3. Target product profile, critical path and pre-clinical development plan

The target product profile (TPP) is a tool used extensively in the pharmaceutical industry for planning research and development (R&D) projects. [89] The TPP can be viewed as a statement of the key features and attributes of the product intended to be developed. Strategic drug development begins with the TPP that then helps delineate the pre-clinical and clinical studies (pathways) necessary to support that particular product profile. [90] TPP is used as a tool when communicating with agencies: as a means to help define the due regulatory requirements that then drives the pre-clinical development plan. Statement of the TPP must be realistic considering resources.

In this study the primary TPP directing development efforts were described (envisioned) as a tumour-targeting, nanoparticulate drug delivery system (NDDS) that has encapsulated a synergistic Fixed-Ratio Drug Combination (FRDC) of a standard chemotherapeutic (paclitaxel) and a Riminophenazine (that is thereby capable of circumventing MDR). The label of “broad-spectrum MDR circumventing anticancer agent” is suggested to describe Riminophenazines.

Within the development portfolio, two additional TPP stipulations were pursued: firstly, imaging capability (through Lipiodol encapsulation in a nanoemulsion) and secondly the development of novel amphiphiles (PVP conjugates) for Riminophenazine encapsulation through strategic alliance with the Institute of Polymer Science (Stellenbosch University).

A list of scientifically and ethically justifiable tests required to prove safety and efficacy for the particular product under development should ideally be outlined with due consideration to the appropriate regulatory guidelines and the regulatory (approval) status of the individual components (drugs and excipients) used in the formulation.

As this project had view (although not scope) to progress through to human trials and eventual commercialization, a holistic view (considering resources available and future development avenues) had to be taken and the practical steps chronologically ordered. The critical path (Figure 3.1.) describes the different steps and activities that must typically be completed at different time points within different focus areas prior to successful commercialization of a drug product. This study concerned the prototype design/discovery and pre-clinical development phases.

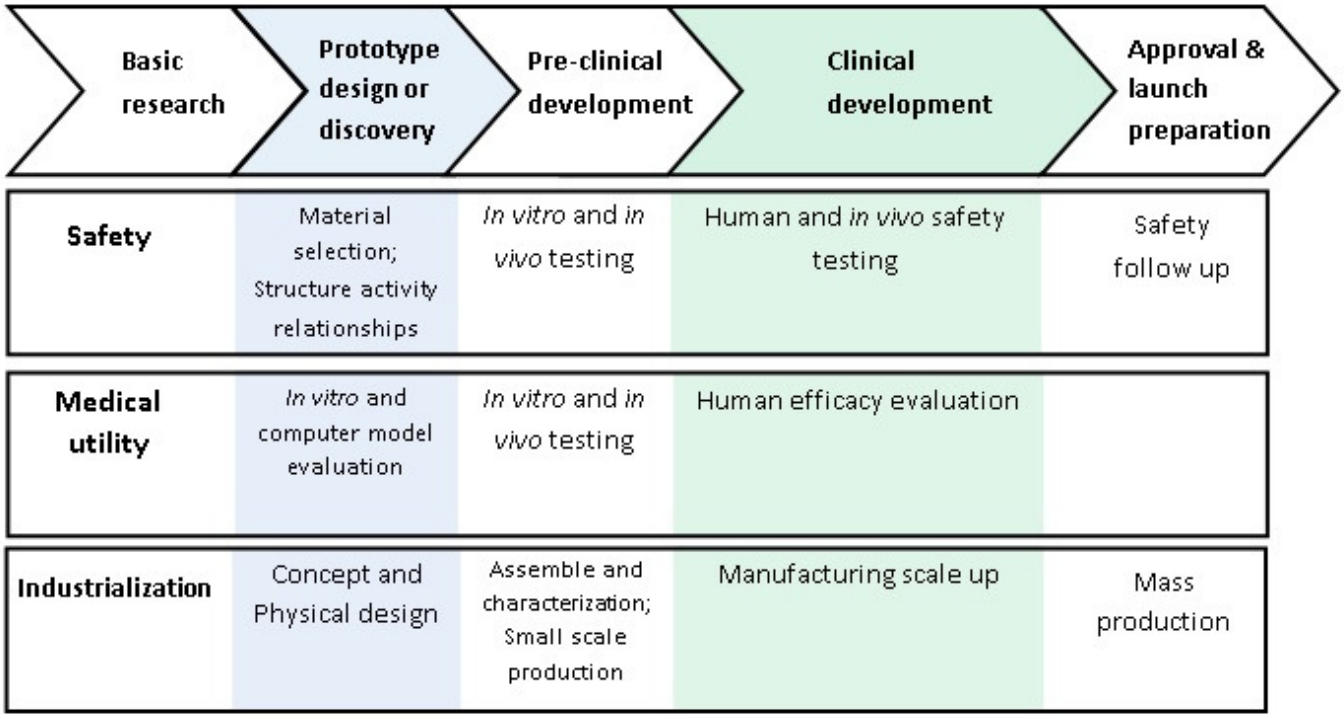

Figure 3.1. A generalised schematic displaying the critical path outlining the different phases of development and the typical actions taken to successfully launch a pharmaceutical product. Adapted from Sadrieh and Miller [91]

Pre-clinical development plan of Riminocelles™

Stage I.

Aim:

- Determine the optimal synergistic FRDC *in vitro* using a Pgp expressing cell line.
- Assess the MDR-circumventing spectrum of Riminophenazines using different ABC expressing neoplastic cells.

Objectives:

- Procure authenticated experimental drugs.
- Purify lead Riminophenazines.
- Determine IC_{50} values of the lead Riminophenazines and standard chemotherapeutics.
- Perform fixed molar ratio combination experiments - determine Combination Indexes (CI) and dose reduction afforded.

Checkpoint:

- Which drug combination possesses the most potential value?

Stage II.

Aim:

- Assemble and characterize a marketable, passively tumour-targeting co-formulation of the lead standard chemotherapeutic and Riminophenazine.

Objectives:

- Characterize and optimize drug loading, particle size, zeta potential and drug retention (under sink conditions) of the developed system.

Stage III.

Aim:

- Evaluate the pre-clinical *in vivo* safety and efficacy of the developed product in accordance with international regulatory guidelines.

Objectives:

- Assess acute toxicity parameters.
- Assess pharmacokinetic and organ accumulation parameters.
- Assess *in vivo* functioning of the developed drug delivery system.
- Evaluate efficacy using a relevant tumour model.
- Assess rodent repeat dose toxicity.

Check points:

- Is a 28-day repeat toxicity study in a non-rodent species warranted?
- Is further evidence of efficacy required?
- Phase II clinical trial?

Pre-clinical development plan of RiminoPLUS™ imaging

Aim:

- Develop a nano-sized emulsion entrapping Lipiodol (contrast oil), suitable for parenteral administration.

Objectives:

- Determine Riminophenazine solubility in Lipiodol.
- Create an excel sheet for simplified pre-calculated titration following the tie lines of a pseudoternary phase diagram.
- Characterise the formulation in terms of size using Dynamic Light Scattering (DLS).
- Evaluate the influence of co-surfactant ratio on size and stability.

4. Riminophenazine procurement, purification and authentication

4.1. Materials

Chemicals and reagents

Riminophenazines

Various batches (synthesised at various laboratories and at different times) of the possible lead Riminophenazines (B4112, B4121, B4125) were made available for investigation in this study. The various batches of interest were allocated unique identifier numbers that indicated their source of origin.

Methanol (MeOH), chloroform ($CHCl_3$) and deuterated chloroform ($CDCl_3$) were purchased from Merck (Darmstadt, Germany).

4.2. Authentication of Riminophenazine purity and integrity

To ensure integrity and validity throughout, it is prudent to authenticate and purify (if required) the investigational drugs prior to *in vitro* and *in vivo* investigations.

As a first step thin layer chromatograms (TLC) of all the different Riminophenazine batches of interest (Figure 4.1.) were run with an optimised mobile phase of Chloroform: Methanol (80:20) using normal phase plates (Alugram® SIL G/UV254, Macherey-Nagel). Qualitative visual comparisons were used to assess the impurity profile and conformity of the various Riminophenazine batches. Unfortunately, the precise date and site of synthesis for several of the stored batches were unavailable. Clofazimine (B663) was shown to be pure. However, several impurities were present for all the other available Tetramethylpiperidine (TMP) derivatives that necessitated purification prior to *in vitro* bioassays. Different batches of the same compound were shown to possess similar contamination profiles. Most of the major impurities (probably precursors or intermediates) were

seen to be more lipophilic and migrated further on a silica TLC plate than the compounds of interest.

After method development with using silica solid phase extraction (SPE) cartridges, normal phase preparative column chromatography was used to purify B4125.1. After preparing a silica (Silica gel 60, Merck, particle size 0.063-0.2 mm) slurry in $CHCl_3$ and carefully filling the column (60 cm x 3 cm) ensuring the removal of air bubbles through gentle taping of the chromatography column glass wall, a small volume of concentrated drug (in $CHCl_3$) was carefully loaded onto the surface of the column (that was protected from disturbance with non-adsorbent cotton wool). Elution of compounds was initiated with 100 ml of $CHCl_3$. Thereafter the % (v/v) of MeOH (in $CHCl_3$) was increased 10% every 100-250 ml. Many fractions (based in part on visual observation) were collected and an aliquot of each fraction again run on TLC (Figure 4.2.) to assess purity.

Clean fractions were pooled, an aliquot dried, reconstituted in $CDCl_3$ and sent to Dr. E. Palmer (Department of Chemistry, University of Pretoria) for ^{1}H and ^{13}C NMR analysis (Bruker Advance 500 MHz) to confirm compound authenticity (Figure 4.3).

To confirm compound purity, a preliminary HPLC method with a wide-polarity, gradient-elution scheme was developed on a C18 column (Figure 4.4 & 4.5). Elution was monitored in series by UV and MS detectors. Detection parameters were broad so as to detect the widest possible range of impurities. Direct infusion, Electrospray Ionization, Mass Spectrometry (ESI MS) was performed and confirmed drug structure through accurate mass and isotopic distribution (Figure 4.6.).

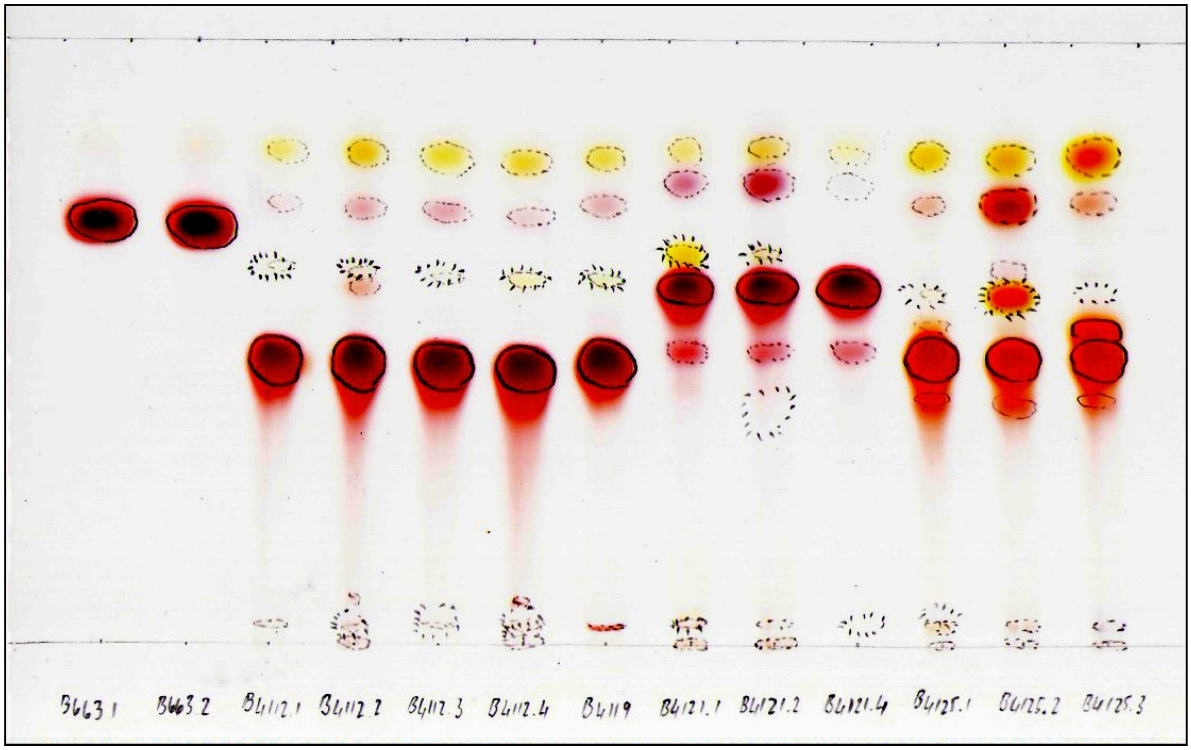

Figure 4.1. TLC chromatogram of the various batches of synthesised TMP-substituted Riminophenazines showing the impurity profile

Batch 1. Origin unconfirmed but used in previous studies; Batch 2. Irish reference sample; Batch 3. Synthesised by Dr. Patricia Gitare (PET laboratories, Little Company of Mary Hospital) in late 2007; Batch 4. Stored at 2-8℃ for prolonged periods. The various batches are denoted with corresponding suffixes.

Developed using $CHCl_3$: MeOH (80:20) on silica plates showed adequate resolution with the major compounds eluting at an R*f* of roughly 0.5. Visualised additionally under UV light - 254 (solid lines) and 360 nm (broken lines).

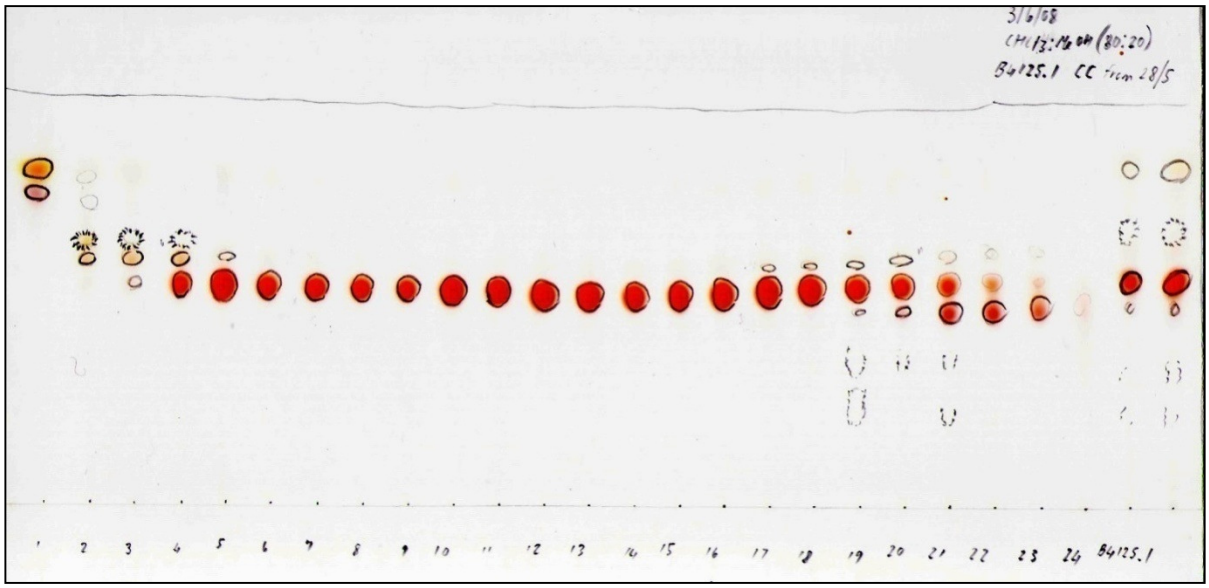

Figure 4.2. TLC chromatogram of the fractions of B4125.1 attained through column chromatography

Developed using $CHCl_3$: MeOH (80:20). Visualised under UV light, 254 and 360 nm.

Twenty four fractions were collected. Fractions 6-16 were pooled and gravimetrically shown to represent 75.8% (w/w) of the original dry starting material

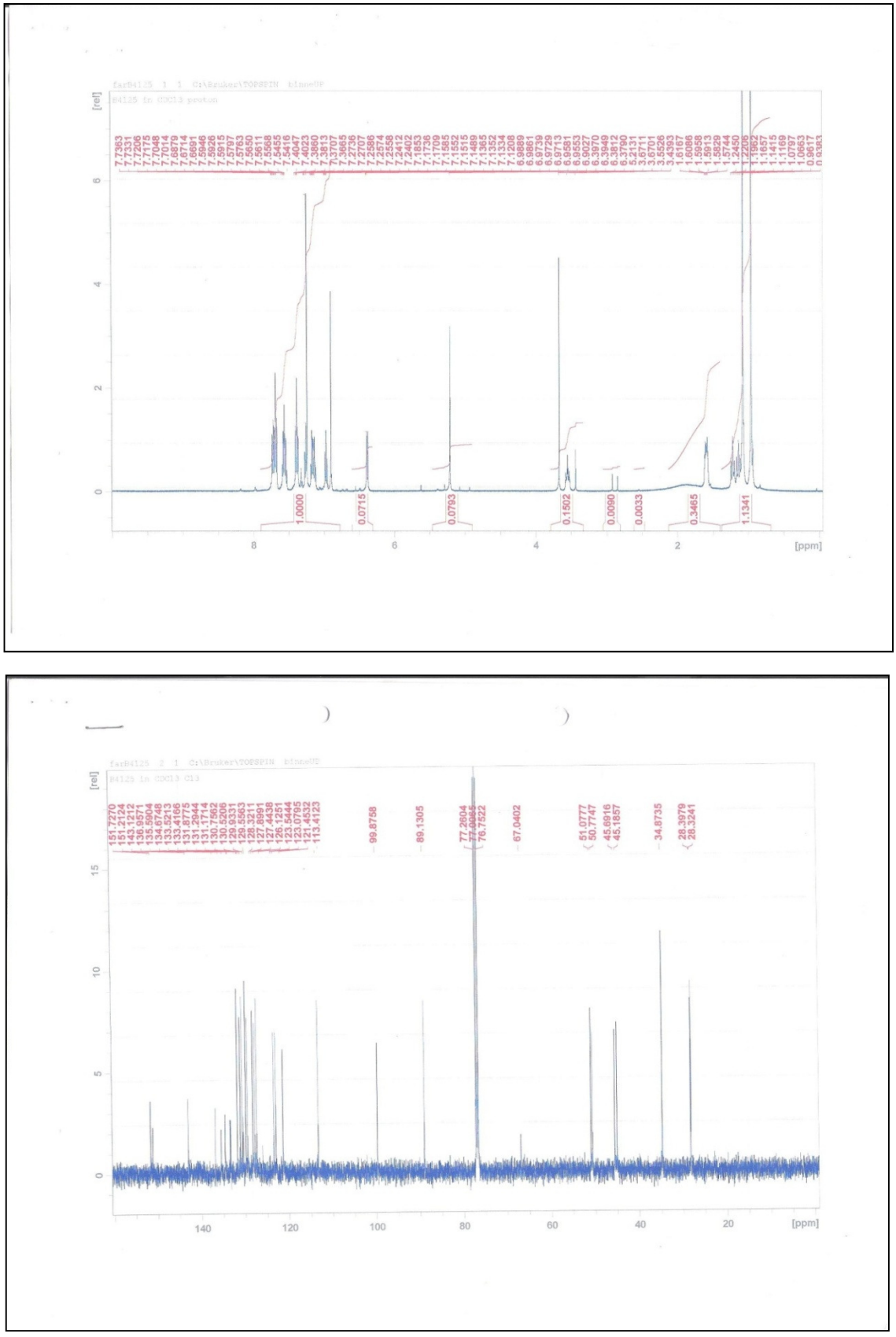

Figure 4.3. ^{1}H (above) and ^{13}C (below) NMR of B4125

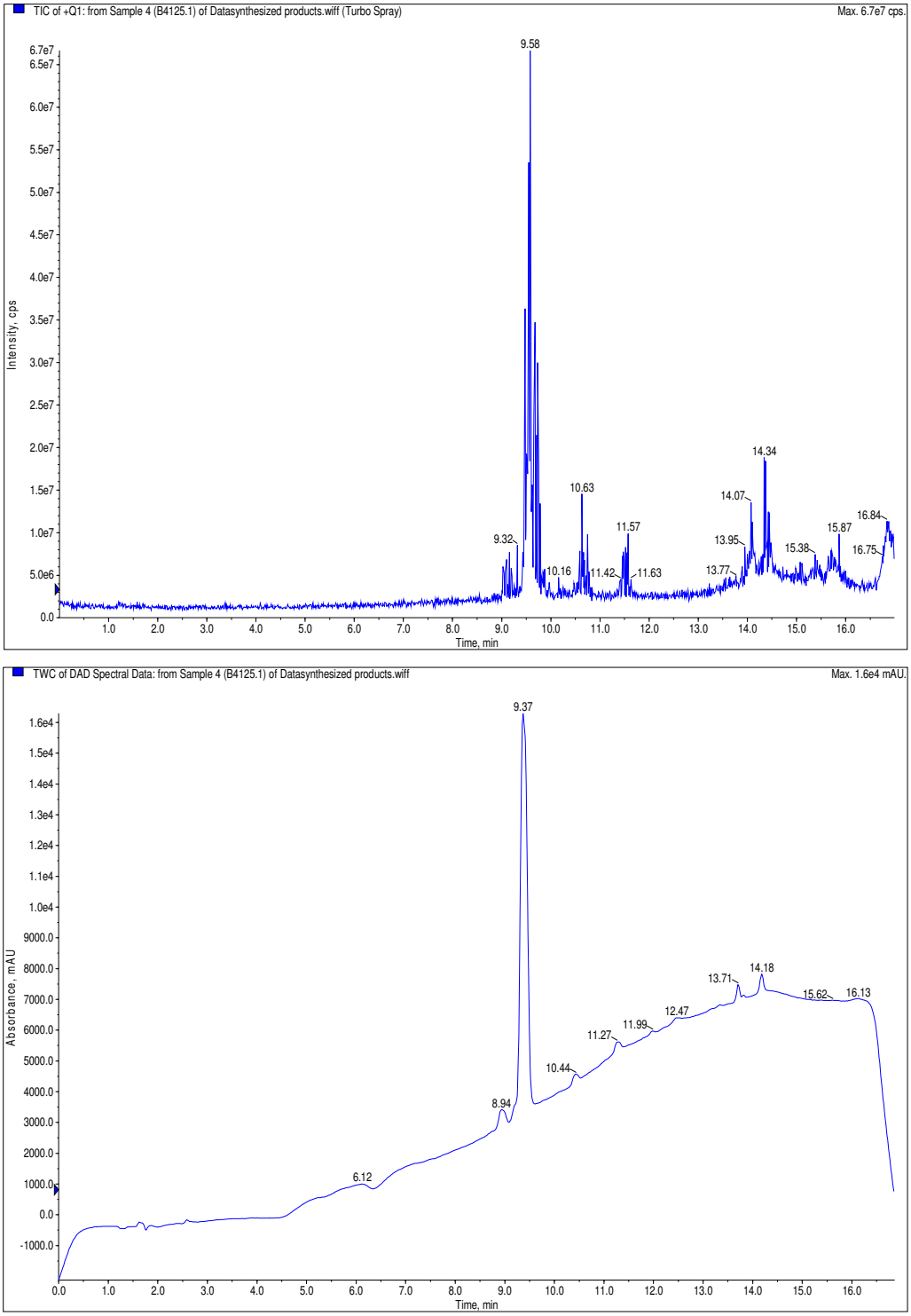

Figure 4.4. B4125.1 with minor impurities as shown through HPLC with MS (above) and UV (below) detection

Mobile phase: A, H_20 (0.1% HCO_2H); B, MeOH (0.1% HCO_2H). Elution: time 0 min, 25% B; time 2 min, 25% B; time 12 min, 100% B; time 14 min, 100% B; 15 min, 25% B; 17 min, 25% B. 5 µl injection of a 10 µg/ml solution in 25% MeOH (0.1% HCO_2H)

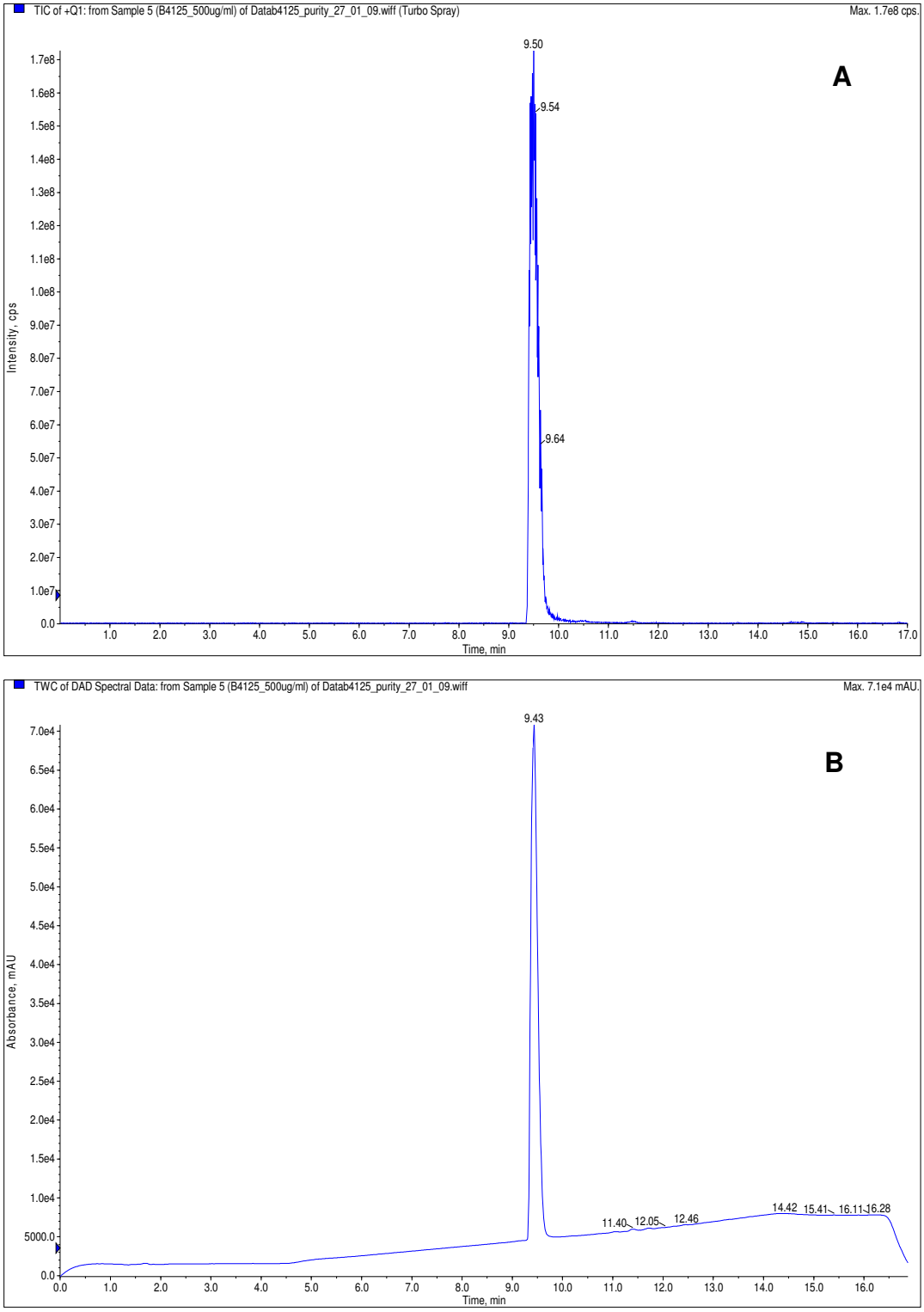

Figure 4.5. Purified B4125 after normal phase preparative column chromatography

A: Total ion chromatogram (100-1200 m/z)

B: Total wavelength chromatogram (UV 250-900 nm)

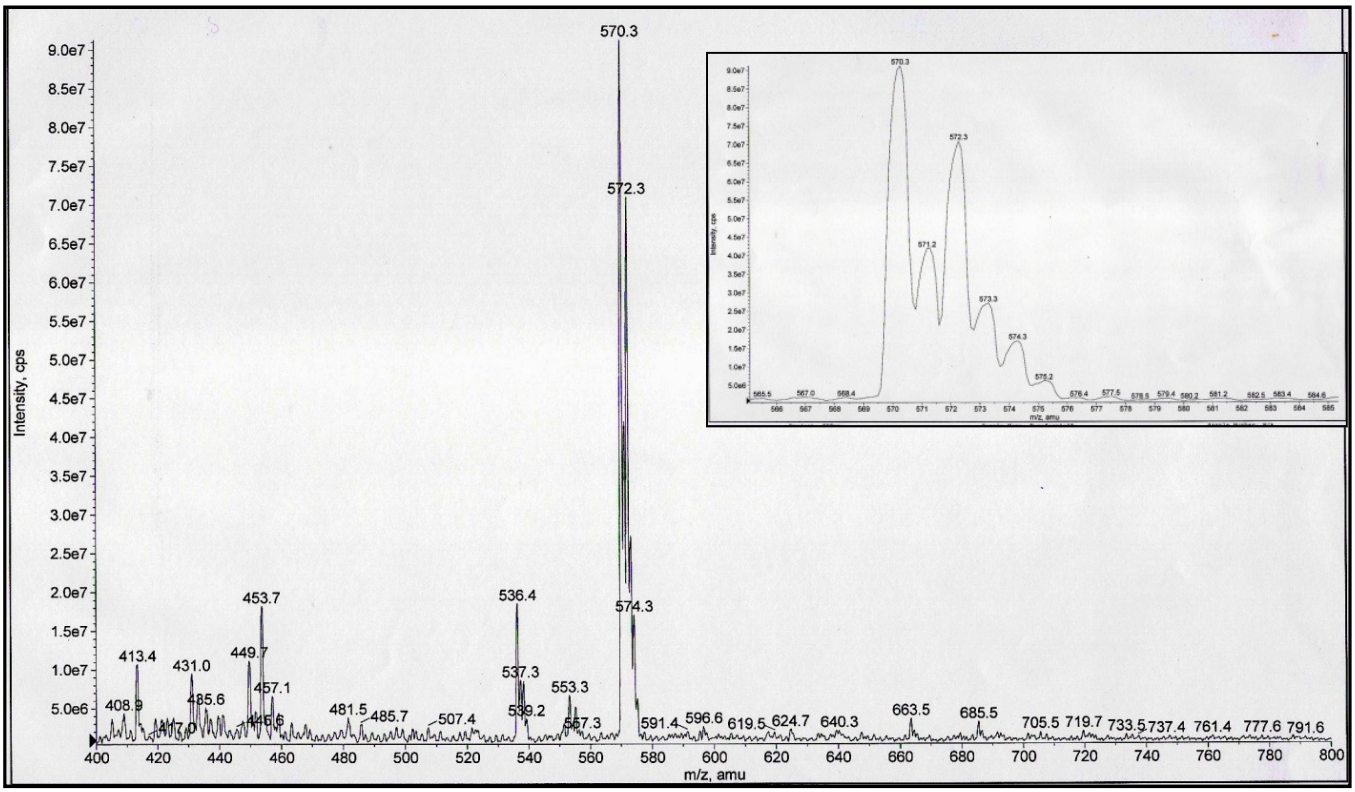

Figure 4.6. Direct infusion of B4125 into the ESI source displaying the expected mass to charge ratio 570.56 m/z and the inset showing the isotopic distribution of the 570.56 peak