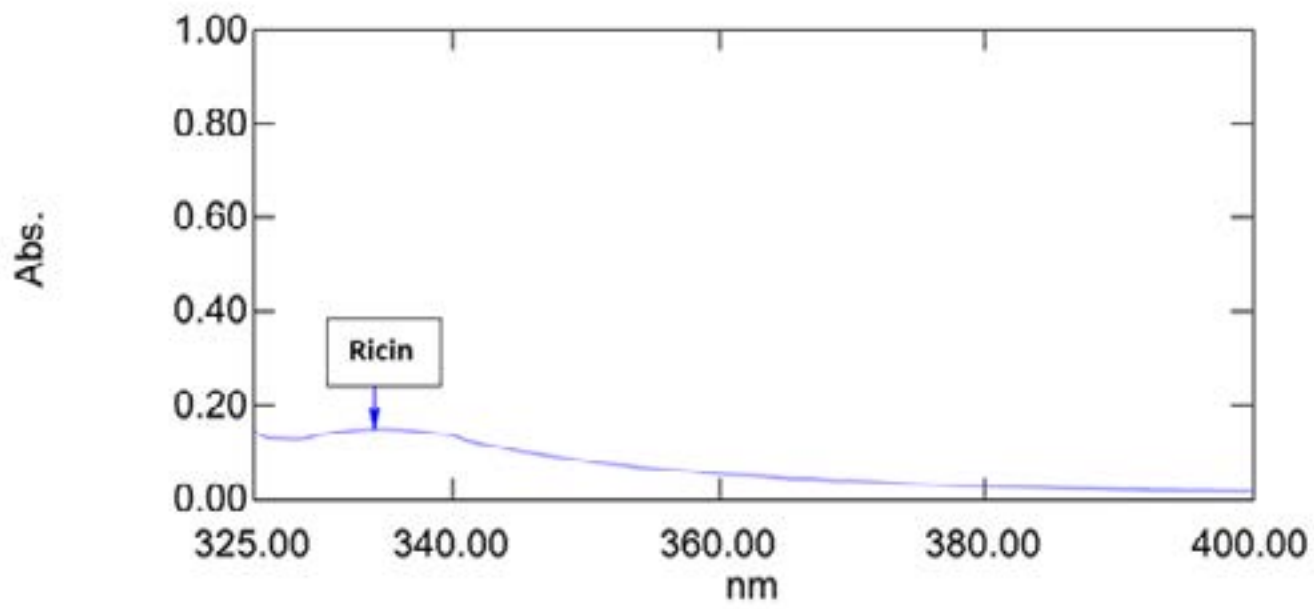


Fig 3: Spectrum obtained in the UV range of UV-Spectrometry