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Microbial enantioselective synthesis of a key intermediate for Diltiazem.

TESI MAGISTRALE IN CHEMICAL ENGINEERING

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Abstract: The production of a key intermediate for the synthesis of Diltiazem was attempted by screening various microorganisms for the reduction of the corresponding precursor. The target product, characterized by (2*S*,3*S*) stereochemistry, was obtained in different amounts and purities. The outcomes of the microbial reactions were characterized in terms of conversion, stereoselectivity, and purity of the product. Upon asymmetric reduction, 40 mg of substrate in N,N-dimethylformamide solution was reduced with up to 99% conversion and >95% enantiomeric excess. In conclusion, microbial asymmetric reduction proved to be an efficient method in terms of conversion, low toxic waste, and lower cost of starting materials.

Key-words: diltiazem; chiral drug; asymmetric reduction; microbial conversion; biotransformation; enzymes;

1. Introduction

1.1 Diltiazem: a description

Diltiazem hydrochloride is a global drug commercially known as Cardizem whose IUPAC name is (2*S*,3*S*)-3-acetoxy-5-[2-(di-methylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one hydrochloride; its structure is shown on Figure 1 below [1]. In the early 1960s, a drug discovery program motivated vast majority of pharmaceutical companies to search for compounds targeting the improvement of coronary circulation for the treatment of cardiovascular diseases and angina. As a result, many drugs have seen light, including the benzothiazepine diltiazem, synthesized by the Japanese company Tanabe [2][3].

This substance belongs to the class of medications of calcium channel-blockers and is used to treat high blood pressure and angina by reducing heart rate and supplying enough oxygen and blood to it. [1]

The key intermediate compound for the synthesis of Diltiazem is a substituted 1,5-benzothiazepine with two chiral centers at C-2 and C-3 positions. Thus, 4 optical isomers are possible, out of which only (2*S*,3*S*) isomer possesses coronary vasodilating activity (Figure 2).

1.2 Conventional synthesis

The production of Diltiazem can be carried out conventionally by asymmetric reduction[4] or optical resolutions of target intermediates[5].

Several alternative routes were investigated due to clinical importance of diltiazem intermediate, which include asymmetric catalytic reduction[6], chemical optical resolution[7], asymmetric dihydroxylation[8], asymmetric hydrocyanation[9], Darzens reaction[10] and Michael addition[11].

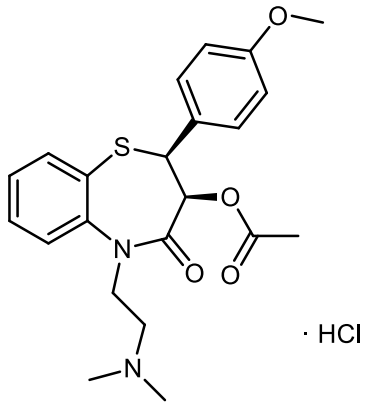

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Figure 1. Diltiazem structure

Biocatalytic strategies have also been proposed for the stereoselective step of the synthesis. In particular, for what concerns the synthesis proposed and patented by the Japanese company Tanabe [5] a stereo controlled reduction step has been individuated as the key passage in the synthesis (Figure 2), permitting to introduce two chiral centers at the same time.

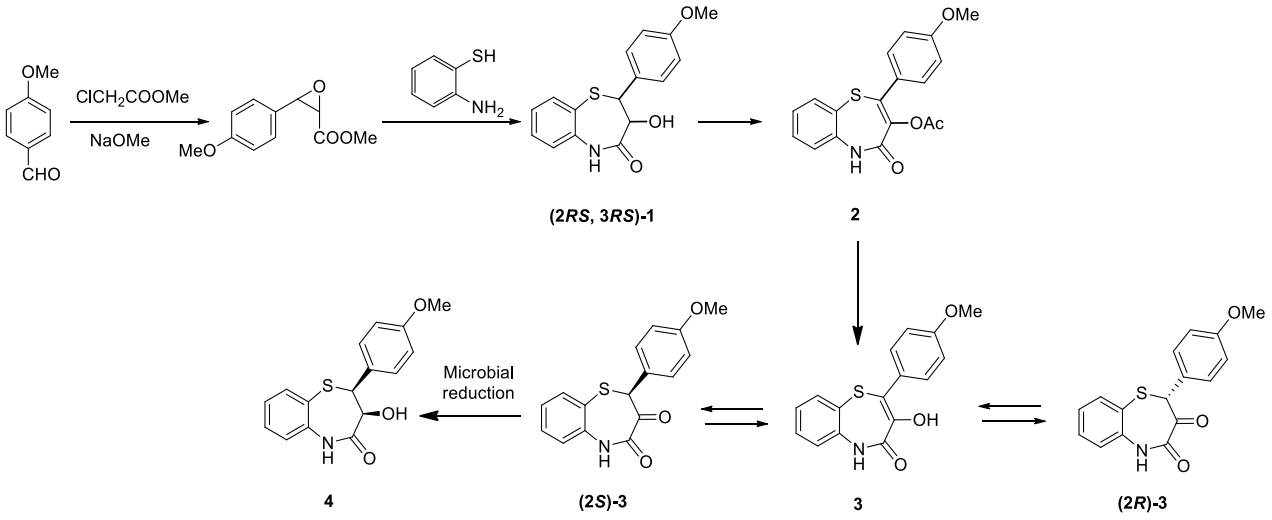


Figure 2. Industrial synthesis patented by Tanabe [12].

Since enzymes able to mediate reductions (i.e. reductases) are ubiquitous in the living organisms, a wide range of yeasts, bacteria, molds and actynomycetes have been investigated for their ability to afford compound **4** starting from the precursor **3** [12][13].

As highlighted in Figure 2, since the ketone undergoes a rapid keto-enol tautomerism, the chiral center adjacent to sulfur atom, at physiological conditions, is continuously racemized, meaning that the reduction is not merely a kinetic resolution, but rather a dynamic kinetic resolution. This allows the system to overcome the 50% limitation typical of conventional kinetic resolutions and potentially permits the quantitative obtainment of the desired product. This dynamic process is essential for achieving high yields and enantiomeric excess in the synthesis of the Diltiazem intermediate.

2. Aim of the work

The aim of this research is to screen a series of microorganisms not previously employed for attempting the selective synthesis of the Diltiazem intermediate 3-hydroxy-2-(4-methoxyphenyl)benzo[b][1,4]thiazepin-4(5H)-one from the corresponding ketone.

In particular, a number of Biosafety Level 1 (BSL-1) microbial agents, already considered or employed in the food industry, have been screened for their ability to efficiently perform the target reaction with high efficiency and stereoselectivity.

3. Results and Discussion

3.1 Characterization of starting material

The starting ketone **3** was an actual industrial intermediate provided by company Flamma SpA in three different batches we labelled as Batch A, Batch B and Batch C: as such, the chemical characterization of the three mixtures was absolutely necessary in order to provide a standardized material for the screening.

Table 1. Composition of the starting material.

Starting material	Ketone (%)	Alcohol (%)	Sulfoxide (%)
Batch A	40.4	43.1	13
Batch B	77.8	1.9	11.1
Batch C	85.1	2.6	3.6

Given the composition of three batches, it was possible to direct employ batch B and C for the screening, whereas batch A required purification.

3.2 Chemical synthesis of rac-*cis*-4

A sample of the racemic product rac-*cis*-4 was necessary as a reference for chiral and non-chiral HPLC analyses. This reference compound was obtained through chemical reduction using sodium borohydride, $NaBH_4$[14][4]. Such a reaction is, naturally, not enantioselective albeit being diastereoselective: the majority of the product is constituted by the racemic *cis* isomer.

In terms of optical purity and characterization, the optical forms of product were detected by 2 peaks at non-chiral HPLC and 2 peaks of alcohols in chiral HPLC reports shown below in Figure 4.

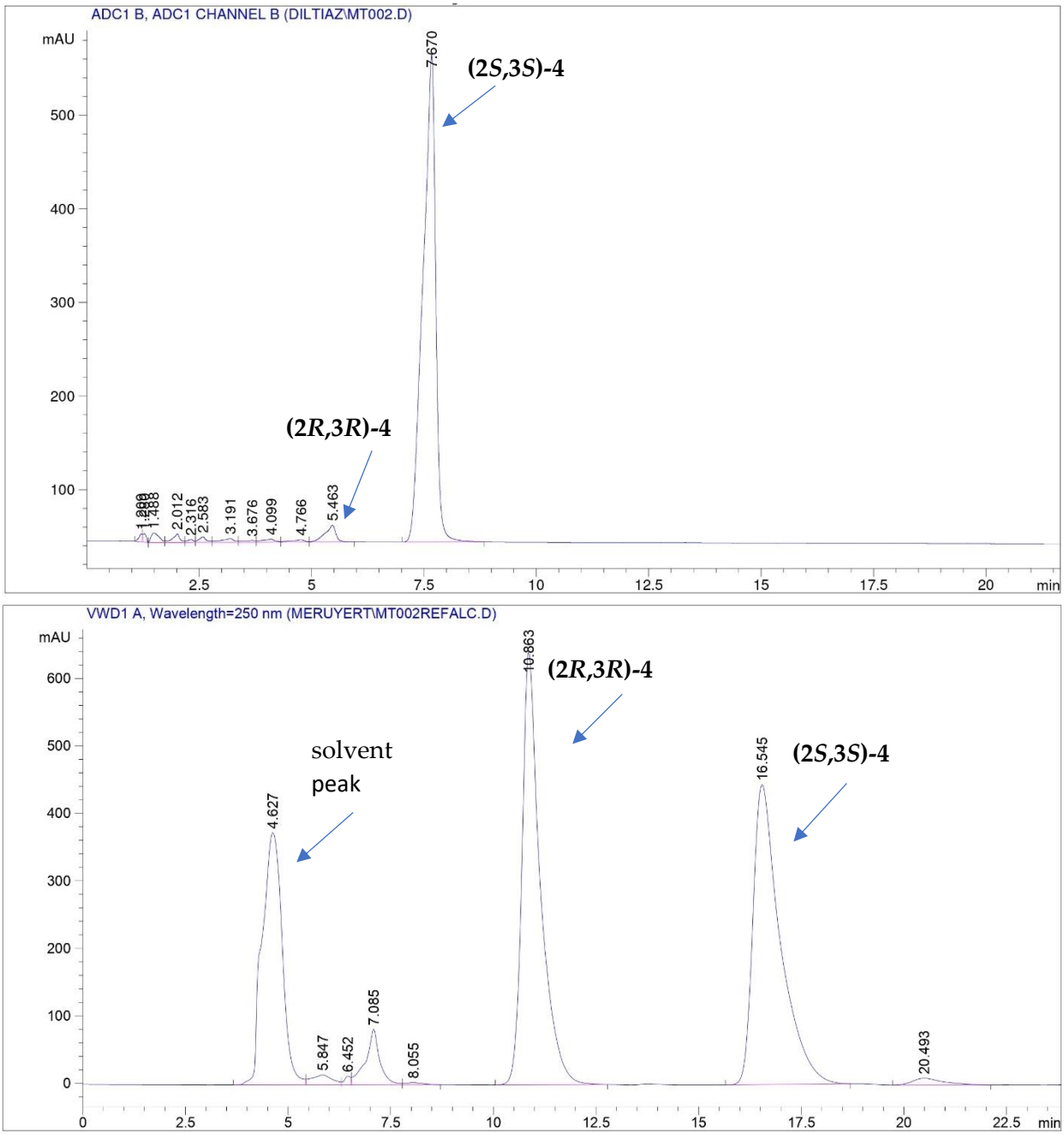


Figure 3. (Top) Non-chiral HPLC signals of chemically synthesized alcohols. (Bottom) Chiral HPLC signals of chemically synthesized alcohols.

3.3 Biotransformations

Due to the fact that starting material is practically insoluble in aqueous solutions, polar organic solvents had to be used to increase the solubility and enable the biotransformation. The best solvent resulted to be N,N-dimethylformamide (DMF); other attempts included dimethyl sulfoxide, methanol, ethanol, acetonitrile or γ-valerolactone. [5].

The starting material was dissolved in DMF to be added to the microorganisms' cultures. When screening was carried out using 13 microorganisms several ones were found to have the capacity of asymmetric reduction of **3**. As a result, starting material was reduced by most of the microorganisms as **4** was selectively produced as shown in Table 1. The *Saccharomyces cerevisiae* was known to give very good results and was added to the screening as a benchmark for evaluating the outcomes.

Microorganisms, used for reducing **3** to **4**, were widely distributed among yeasts belonging to the genus *Candida, Pichia, Saccharomyces, Rhodosporidium,* bacteria belonging to the genus *Bacillus* and mold belonging to the genus *Penicillium.* The culture of above listed microorganisms can be cultured in corresponding to it mediums, containing carbon sources, nitrogen sources or salts, at temperatures from 20 to 40 °C under aerobic conditions and constant shaking. The concentration of the substrate was optimally chosen at 40 mg/1.2 ml according to previous studies and kept for 24 h and 48 h at 24 °C. After the reaction was completed, the desired optically active compound was obtained and the highest enantiomeric selectivity was observed in *Saccharomyces cerevisiae, Pichia pastoris, Torulaspora delbrueckii, Saccharomyces bayanus, Pediococcus pentosaceus and Lactobacillus rhamnosus*.

Among them, *Saccharomyces cerevisiae* had the highest capability of asymmetric reduction with high optical purity, 99% conversion, >95% enantiomeric excess. Interestingly, *Penicillium camemberti* gave the opposite enantiomer as the major product.

Table 2. Screening of microorganisms for asymmetric reduction.

	Microorganism	Strain	Conversion (%)	Sulfoxide (%)	d.e. (%)	e.e. (%)
1	*Saccharomyces cerevisiae*	Type II YSC2	99.9	3.7	95	97
2	*Torulaspora delbrueckii*	DSM 70483	95.2	4.2	99.2	96.4
3	*Pichia pastoris*	DSM 70382	96.1	9.9	-	97.6
4	*Starmerella bombicola*	DSM 27465	62.7	26.1	40.6	96
5	*Geotrichum candidum*	DSM 10452	54.6	52.8	96.8	-
6	*Penicillium camemberti*	DSM 1233	96.1	86.6	80.9	- 56.6
7	*Cryptococcus curvatus*	DSM 70022	69.7	88.7	68.4	-
8	*Yarrowia lipolytica*	DSM 70562	87.3	81.4	71.5	-
9	*Lactobacillus rhamnosus*	DSM 53103	91.6	47.1	- 71.7	98
10	*Lipomyces starkeyi*	DSM 70295	97.6	24.5	2.4	89.3
11	*Rhodosporidium toruloides*	DSM 4444	34.8	97.8	61.7	-
12	*Pediococcus pentosaceus*	DSM 20336	89.1	25.7	91.2	99
13	*Saccharomyces bayanus*	DSM 70412	100	1.9	46.7	96.5

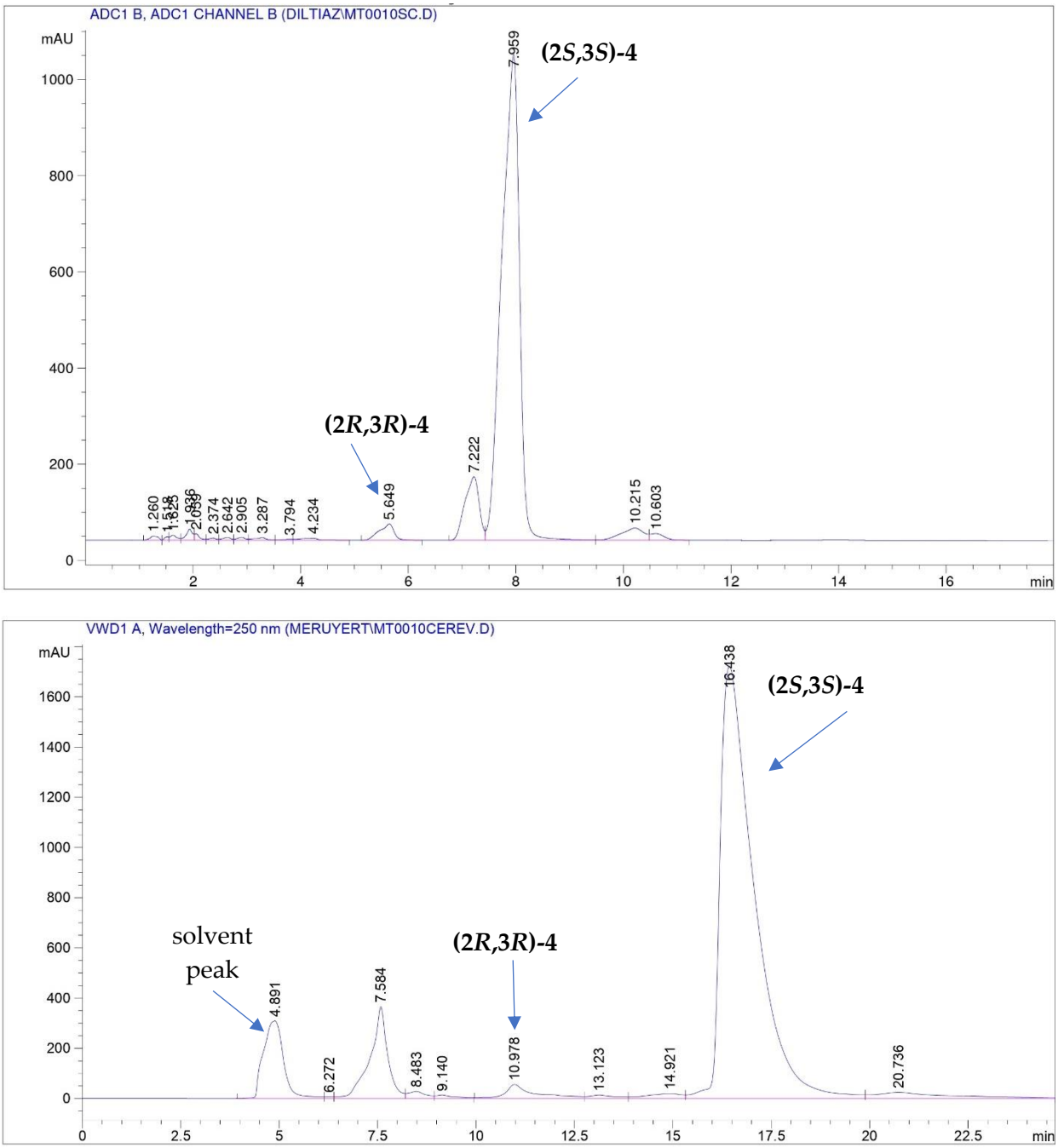


Figure 4. (Top) Non-Chiral HPLC signals of *Saccharomyces cerevisiae* alcohol product. (Bottom) Chiral HPLC signals of *Saccharomyces cerevisiae* alcohol product.

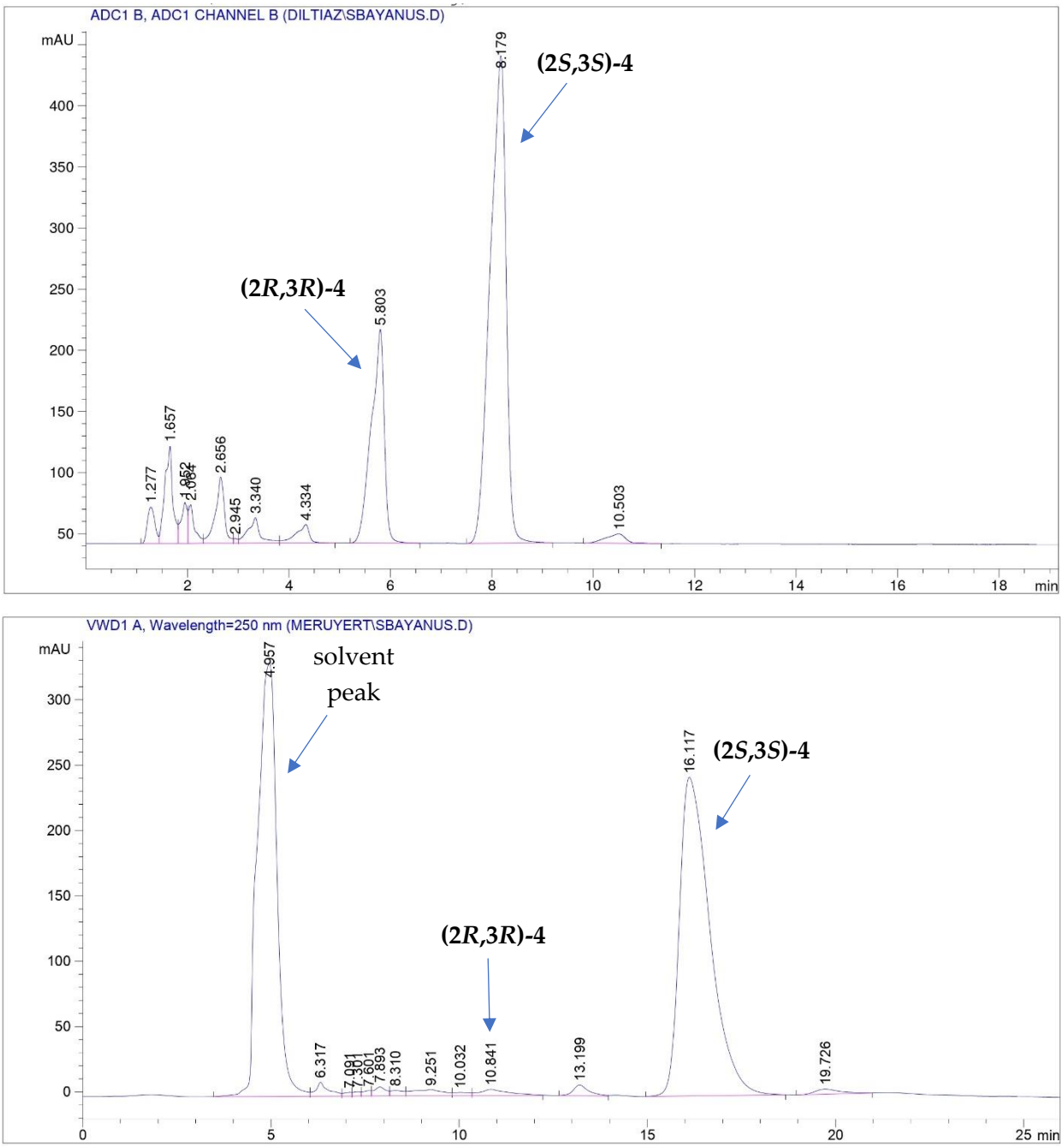
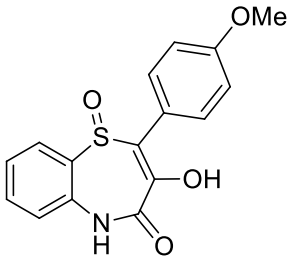


Figure 5. (Top) Non-Chiral HPLC signals of *Saccharomyces bayanus* alcohol product. (Bottom) Chiral HPLC signals of *Saccharomyces bayanus* alcohol product.

3.4 Identification of by-products

While attempting to increase the conversion yield, it has been found that the formation of by-products lowered the conversion of **3** to **4**, the main by-products being sulfoxide compound and *trans* alcohol form. In particular, the sulfoxide was detected by non-chiral HPLC analysis and further separated from *Yarrowia lipolytica* biotransformation mixture by chromatography. The by-product was obtained in crystalline form and characterized by HPLC and NMR analysis (Figure 6).



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3-hydroxy-2-(4-methoxyphenyl)benzo[*b*][1,4]thiazepin-4(5*H*)-one 1-oxide

Figure 6. Sulfoxide **6** structure.

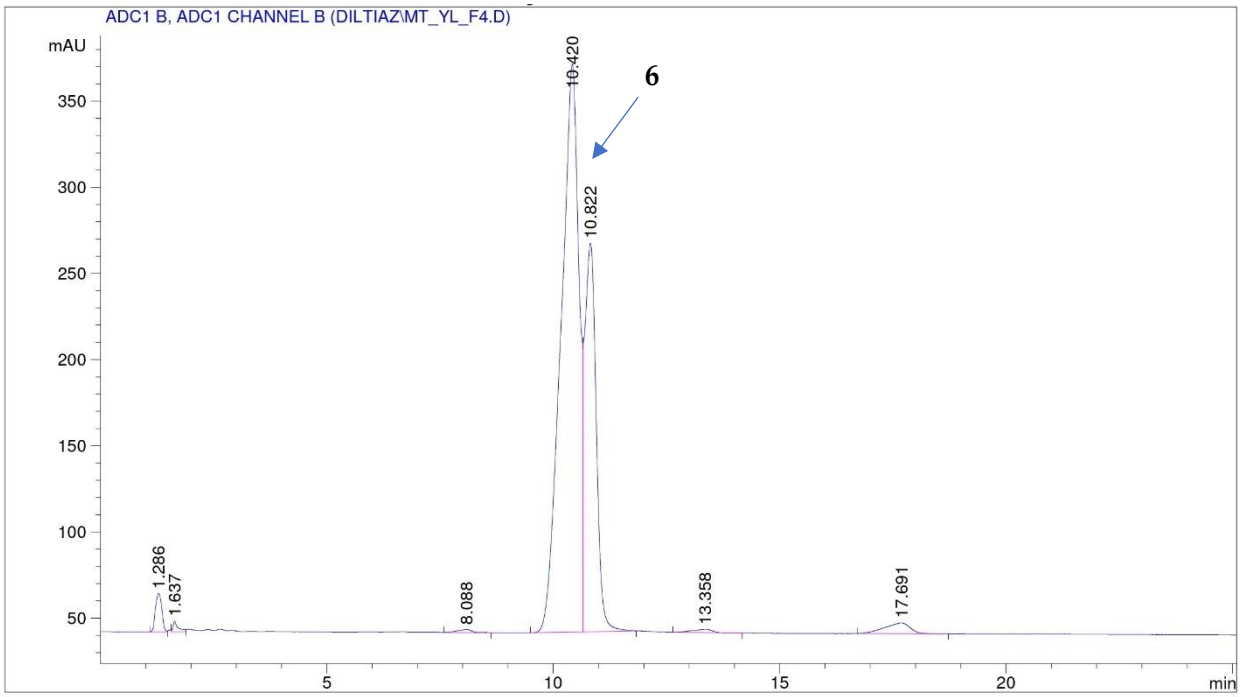

Figure 7. Non-chiral HPLC signal of sulfoxide by-product **6** separated from *Yarrowia lipolytica* biotransformation extract.

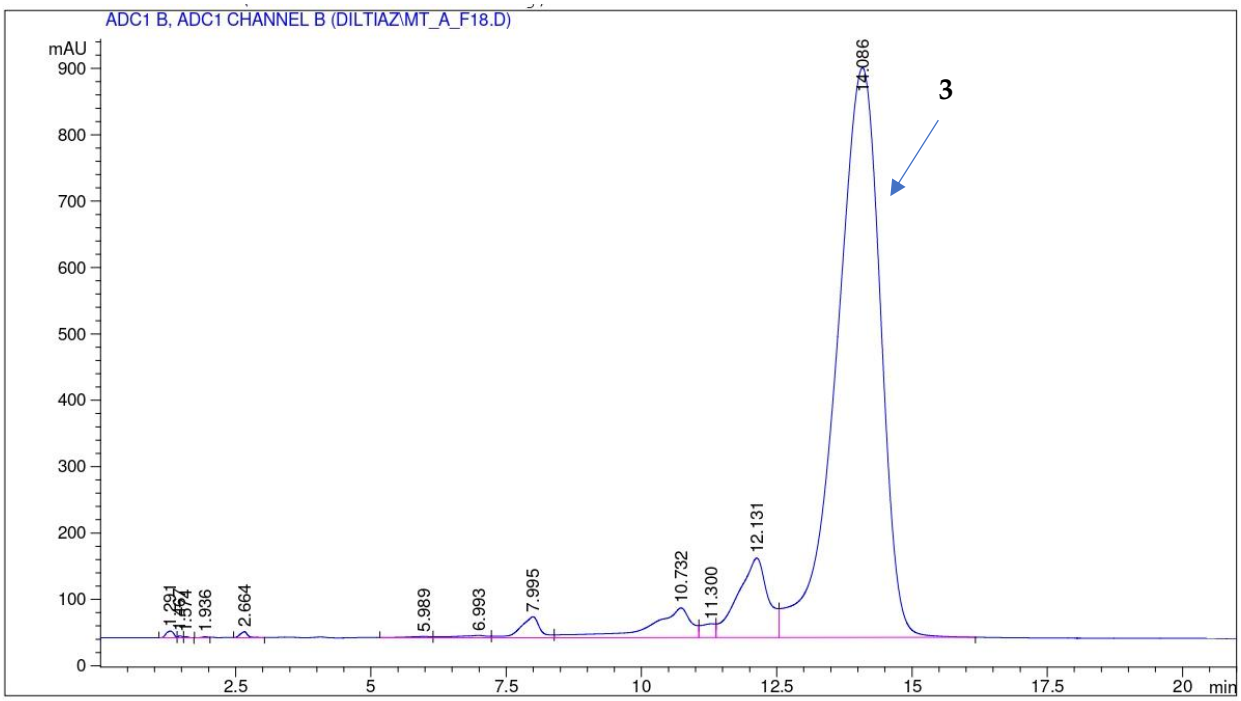

Figure 8. Non-chiral HPLC of purified ketone **3** fraction.

3.5 Oxidation of alcohol (batches A and C)

As mentioned in Paragraph 3.1, Batch A was a roughly 1:1 mixture of ketone and alcohol. Therefore, the mixture has been purified by chromatography and the racemic alcohol rac-*cis*-4 was subjected to chemical oxidation to provide further material for the biotransformation. After a partially successful attempt with 2-iodoxybenzoic acid (IBX), affording the ketone in 56% conversion, the best conditions were found to be those mentioned in [12], which permitted to produce the pure ketone in quantitative yield. In particular, the racemic alcohol rac-cis-**3** underwent oxoacylation by a mixture of DMSO, Ac_2O and pyridine to afford the acylated enol **2**.

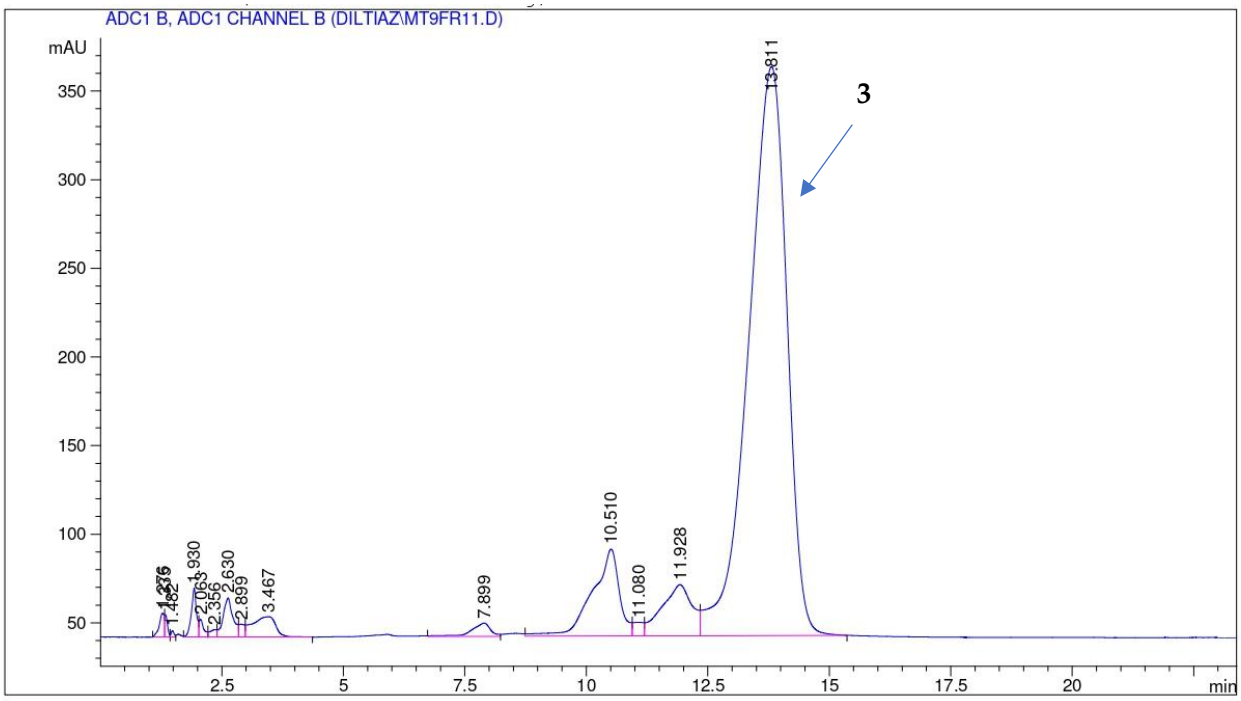

Figure 9. Non-chiral HPLC of obtained ketone **3** from oxidation of batch C.

4. Materials and Methods

4.1 General methods

Biotransformations were run in a INNOVA 42 Thermoshaker (New Brunswick Scientific, Enfield, CT, USA). Growth media sterilization was carried out with LIARRE JUNO Vertical-Lab Clave.

GC-MS analysis employed a HP-5MS column (30 m x 0.25 mm x 0.25 μm, Agilent Technologies Italia S.p.A., Cernusco sul Naviglio, Italy). The following temperature program was employed: 60 °C (1 min), 6 °C/min, 150 °C (1 min), 12 °C/min, 280 °C (5 min). Samples were derivatized with a silylating mixture consisting of pyridine, hexamethyldisilazane and trimethylchlorosilane 9:3:1 in volumetric ratio. In a GC vial 10 mg of compound were treated with 150 μL of silylating mixture and left 30 mins at room temperature before analysis.

TLC analyses were performed on Macherey-Nagel precoated TLC sheets Polygram SIL G/UV254 purchased from Chimikart s.r.l. (Naples, Italy).

The characterization of products was performed through ^{1}H-NMR and ^{13}C-NMR on a 400 MHz spectrometer (Bruker, Billerica, MA, USA) in $CDCl_3$ solution. The chemical shift scale was based on tetramethyl silane.

Optical purity was measured by HPLC (non-chiral and chiral) using Kinetex C18 (150 × 2.1 mm, 2.6 μm) column and Chiracel OD-H column (150 × 4.6 mm, 5 μm, Daicel Chemical industries, Ltd, Tokyo) under the following conditions:

Non chiral HPLC: the sample was dissolved in methanol, eluent was KH_2PO_4 10 mM pH = 3 and acetonitrile (7:3), flowrate = 1 ml/min, wavelength 250 nm, temperature 40 °C.

Chiral HPLC: the sample was dissolved in ethyl acetate, eluent was hexane: isopropanol (8:2), flowrate = 0.8 ml/min, wavelength 250 nm, room temperature.

4.2 Chemicals

Starting material was provided by Flamma SpA (Chignolo D'Isola, Italy). All other chemicals were bought from Fisher Scientific (Segrate, Italy) and were of reagent grade.

4.3 Chemical reduction of 2-(4-methoxyphenyl) benzo[b][1,4]thiaepine-3,4(2H,5H)-dione:

A sample of Batch C (200 mg, 1 mmol) was dissolved in 9:1 ethanol/water (10 ml) in an ice bath at 0 °C; then, $NaBH_4$ (38 mg, 0.75 mmol) was added, keeping ice bath for 10 minutes and keeping the mixture at room temperature for another 30 min. The reaction was monitored by TLC (dichloromethane/methanol 95:5) until the starting material had disappeared. After completion of the reaction, the product was extracted with 0.1 N HCl and ethyl acetate; the organic phases were brought together, dried with sodium sulfate and evaporated; the crude residue was 160 mg. Its identity was characterized by non-chiral HPLC and chiral HPLC, NMR (H and C) spectroscopy. Non chiral HPLC, retention times: 5.5 min for *trans*-**4**, 7.6 min for *cis*-**4**. Chiral HPLC, retention time: 10.8 min for *trans*-**4**, 16.5 min for *cis*-**4**. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.84 (s, 1H), 7.68 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.53 – 7.44 (m, 2H), 7.40 (td, *J* = 7.7, 1.6 Hz, 1H), 7.23 (dd, *J* = 7.6, 1.4 Hz, 1H), 7.12 (dd, *J* = 7.8, 1.4 Hz, 1H), 6.94 – 6.82 (m, 2H), 5.08 (d, *J* = 6.8 Hz, 1H), 4.48 (dd, *J* = 9.1, 6.8 Hz, 1H), 3.81 (s, 3H), 2.92 (d, *J* = 9.4 Hz, 1H) (Figure 10).

4.4 Microorganisms growth

A 250 ml flask for each biotransformation was sterilized in autoclave. Next, the medias were also prepared and sterilized according to recipe and type of strain as in Table 3.

YUM media: 1 L deionized water, yeast extract (3 g/L), malt extract (3 g/L), glucose (10 g/L) and peptone from soybeans (5 g/L).

MRS broth media: 1 L deionized water, peptone (10 g/L), meat extract (8 g/L), yeast extract (4 g/L), D(+)-Glucose (20 g/L), dipotassium hydrogen phosphate (2 g/L), triammonium citrate (2 g/L), sodium acetate (5 g/L) magnesium sulfate (0.2 g/L), manganous sulfate monohydrate (0.05 g/L), sodium thioglycolate (1.2 g/L), cysteine (1.8 g/L), resazurin (1 mg), and filled with N_2.

Table 3. Microorganisms and medium used for them.

	Microorganism	Medium	Temperature (°C)
1	*Saccharomyces cerevisiae*	YUM	24
2	*Torulaspora delbrueckii*	YUM	24
3	*Pichia pastoris*	YUM	24
4	*Starmerella bombicola*	YUM	24
5	*Geotrichum candidum*	YUM	24
6	*Penicillium camemberti*	YUM	24
7	*Cryptococcus curvatus*	YUM	24
8	*Yarrowia lipolytica*	YUM	24
9	*Lactobacillus rhamnosus*	MRS	24
10	*Lipomyces starkeyi*	YUM	24
11	*Rhodosporidium toruloides*	YUM	24
12	*Pediococcus pentosaceus*	MRS	37
13	*Saccharomyces bayanus*	YUM	24

Some microorganisms needed to be cultured from pre-inoculum form. Under sterile biohood, petri dish containing spores of microorganisms was opened, then using 1-2 ml of sterile water rinsed on top of surface and gently scratched to collect all spores and then brought with pipette to new sterile tube. The presence of spores was checked under a microscope.

After sterilization, each flask was filled with 100 ml of medium. Each pre-inoculum of microorganism was taken as 2 ml and placed into each flask containing medium. In the case of *Penicillium camemberti*, its pre-inoculum contained solid pieces, so it was taken solid with 0.5 ml. Each flask was properly closed with cork and taped with film. All flasks with YUM medium microorganisms are capable of growth at room temperature, they were placed under constant shaking into incubator for 24-48 h. MRS medium microorganisms were put at higher temperature (37 °C).

4.5 Biotransformation of microorganisms by 2-(4-methoxyphenyl) benzo[b][1,4]thiaepine-3,4(2H,5H)-dione

After the strains were grown in the given mediums, the reaction was initiated under sterile conditions by addition of substrate: solution was prepared using 400 mg of ketone **3** and 12 ml of DMF. In each flask of microorganism was added 1.2 ml (containing 40 mg of substrate), sealed and left for 48 h at 25 °C under constant shaking (300 strokes per minute). After 24 h the reaction was checked by TLC hexane: ethyl acetate (6:4). In the case of *Lactobacillus rhamnosus* and *Pediococcus pentosaceus*, MRS broth volume was 200 ml and 80 mg of substrate were injected into flask with silicone cap.

Upon reaction completion, the mixture was added with ethyl acetate (40 ml), and mixed. The cells were easily removed by adding some Celite directly into flask reaction and further by filtration of a Celite pad in a Buchner funnel, the cake was further washed with ethyl acetate and water. The phase separation was facilitated by addition of NaCl. The desired organic phase was collected and dehydrated by anhydrous sodium sulfate and evaporated in rotary evaporator. The extract product was weighted and further used in analytical characterization by non-chiral HPLC in general: retention time, 5.5 min for (trans)-4, 7.5-7.8 min for (cis)-4, 10.1-10.7 min sulfoxide impurity, 13-14 min starting material (if unconverted). Chiral HPLC: retention time, 4.6-4.9 min for AcOEt sample solvent, 10.8 min for (trans)-**4**, 14.2-15 min for **3** (present if unconverted fully). 16.5-17.2 min for (cis)-**4**.

4.6 Sulfoxide separation

Yarrowia lipolytica biotransformation extract (20 mg) was dissolved in acetone (1 ml) and silica fpr chromatography (100 mg) was added; the mixture was dried in rotary evaporator and chromatographically separated. The product was weighted (6 mg), characterized by H-NMR (Figure 11) and further used in analytical characterization by non-chiral HPLC: retention time, 10.4-10.6 min.

4.7 Purification of starting material

Starting material from batch A (1 g) was dissolved in acetone (2 ml) and silica for chromatography (5 g) was added, then it was dried in rotary evaporator till powder, and purified by column chromatography. The resulting product was weighted and further used in analytical characterization by non-chiral HPLC: retention time, 13.9 min for **3**.

4.8 Oxidation of alcohol in starting material (IBX method)

According to literature method [15], the rac-*cis*-**4** alcohol (82 mg, 272.1 μmol) was dissolved in 1 ml DMSO; in a separate Eppendorf tube IBX (114 mg, 408.1 umol, 1.5 eq.) was dissolved in 1ml DMSO, then both solutions were mixed in small round bottom flask with magnetic stirring at room temperature. The reaction was monitored by TLC every 30 minutes until the starting material was fully converted. After 2 h, the additional IBX (57 mg) was added to the reaction. As a result, the reaction was completed in 4-5 hours. The reaction was stopped by addition of water and ethyl acetate. It formed white foam and precipitate as IBX is not soluble in water, therefore the solution was filtered through Buchner funnel and IBX was recovered as a powder. The collected solution was further extracted through usual phase separation with water and ethyl acetate, washed 3 times with water to eliminate methyl sulfoxide formed. The desired organic phase was collected and dehydrated by anhydrous sodium sulfate and the ethyl acetate was removed in rotary evaporator. The extract product gave a mass of 53.1 mg of ketone, 65% yield. The product was weighted and

further used in analytical characterization by non-chiral HPLC in general: retention time, 13.9 min for **3**.

4.9 Oxidation of alcohol in starting material (Swern oxidation)

According to the literature [14], substrate rac-*cis*-**3** from batch C (200 mg) was dissolved in DMSO (500 μl) and Ac_2O (100 μl), was added with catalytic amount of pyridine (5 μL) in a small round flask with stirring bar. The reaction was monitored by TLC until the starting material was fully converted in span of 4-5 hours. After the reaction finished, HCl (10 ml) was added to eliminate pyridine and AcOEt (10 ml) was added for extraction of organic phase. The organic phase was dried on sodium sulfate and evaporated. The crystalline material of 170 mg, 85% yield was collected and tested on TLC and characterized. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.89 (s, 1H), 7.67 – 7.57 (m, 3H), 7.43 – 7.34 (m, 1H), 7.20 (ddd, *J* = 8.2, 6.2, 1.5 Hz, 2H), 6.93 – 6.85 (m, 2H), 3.84 (s, 3H), 2.06 (s, 3H) (Figure 12).

A suspension of **2** (170 mg) in MeOH (1 ml) was mixed and then solution of NaOH (300 μl, 4 M) in H_2O (300 μl) was added. The solution was allowed to stir at room temperature for 2 h. The reaction was neutralized by the addition of HCl (5-10 ml) and extracted with AcOEt. The product was weighted and further used in analytical characterization by non-chiral HPLC in general: retention time, 14 min for **3**. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.97 (s, 1H), 7.70 (dd, *J* = 7.7, 1.2 Hz, 1H), 7.44 (td, *J* = 7.7, 1.5 Hz, 1H), 7.25 (td, *J* = 6.6, 6.2, 1.3 Hz, 1H), 7.23-7.18 (m, 3H), 6.89 – 6.85 (d, J = 6.9 Hz, 2H), 5.44 (s, 1H), 3.79 (s, 3H) (Figure 13) .

5. Conclusion

The asymmetric reduction of ketone **3**, precursor of Diltiazem, was successfully demonstrated in laboratory scale employing a range of microorganisms. Some microorganisms permitted to achieve high conversions, low amounts of byproducts and very good stereoselectivity. In order to improve the results, we want to intensify the process by increasing the amount of substate for biotransformation and work on a larger scale. Other variables (temperature, solvents, immobilization) will also be investigated.

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9. Appendix A

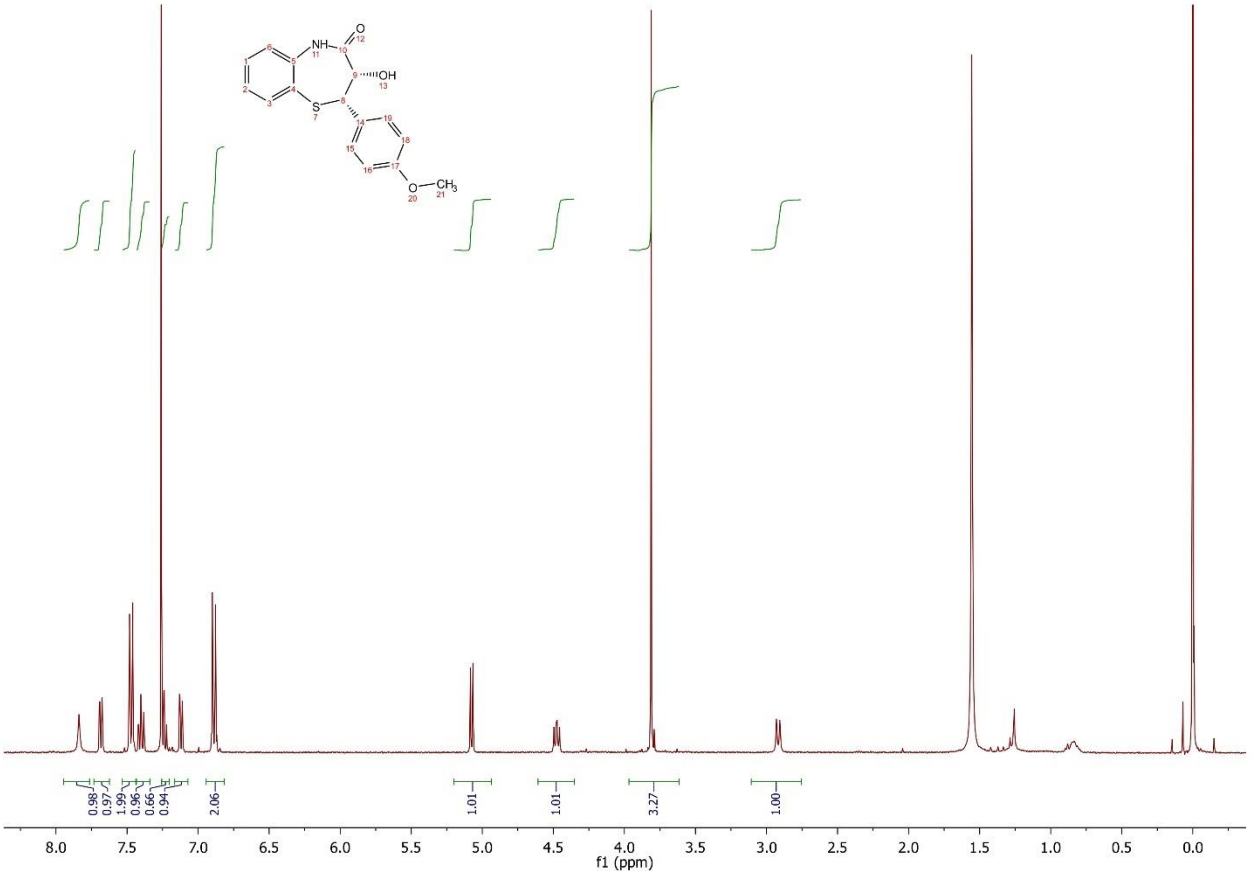

Figure 10. ^{1}H NMR characterization of chemically synthesized alcohol **4**.

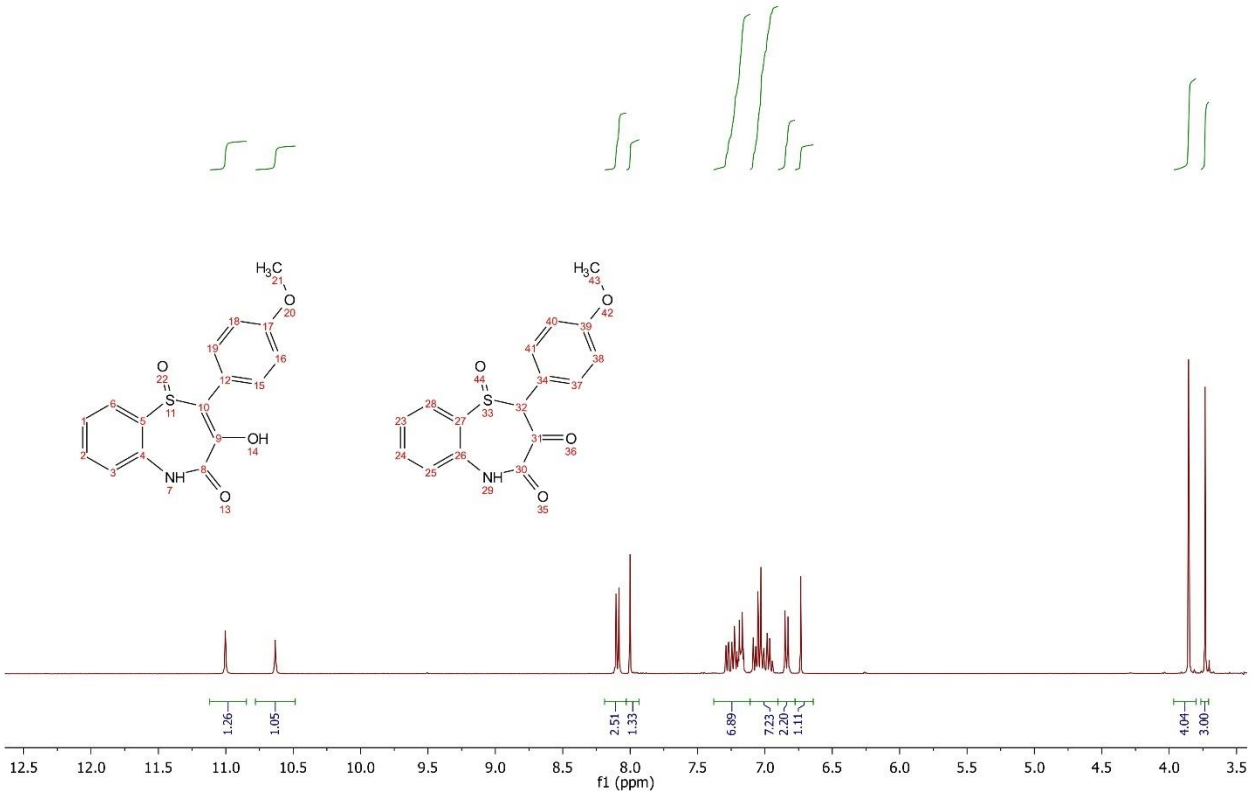

Figure 11. ^{1}H NMR characterization of sulfoxide by-product from *Yarrowia lipolytica* mixture.

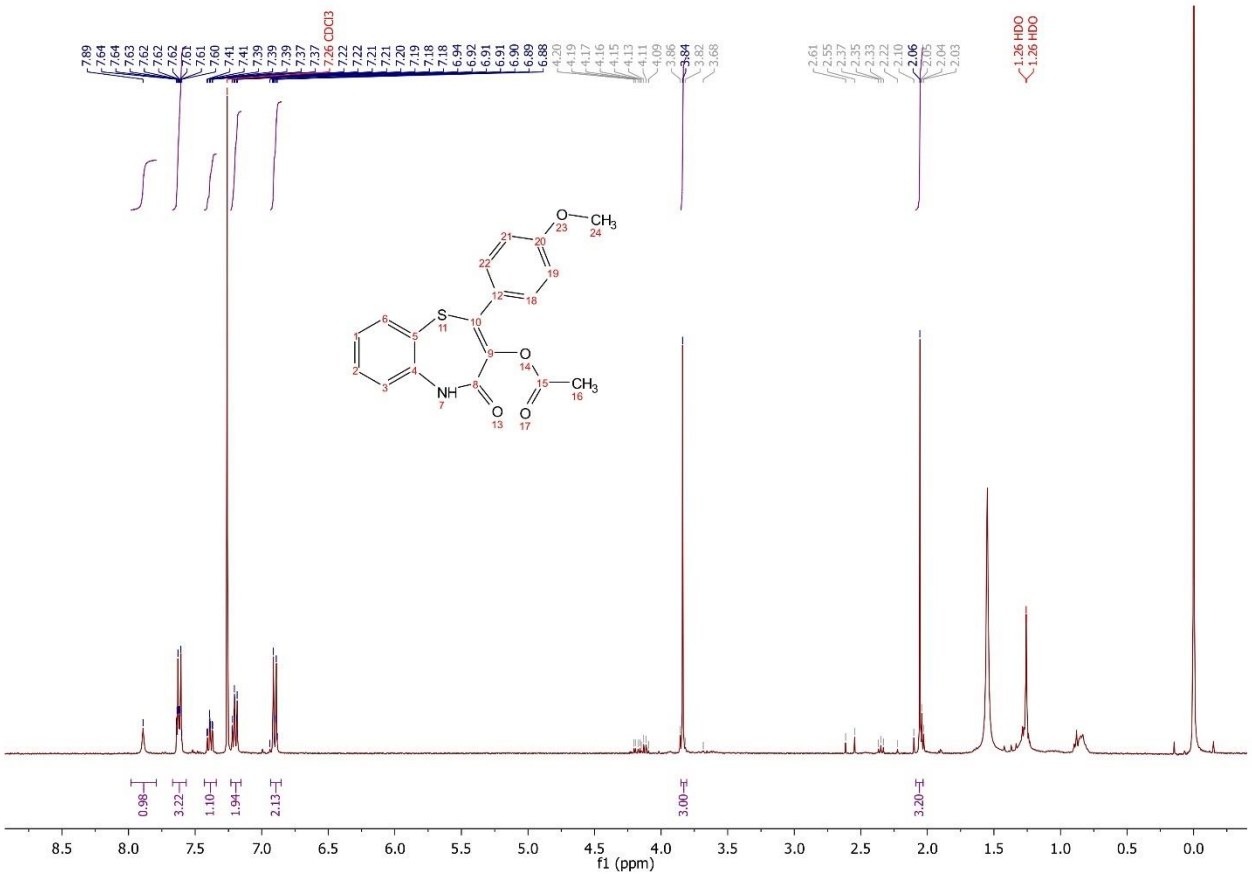

Figure 12. ^{1}H NMR characterization of oxoacylation of batch C starting material

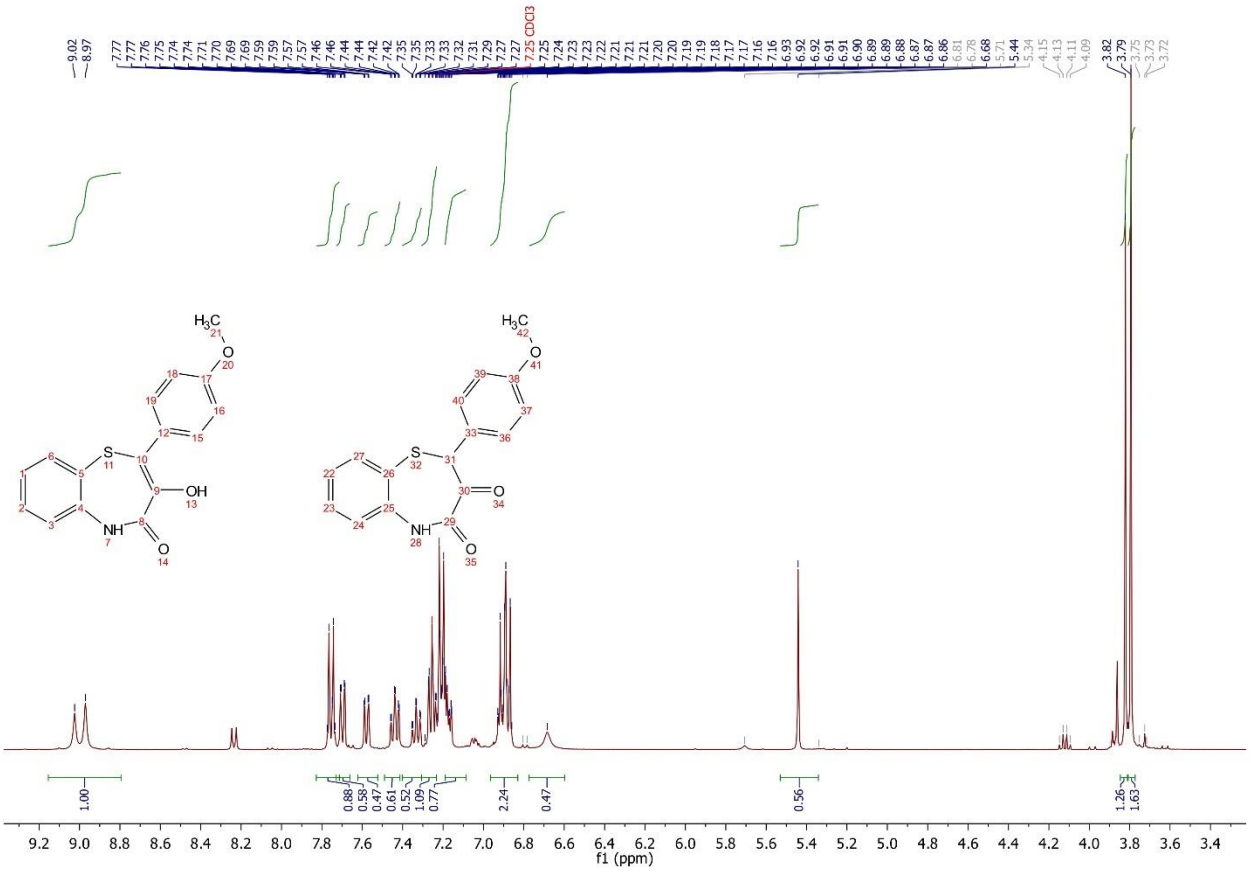

Figure 13. ^{1}H NMR characterization of ketone **3**.

10. Appendix B

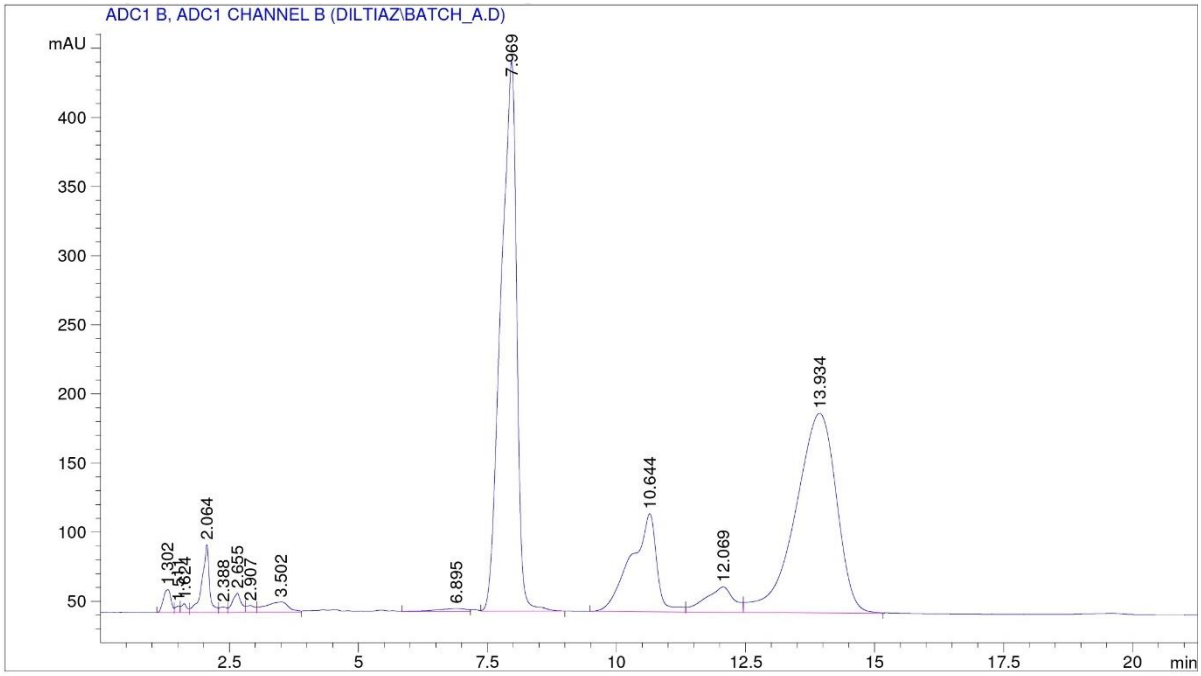

Figure 14. Non-chiral HPLC Batch A ketone starting material.

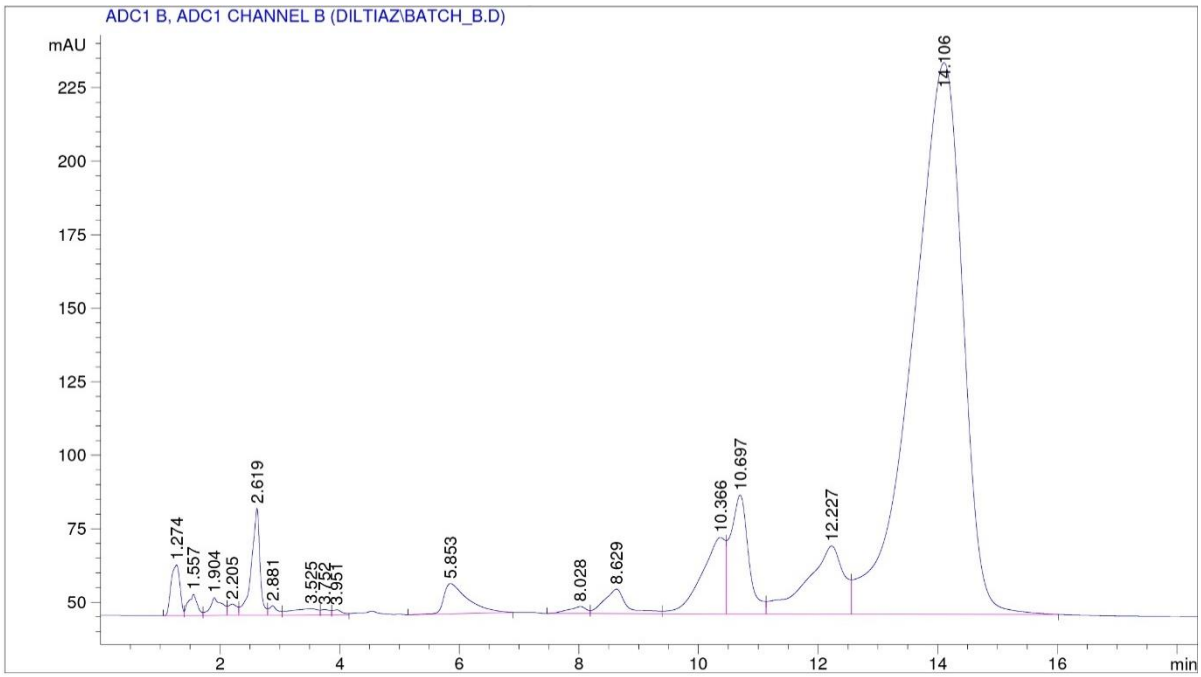

Figure 15. Non-chiral HPLC Batch B ketone starting material.

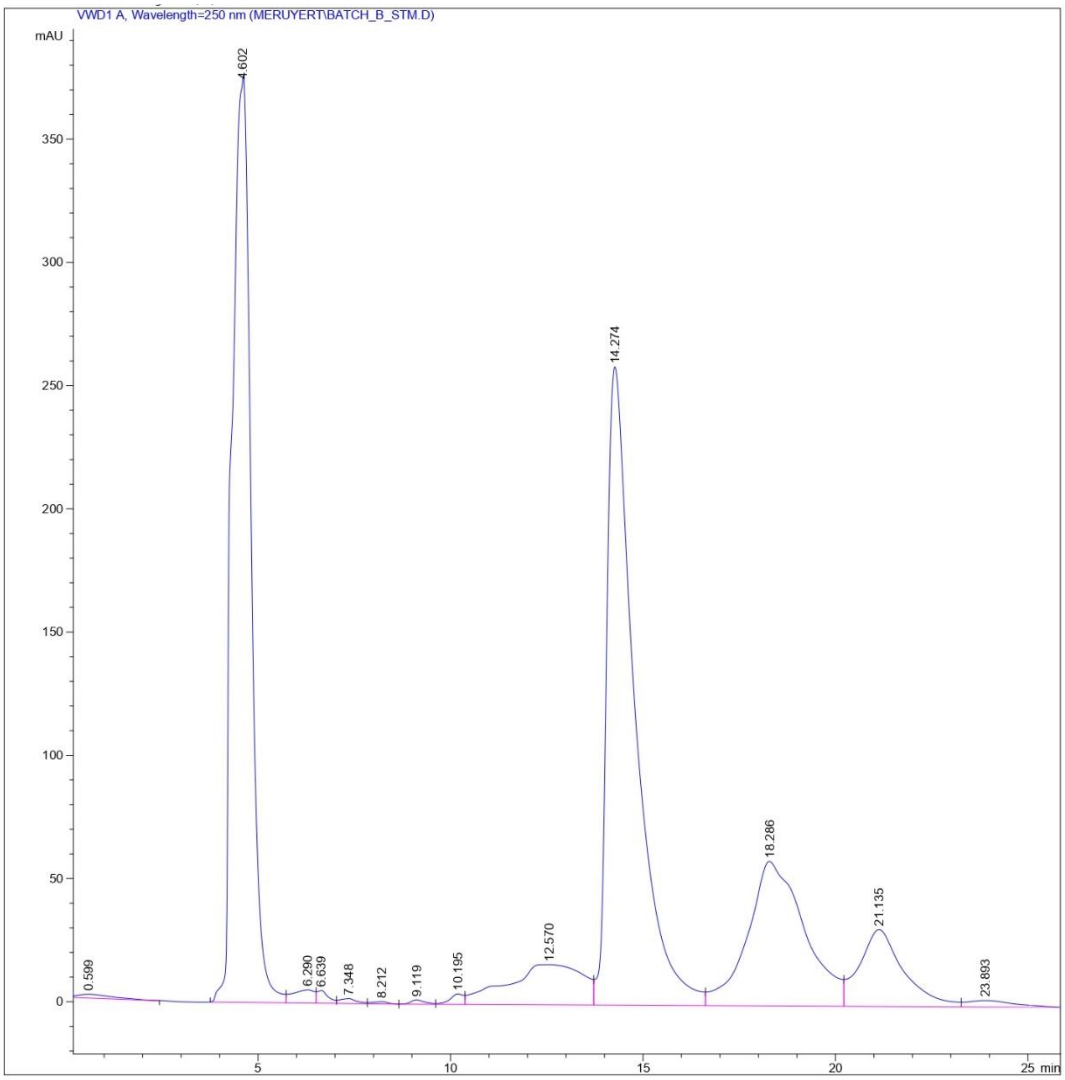

Figure 16. Chiral HPLC Batch B ketone starting material.

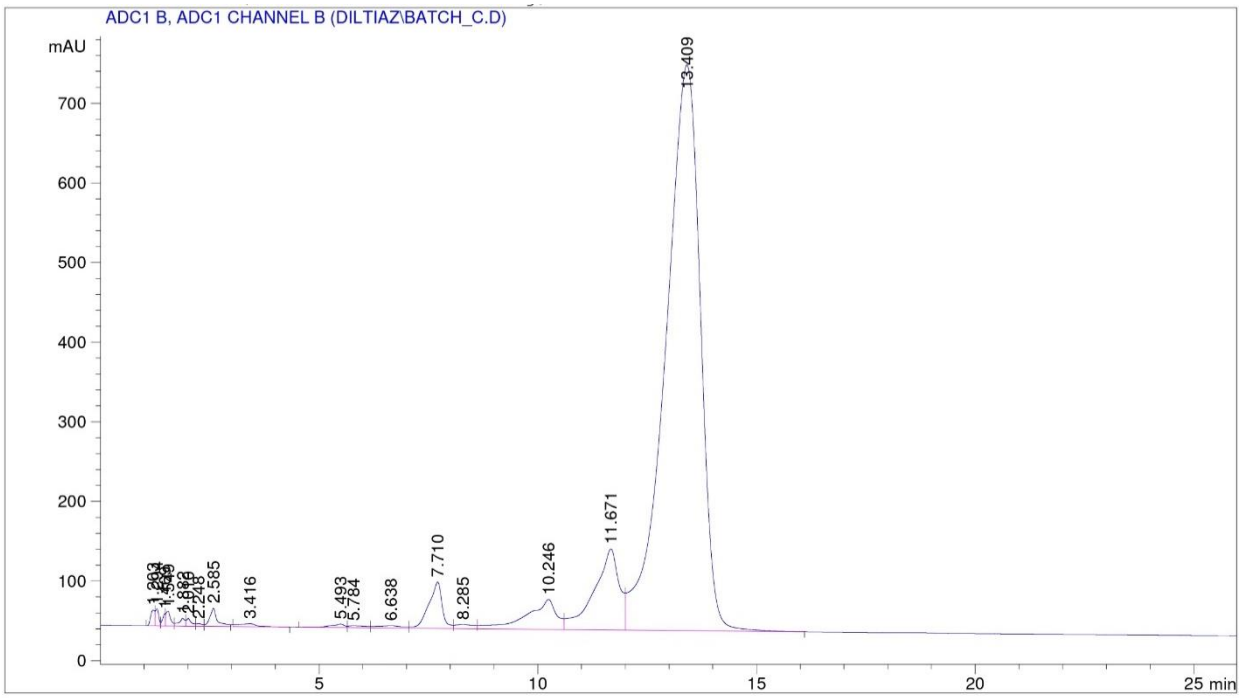

Figure 17. Non-chiral HPLC Batch C ketone starting material.

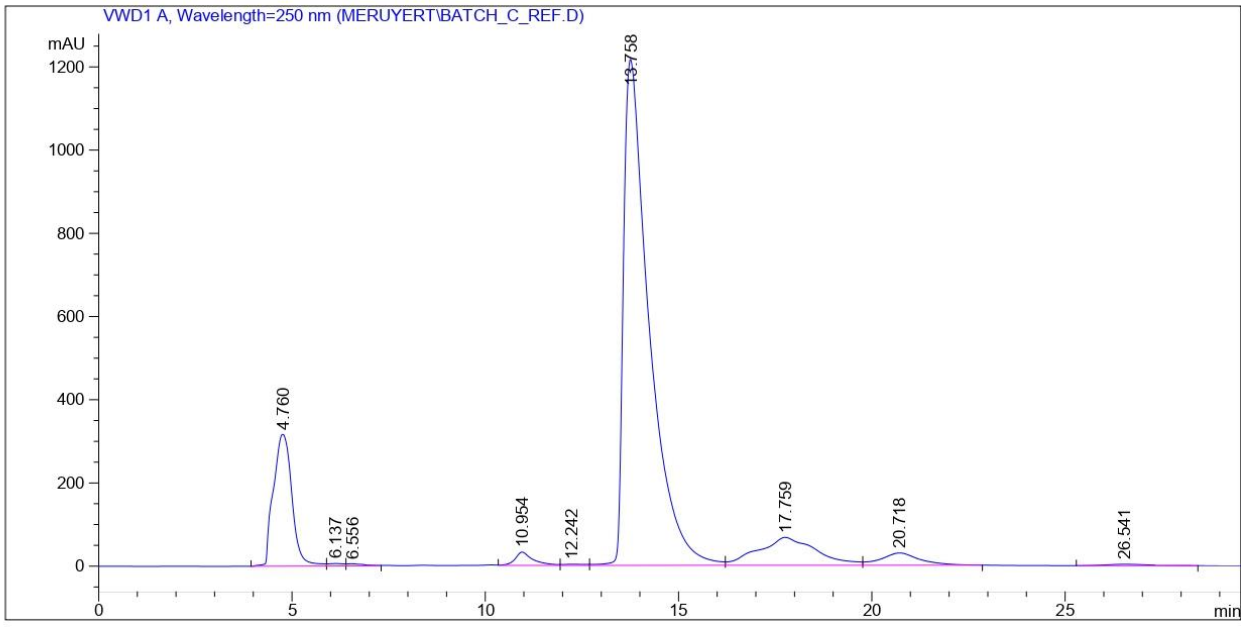

Figure 18. Chiral HPLC Batch C ketone starting material.

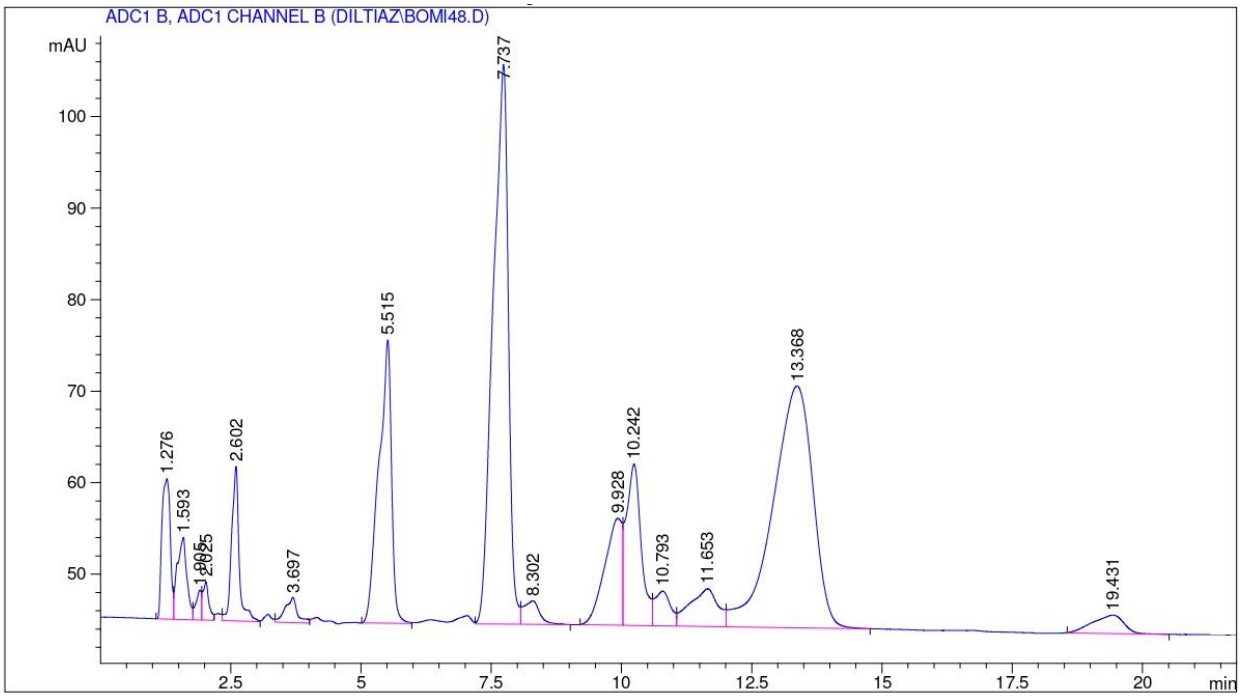

Figure 19. Non-chiral HPLC *Starmerella bombicola* biotransformation product extract.

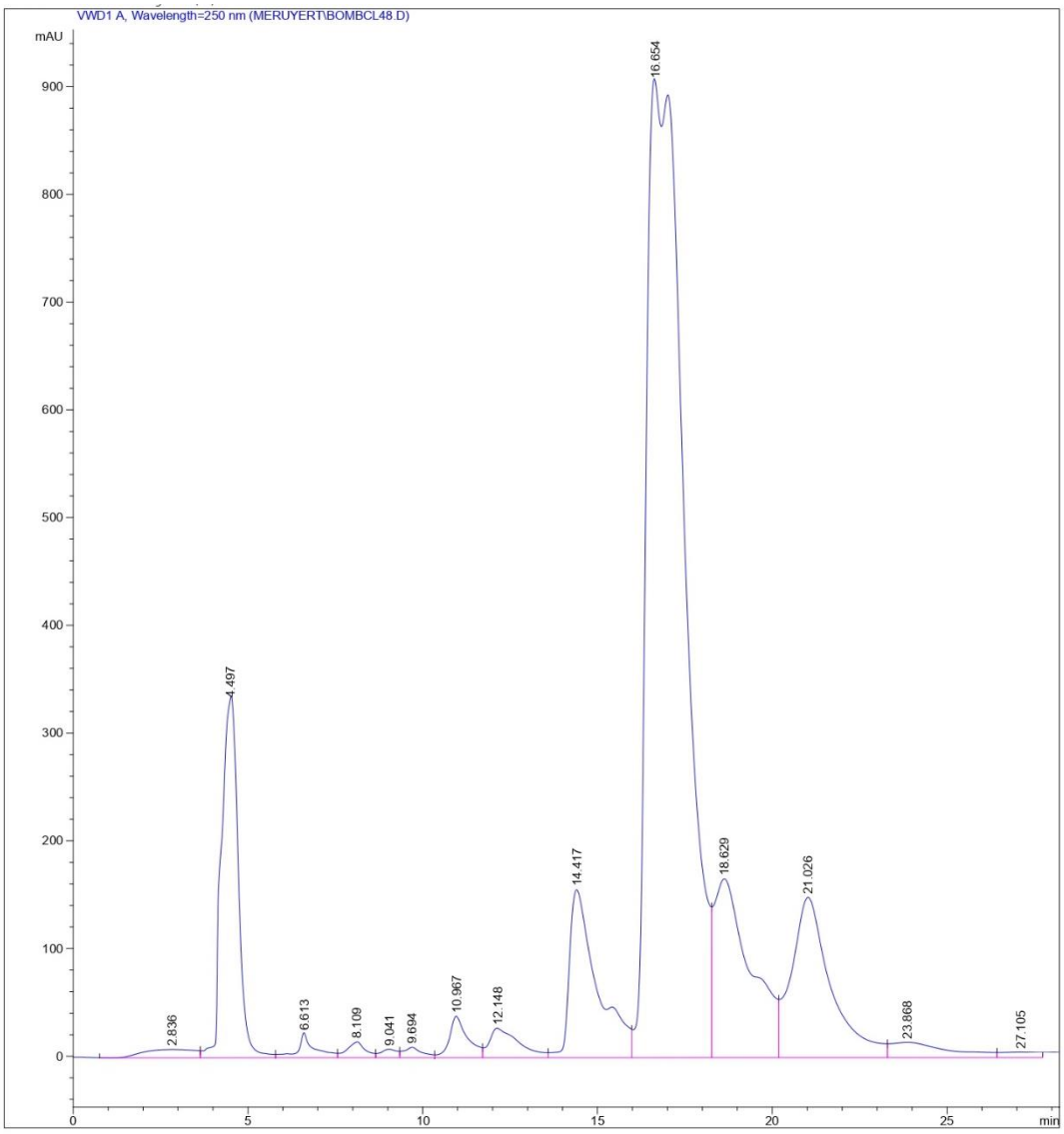

Figure 20. Chiral HPLC *Starmerella bombicola* biotransformation product extract.

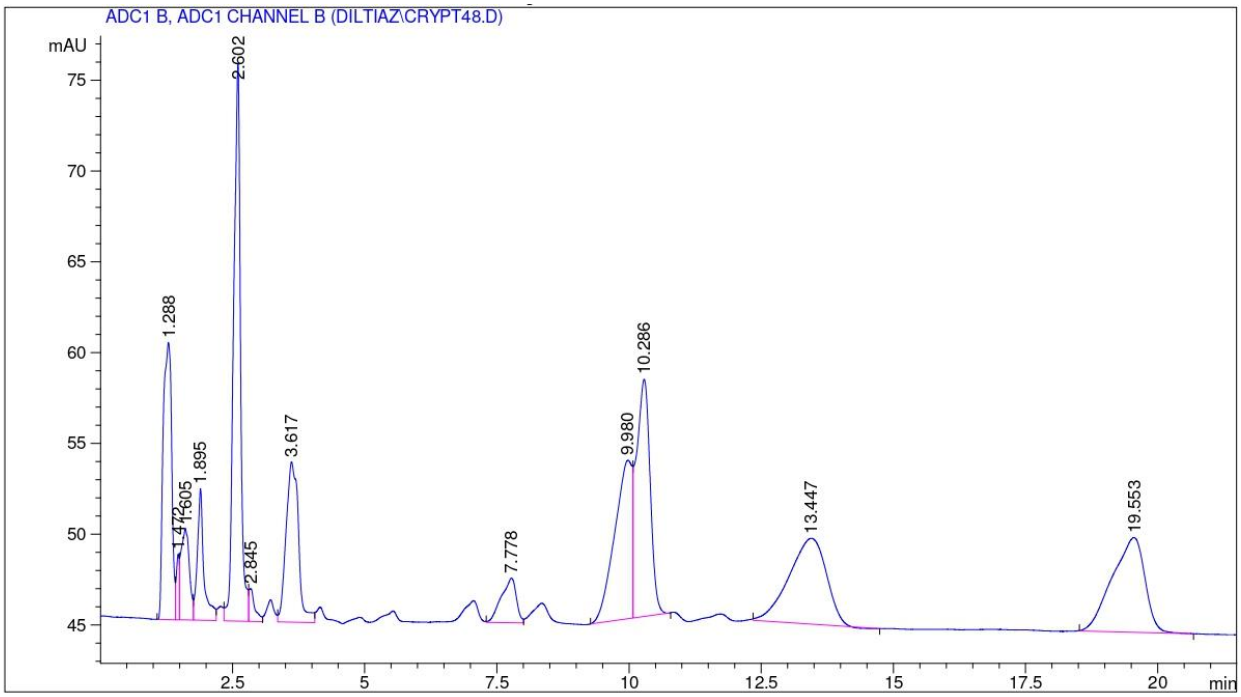

Figure 21. Non-chiral HPLC *Cryptococcus curvatus* biotransformation product extract.

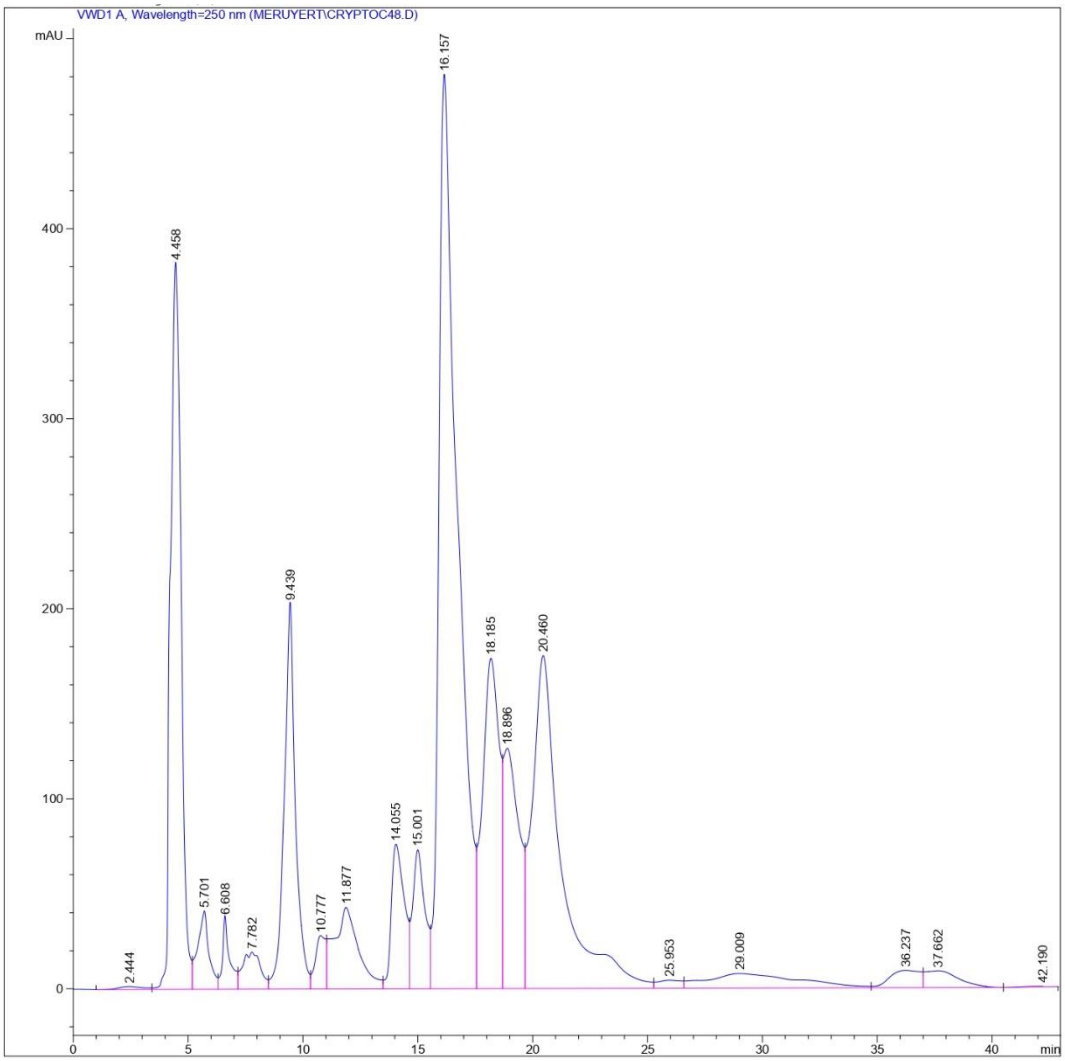

Figure 22. Chiral HPLC *Cryptococcus curvatus* biotransformation product extract.

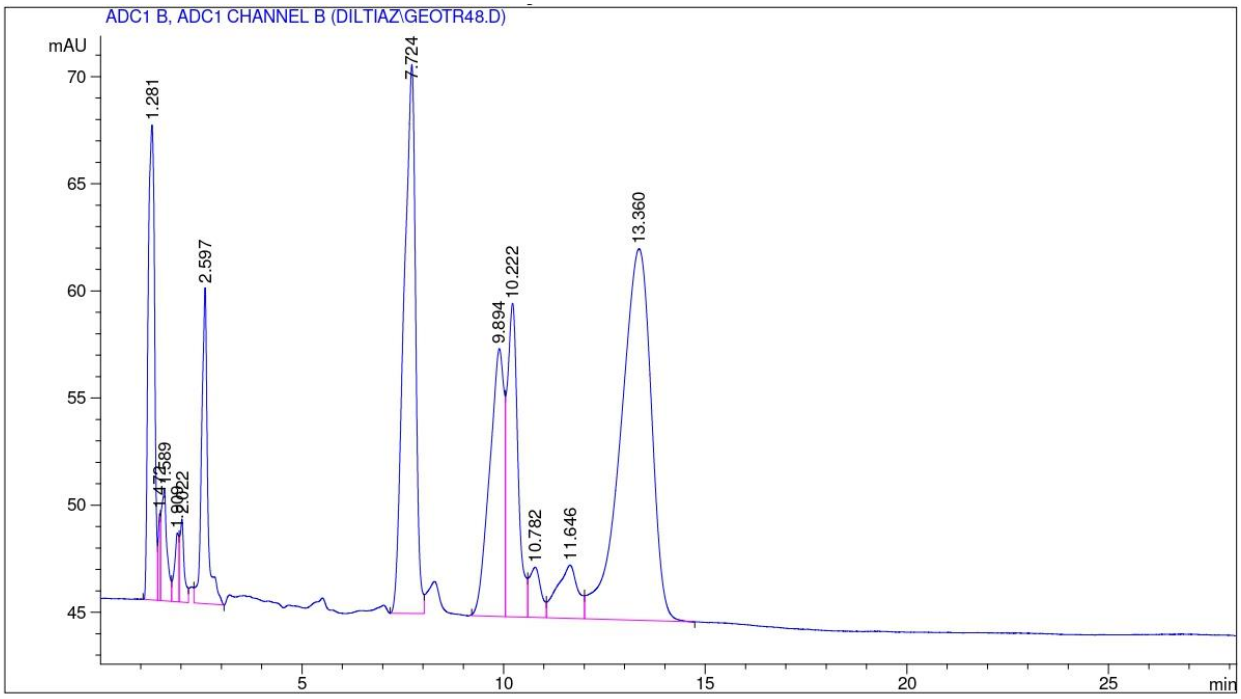

Figure 23. Non-chiral HPLC *Geotrichum candidum* biotransformation product extract.

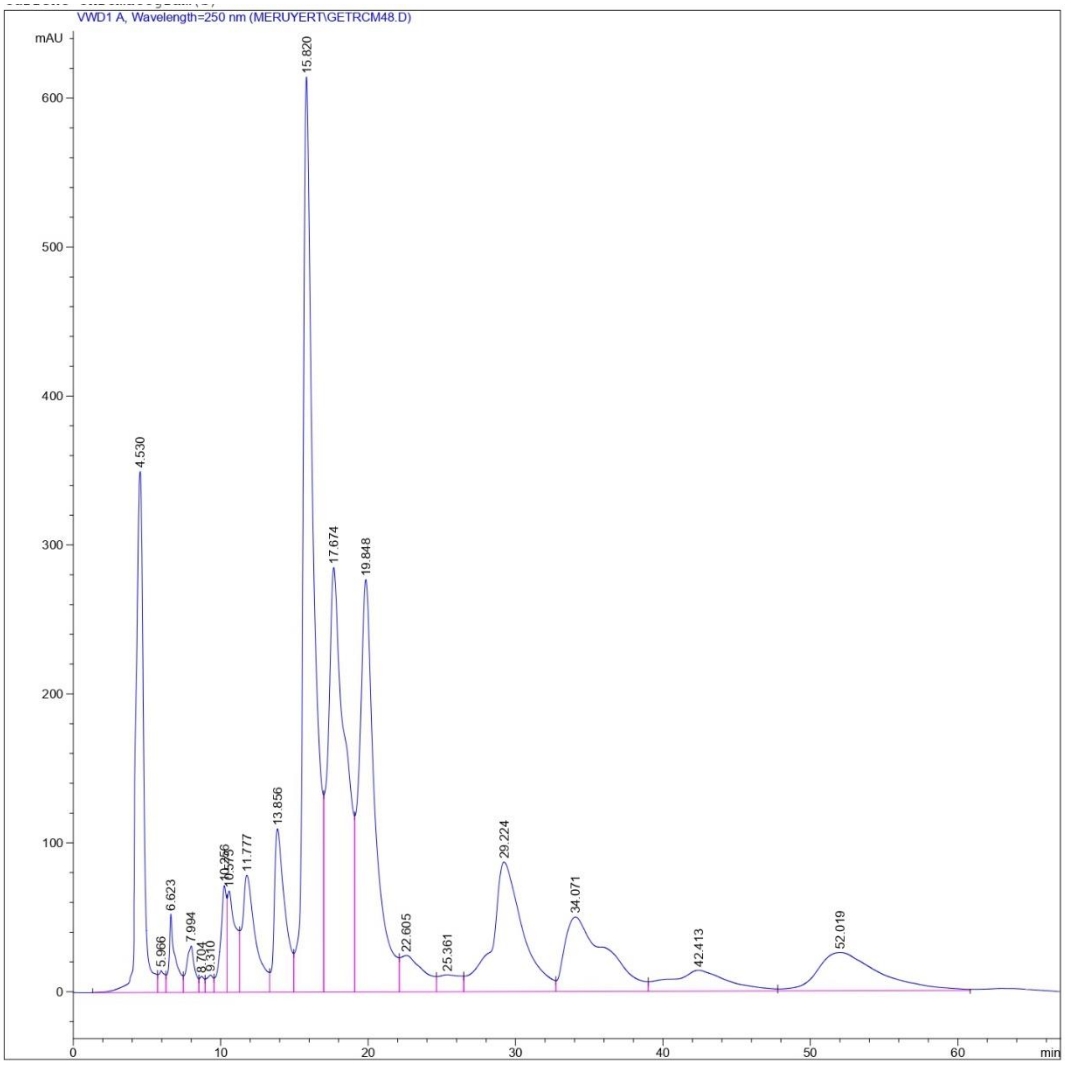

Figure 24. Chiral HPLC *Geotrichum candidum* biotransformation product extract.

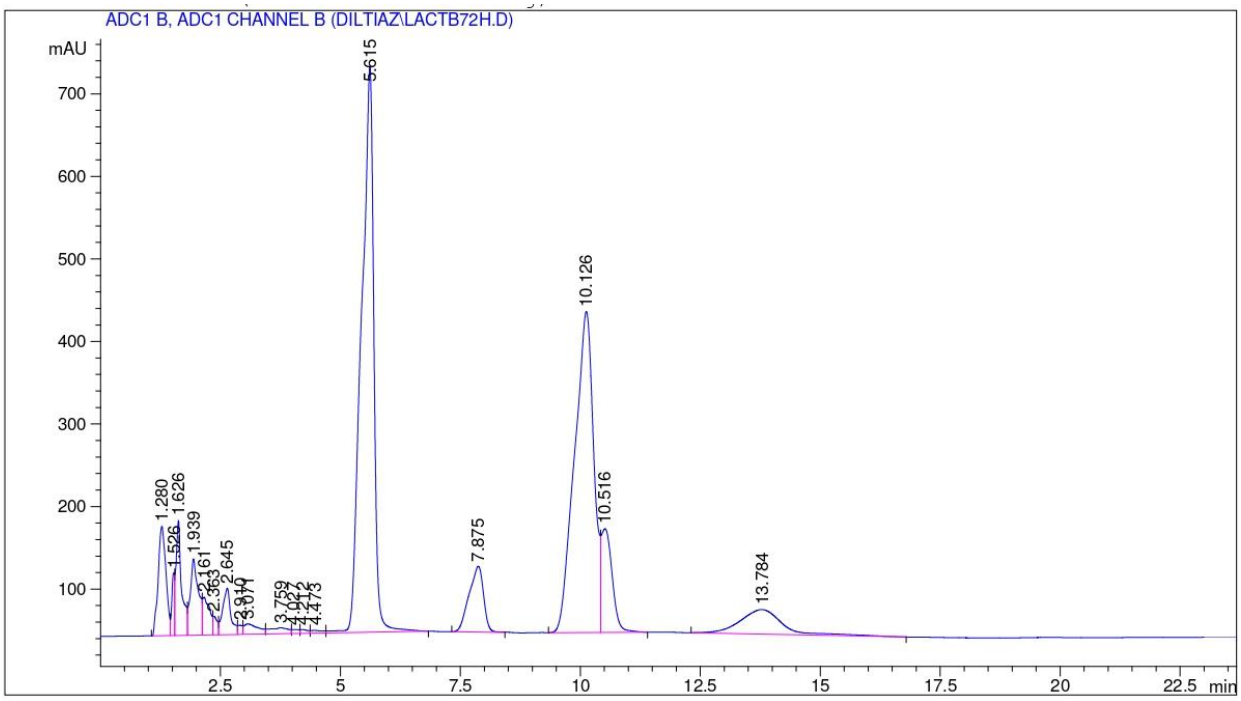

Figure 25. Non-chiral HPLC *Lactobacilus rhamnosus* biotransformation product extract.

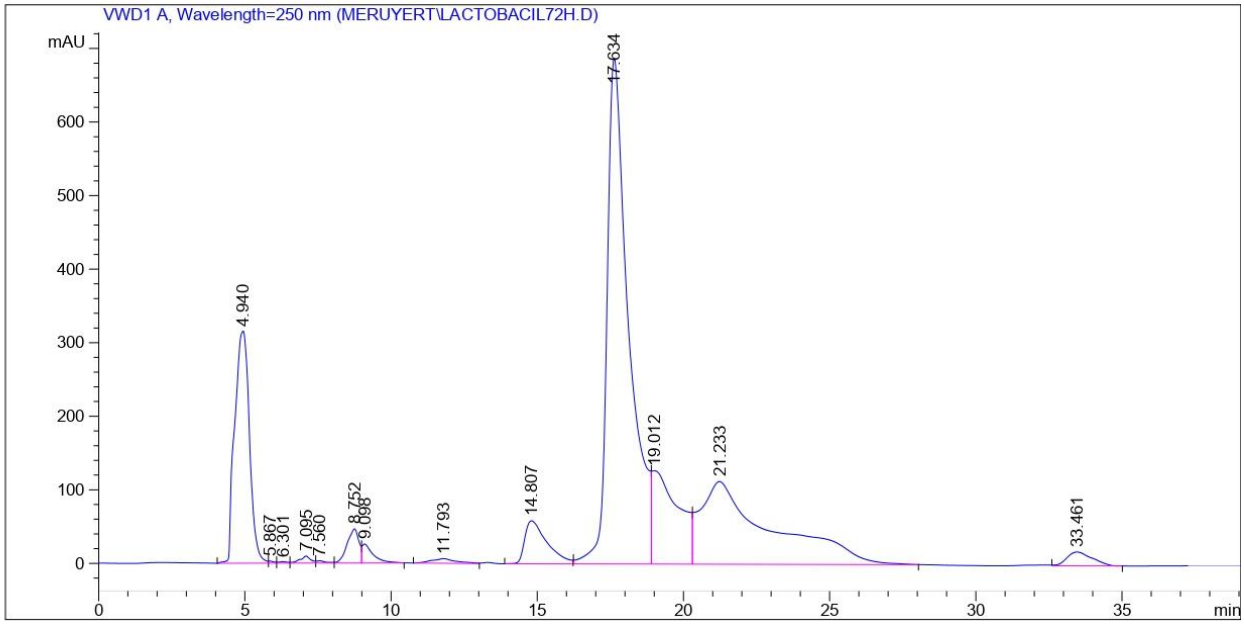

Figure 26. Chiral HPLC *Lactobacillus rhamnosus* biotransformation product extract.

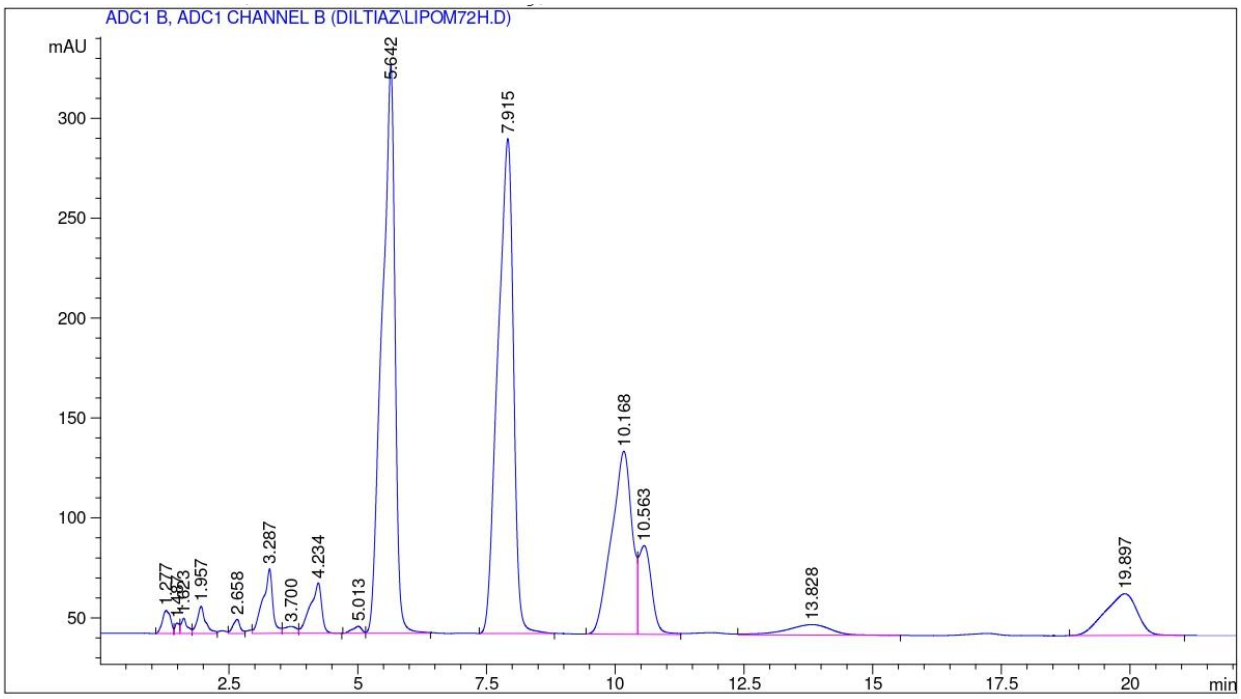

Figure 27. Non-chiral HPLC *Lipomyces starkeyi* biotransformation product extract.

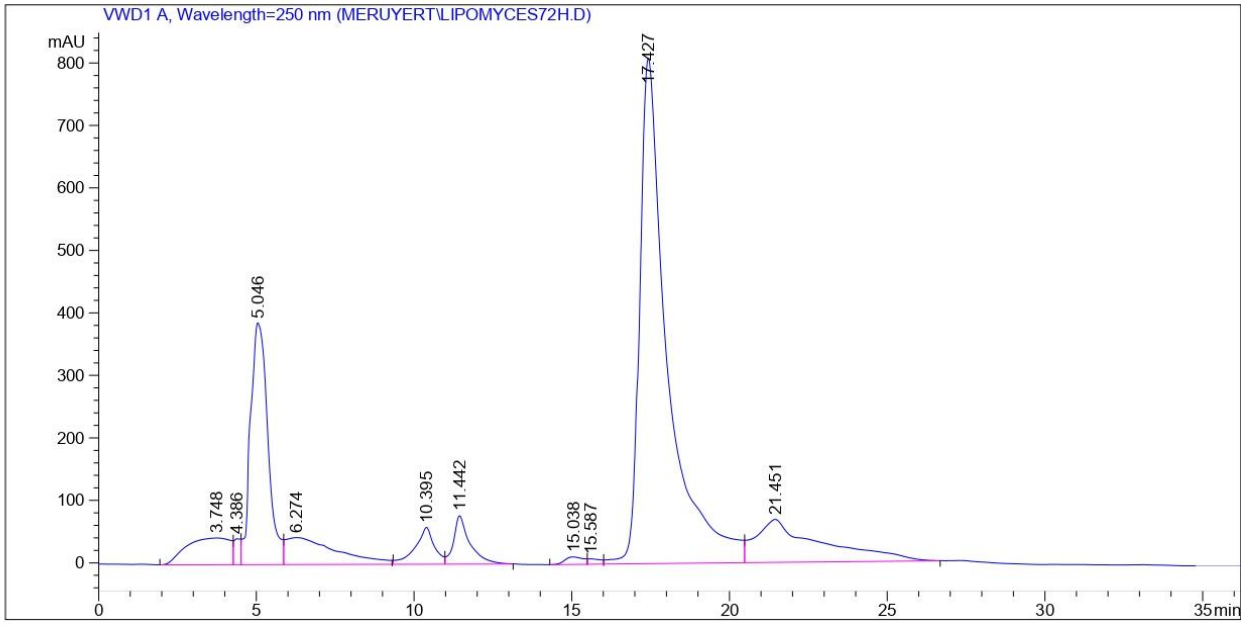

Figure 28. Chiral HPLC *Lipomyces starkeyi* biotransformation product extract.

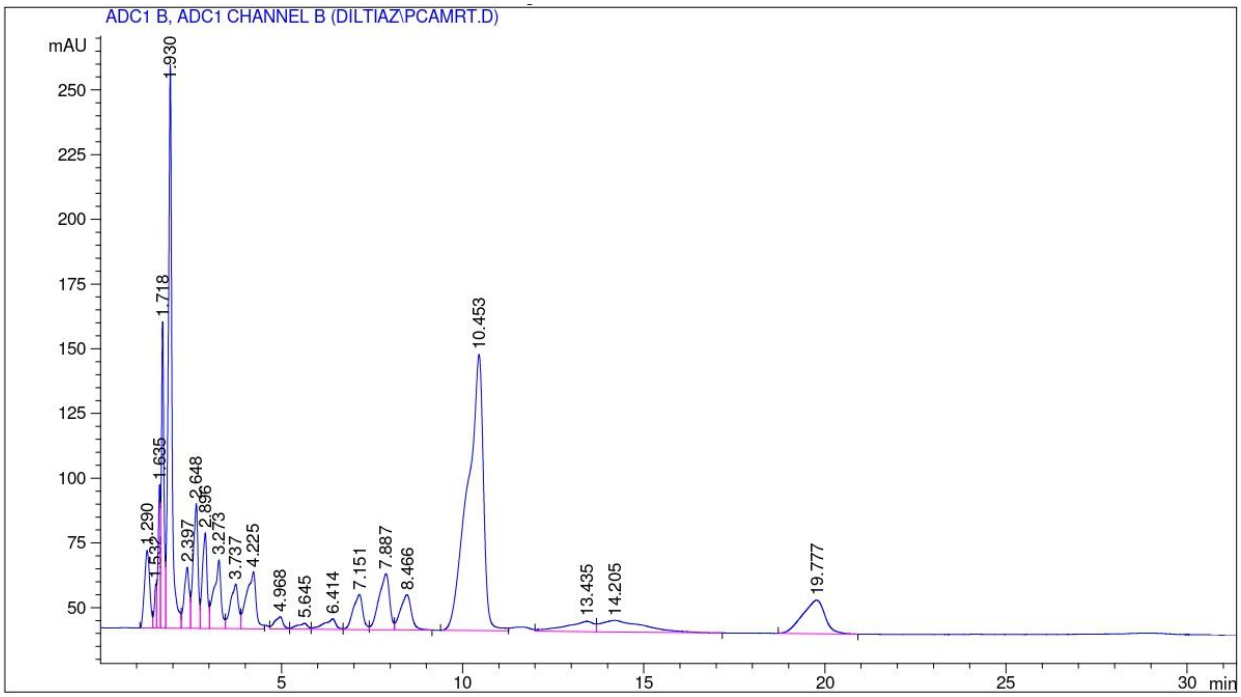

Figure 29. Non-chiral HPLC *Penicillium camemberti* biotransformation product extract.

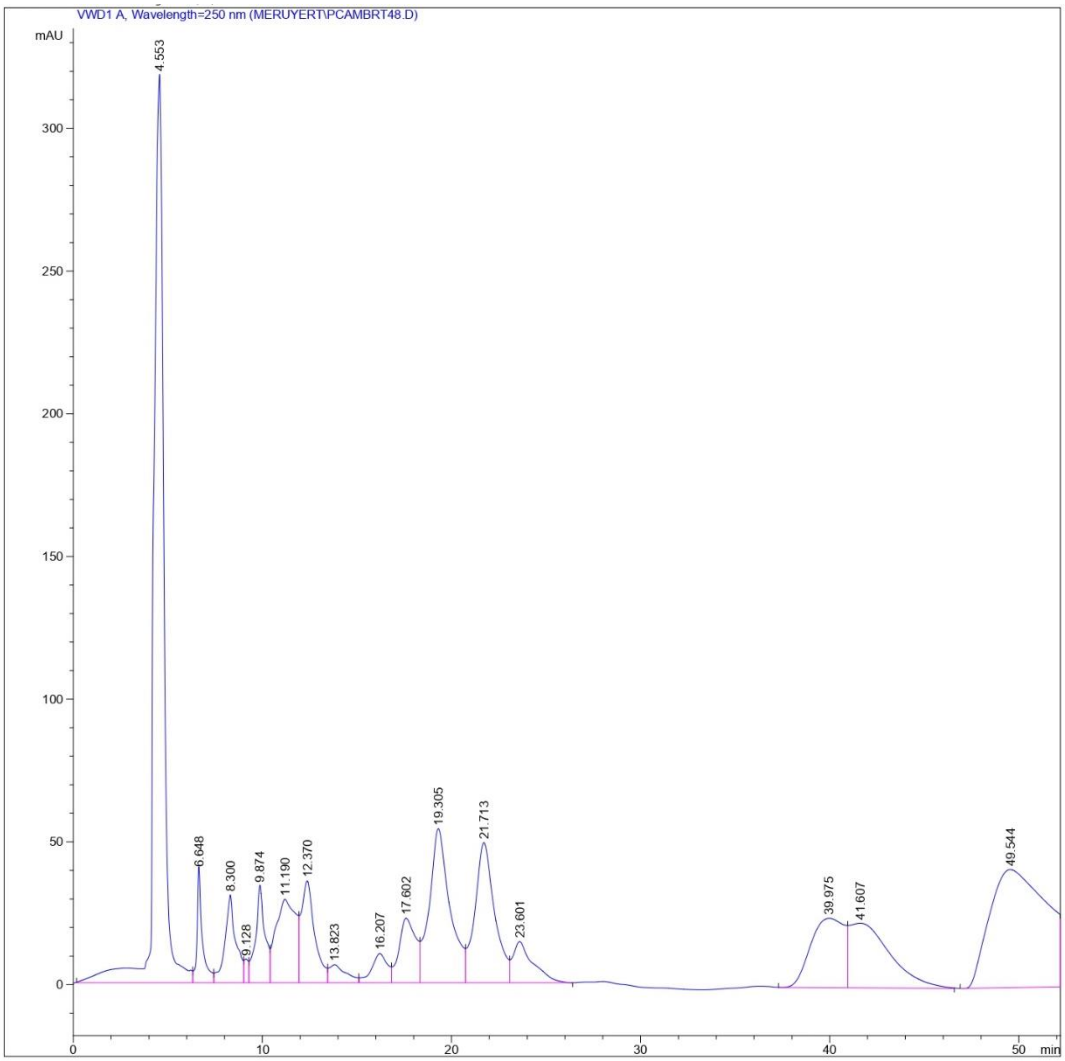

Figure 30. Chiral HPLC *Penicillium camemberti* biotransformation product extract.

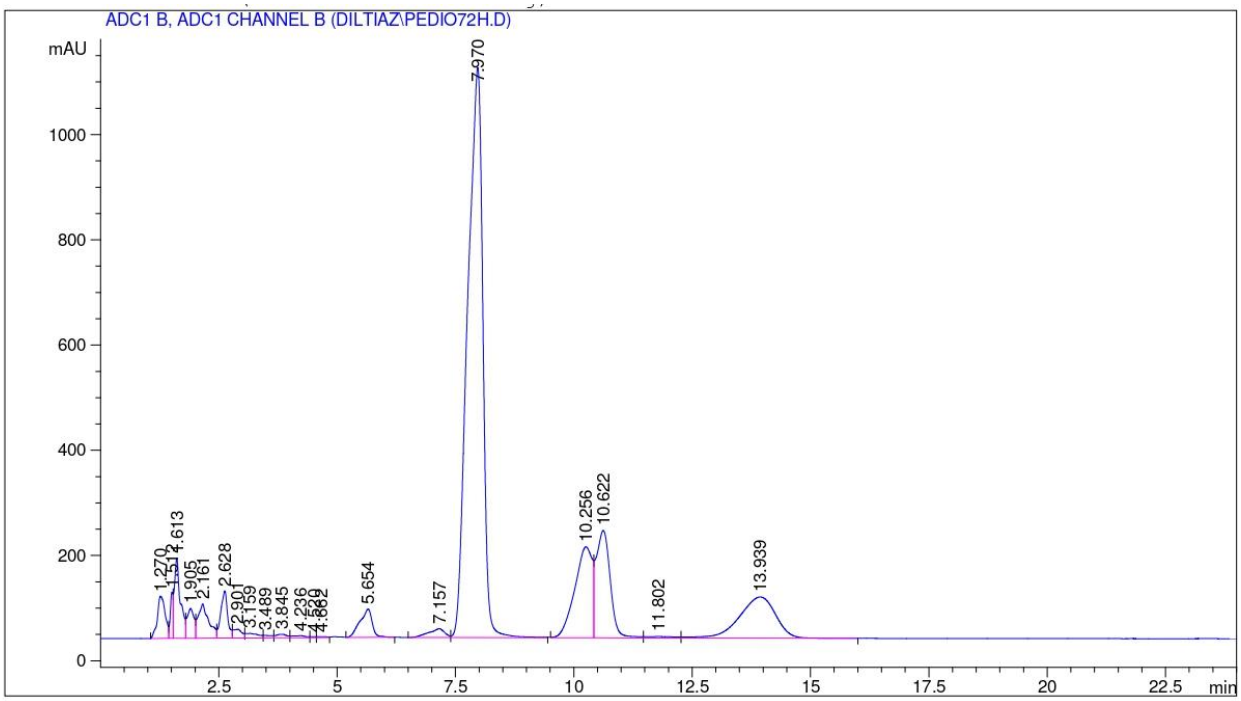

Figure 31. Non-chiral HPLC *Pediococcus pentosaceus* biotransformation product extract.

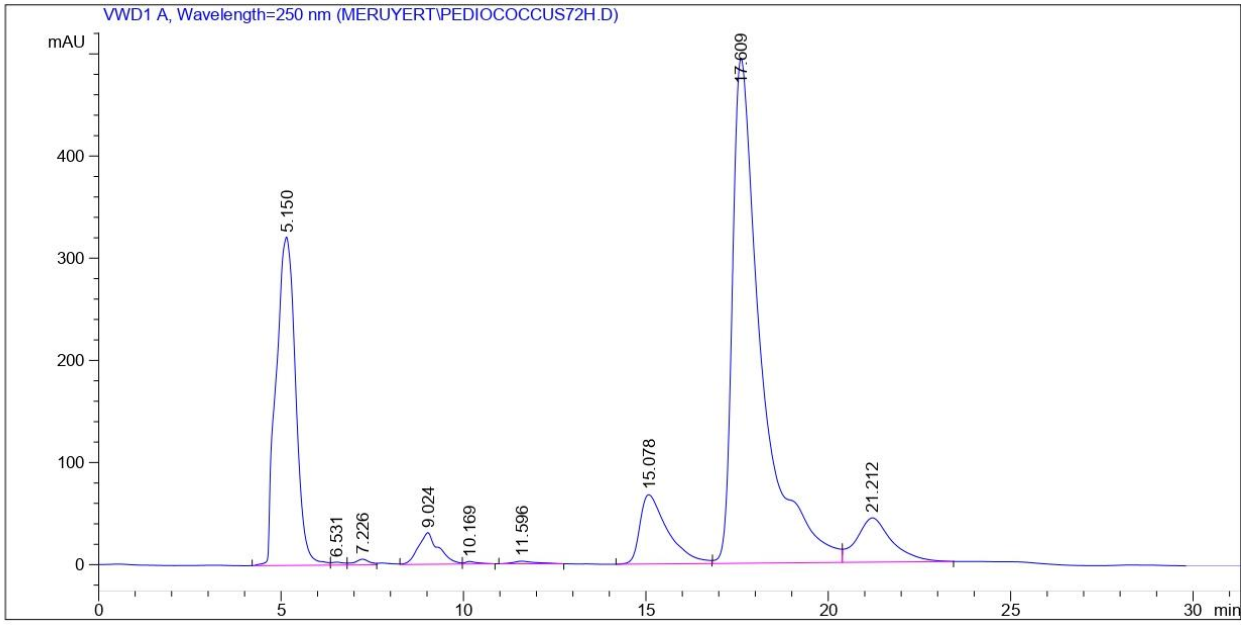

Figure 32. Chiral HPLC *Pediococcus pentosaceus* biotransformation product extract.

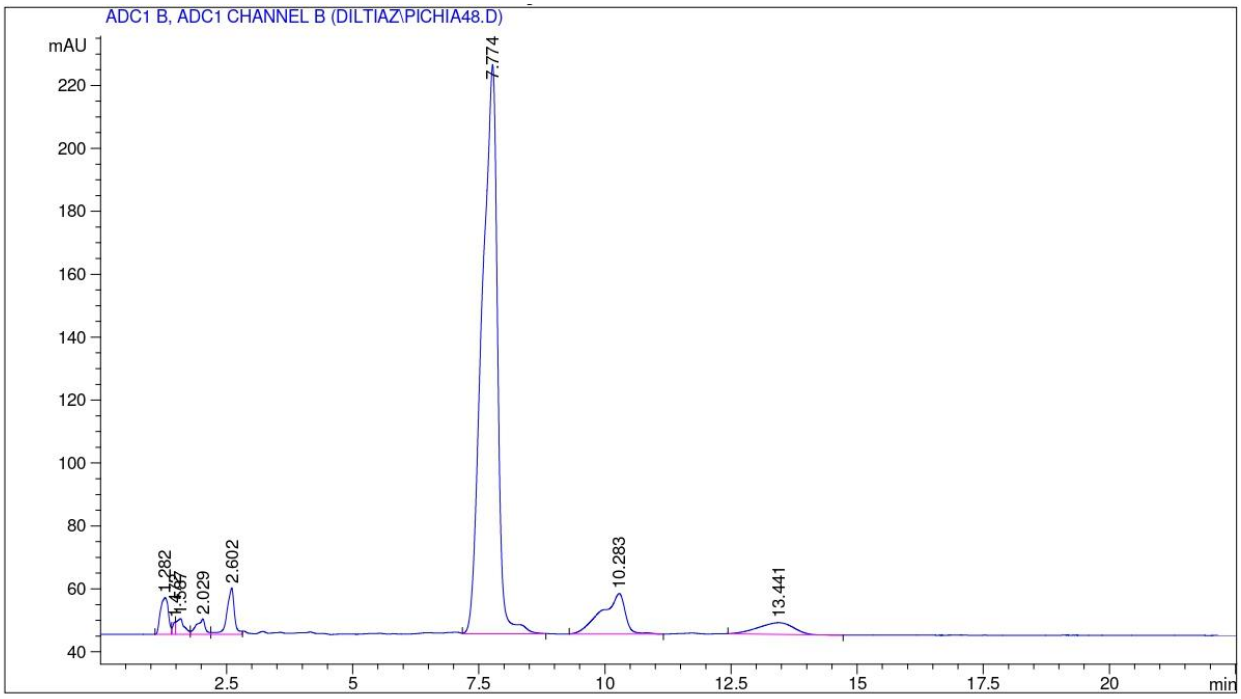

Figure 33. Non-chiral HPLC *Pichia pastoris* biotransformation product extract.

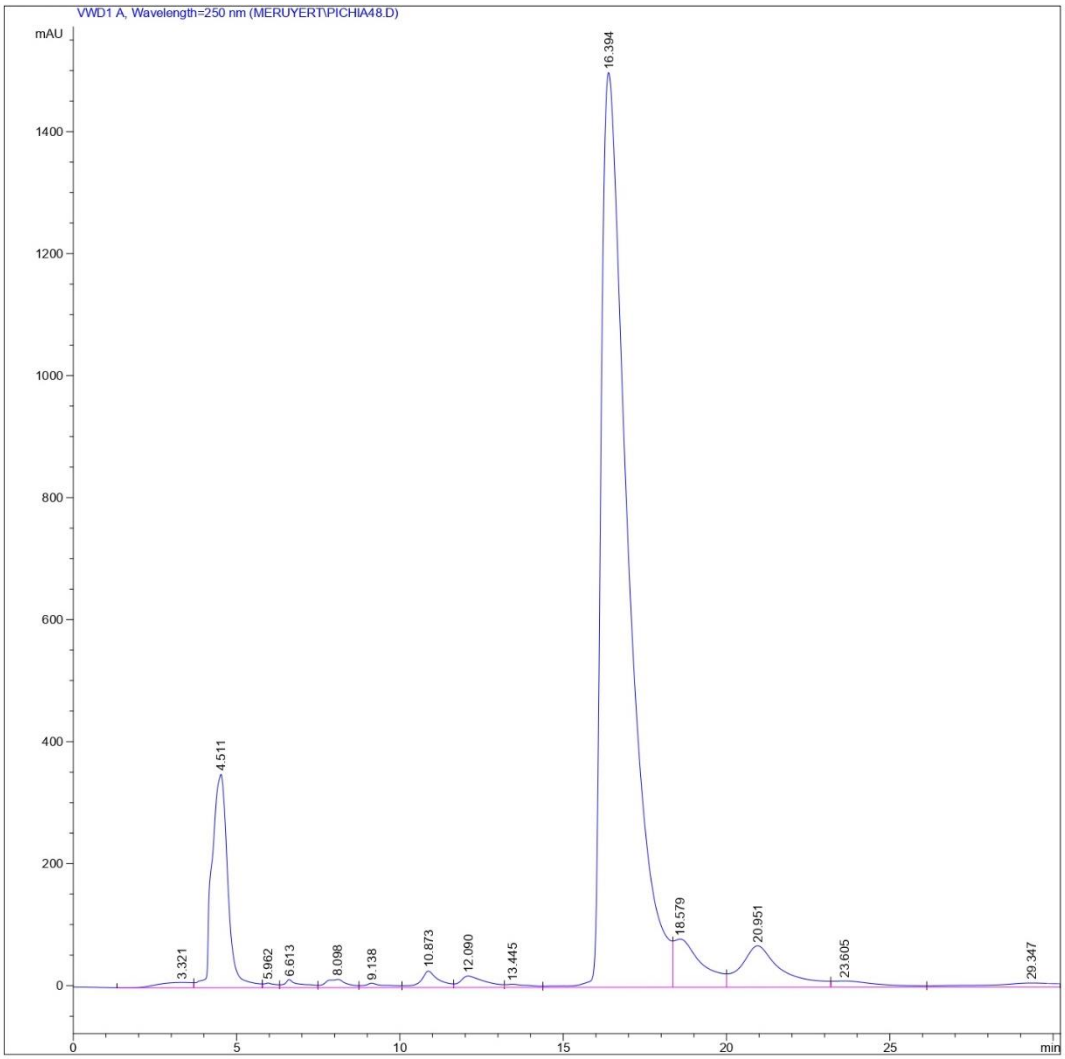

Figure 34. Chiral HPLC *Pichia pastoris* biotransformation product extract.

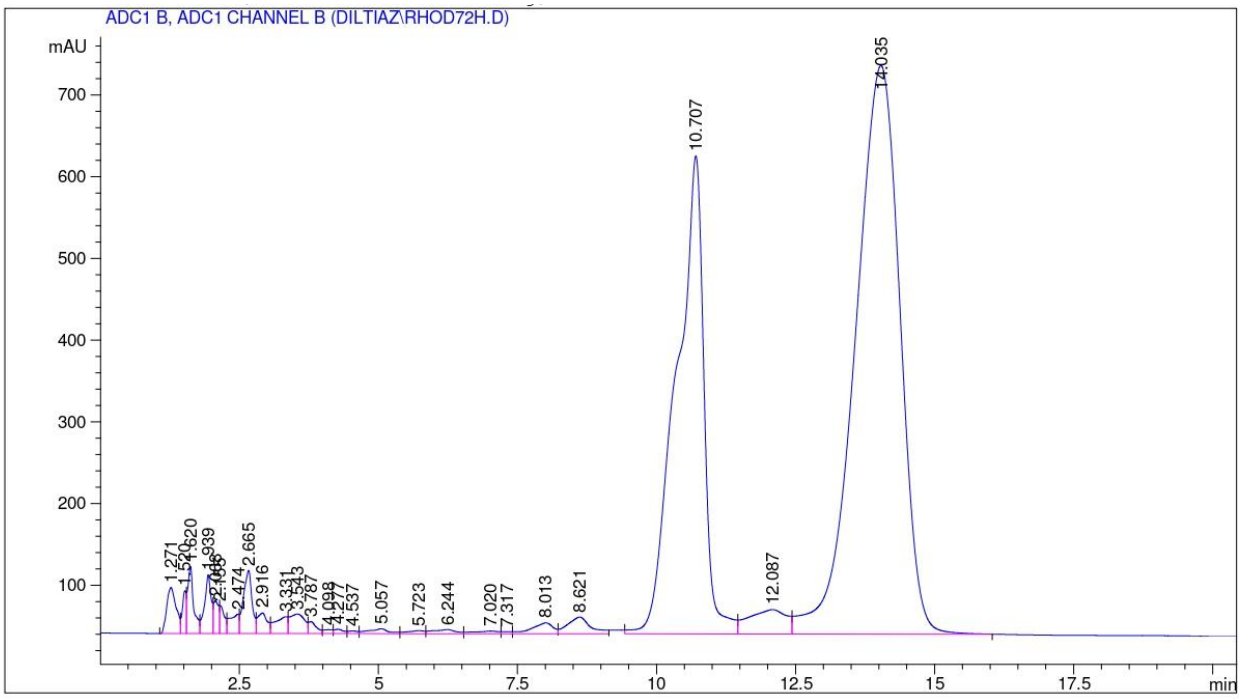

Figure 35. Non-chiral HPLC *Rhodosporidium toruloides* biotransformation product extract.

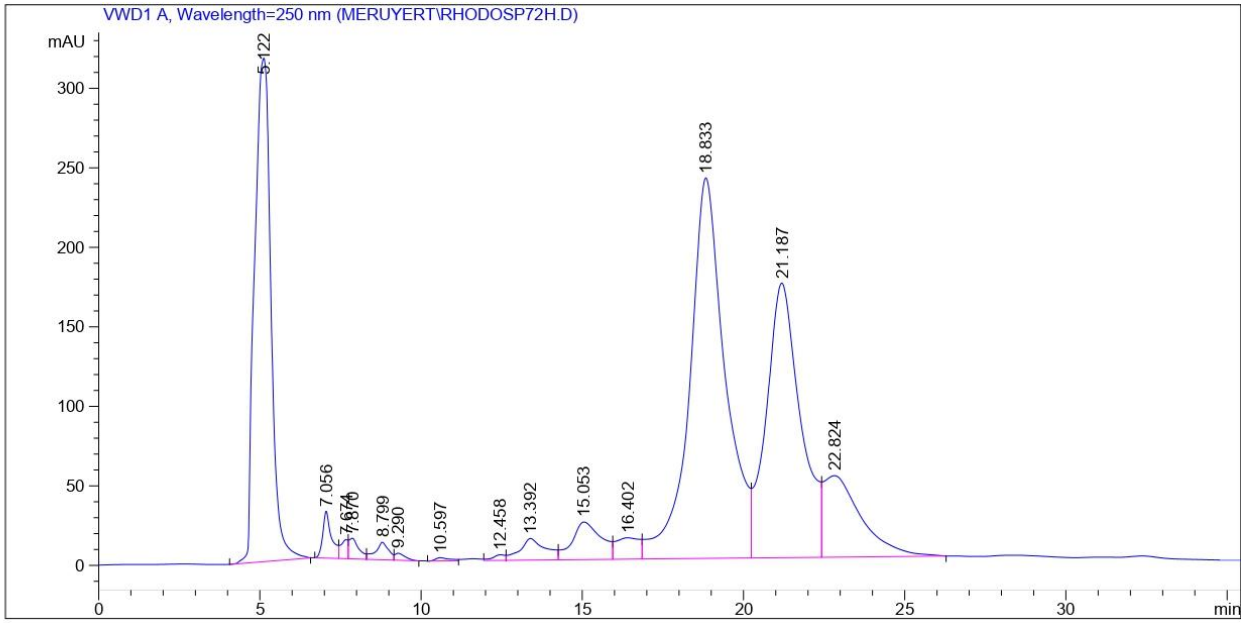

Figure 36. Chiral HPLC *Rhodosporidium toruloides* biotransformation product extract.

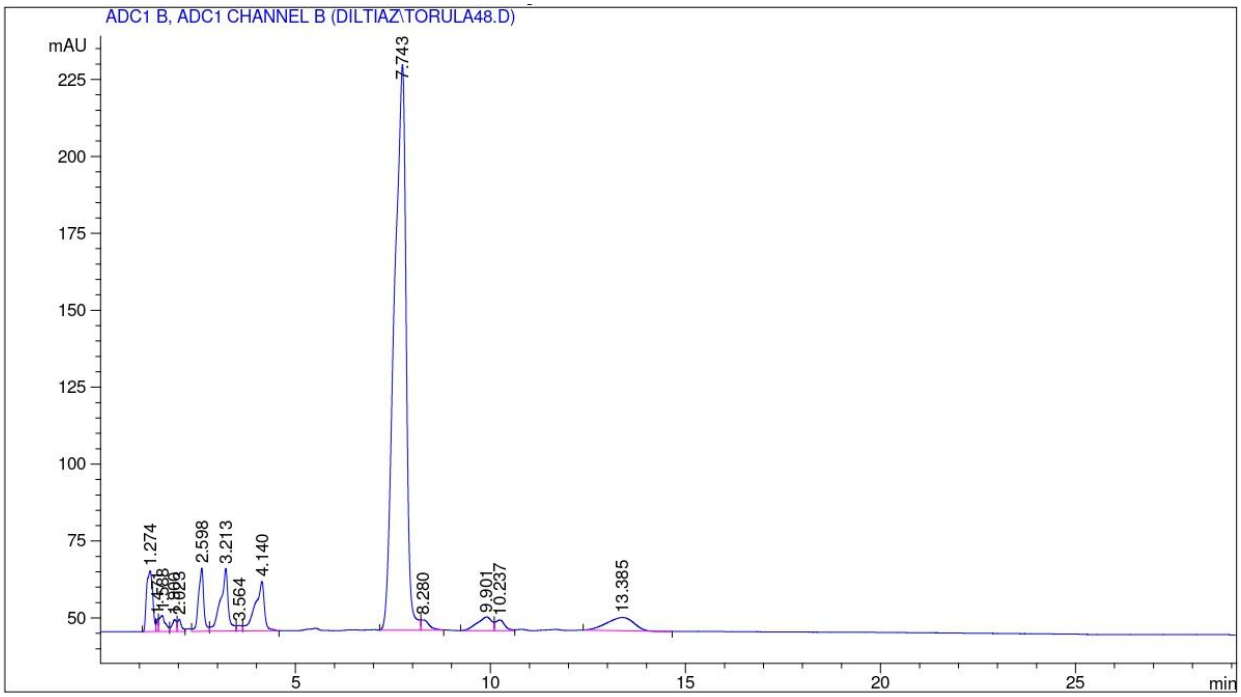

Figure 37. Non-chiral *Torulaspora delbrueckii* biotransformation product extract.

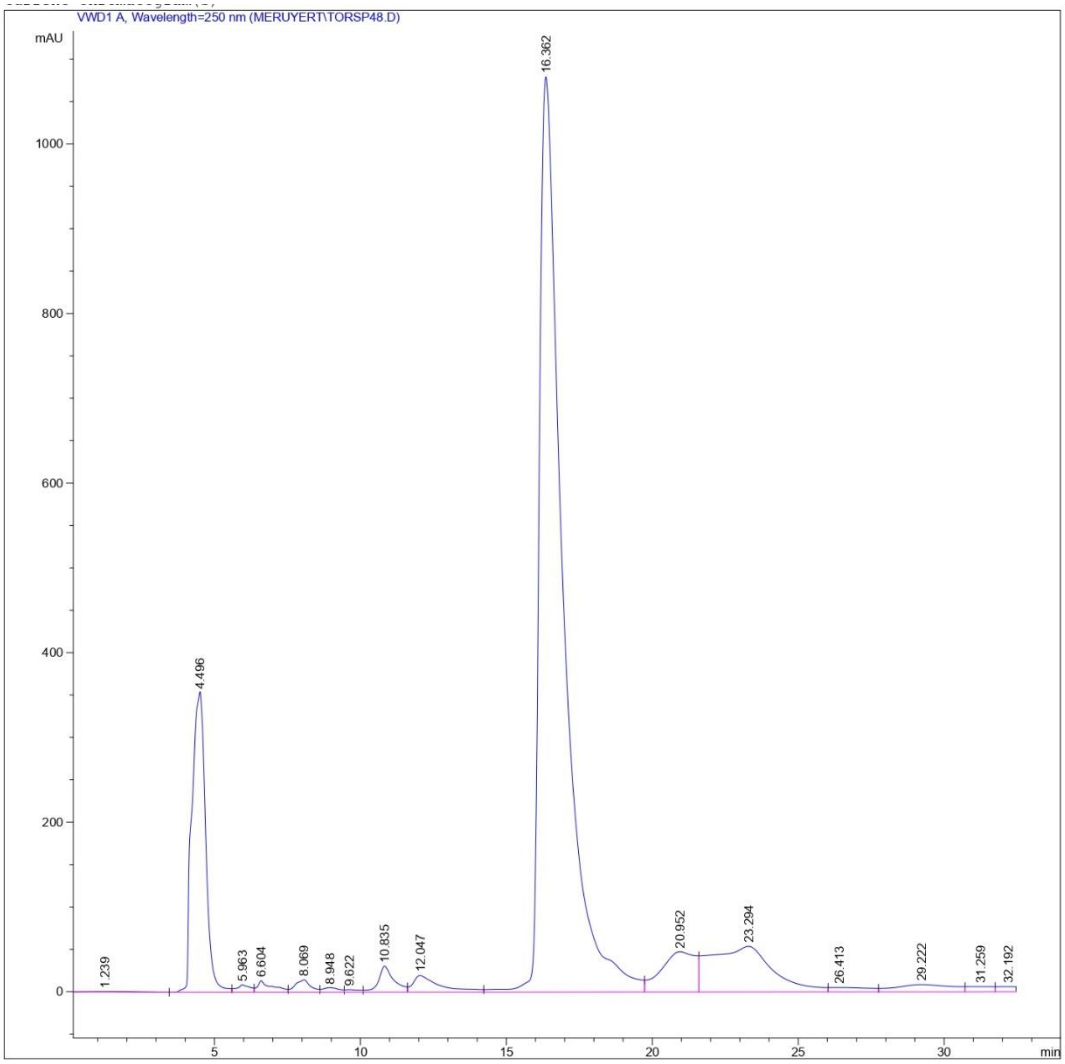

Figure 38. Chiral HPLC *Torulaspora delbrueckii* biotransformation product extract.

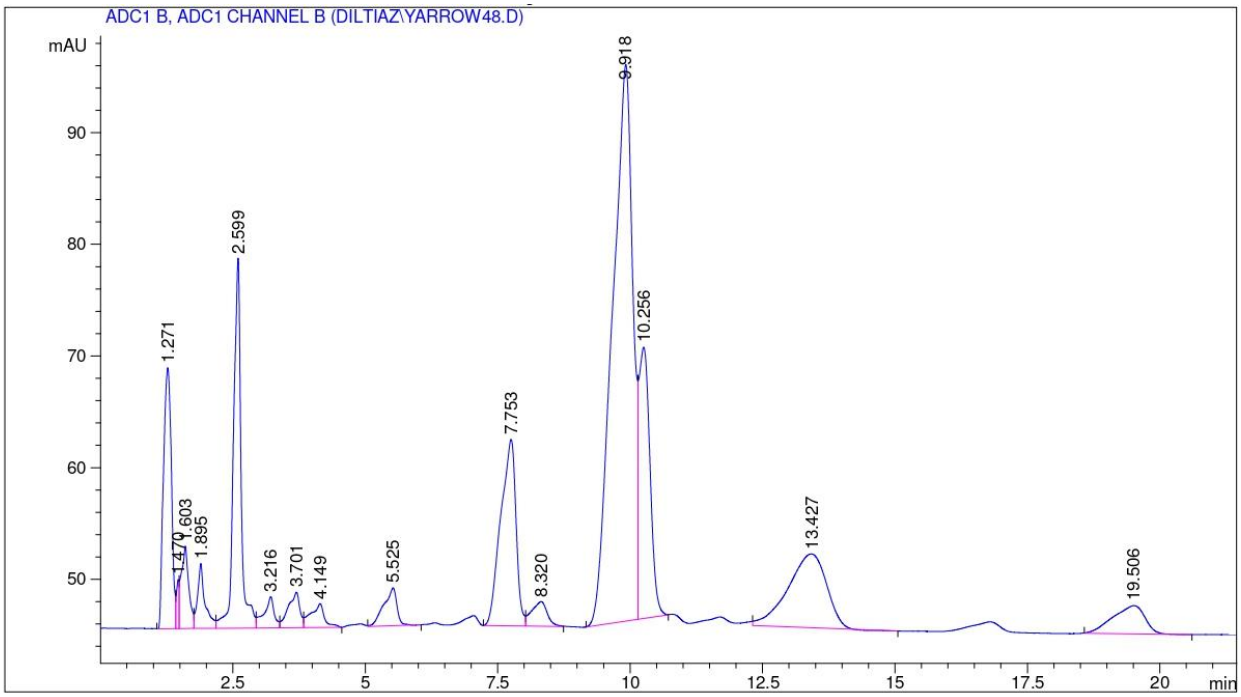

Figure 39. Non-chiral HPLC *Yarrowia lipolytica* biotransformation product extract.

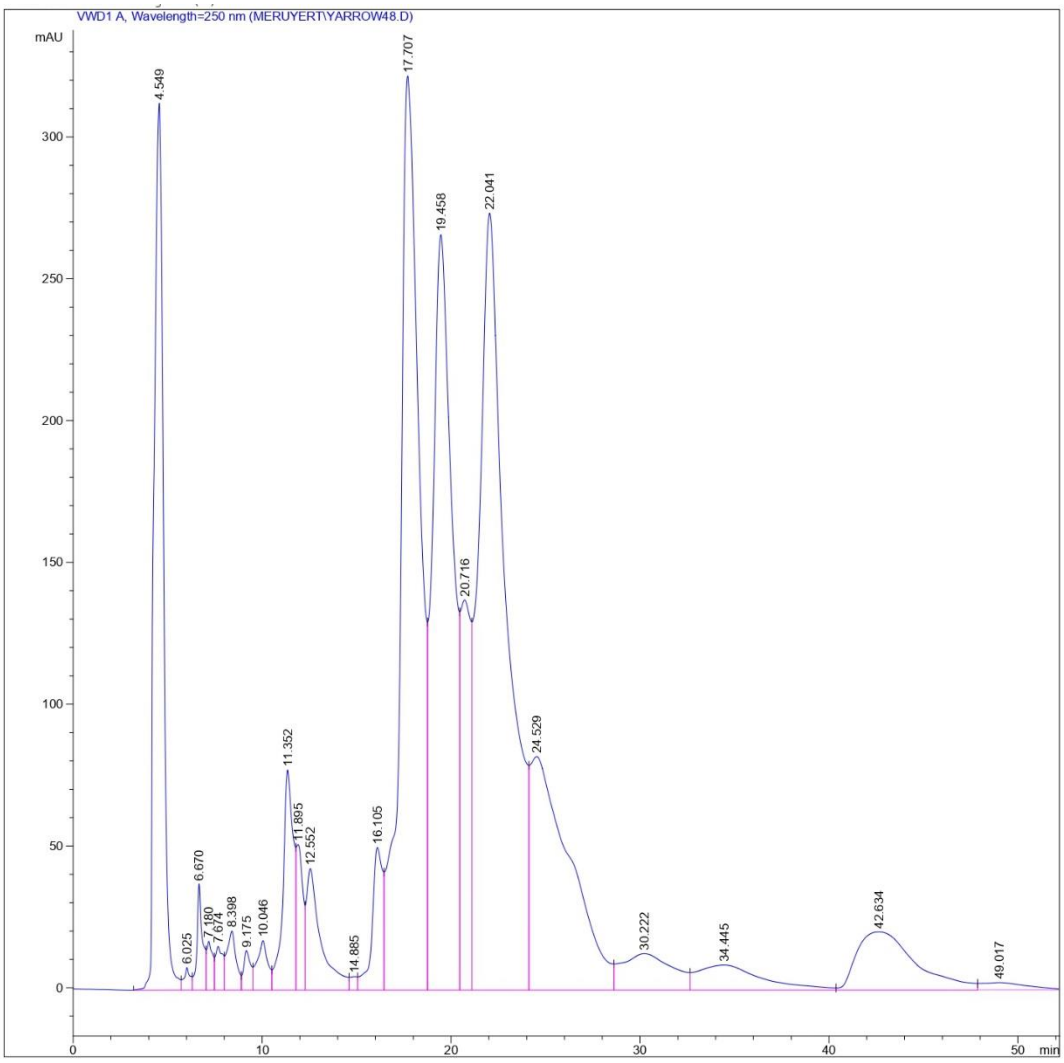

Figure 40. Chiral HPLC *Yarrowia lipolytica* biotransformation product extract.

11. Abstract in lingua italiana

Oggetto della tesi è stata la produzione biocatalitica di un intermedio chiave per la sintesi del diltiazem, attraverso la riduzione del corrispondente precursore mediata da microorganismi. Il prodotto delle biotrasformazioni è stato caratterizzato a livello di resa e purezza stereochimica. I risultati delle reazioni sono stati valutati in termini di conversione, stereoselettività e chemoselettività. In particolare, 40 mg di substrato in soluzione di N,N-dimetilformammide (DMF) sono stati ridotti con conversioni fino a 99% e con eccessi enantiomerici >95%. In conclusione, la riduzione asimmetrica microbica si è rivelata un metodo efficiente dal punto di vista della conversione, del costo del biocatalizzatore, della bassa produzione di rifiuti tossici e delle blande condizioni di reazione.

12. Acknowledgements

The completion of this study could not have been possible without the expertise of my supervisor Davide Tessaro. I would like to express my sincere gratitude for his constant support, training during my research, his knowledge, motivation, and patience, which helped me to finish my thesis with success.

Besides my supervisor, I would like to thank co-advisor of this research Stefano Serra for his expertise with microorganism growth and guidance during biotransformation reactions. Finally, I thank all members of the BioCatLab team, for their welcomeness, team spirit, extensive knowledge and expertise in the field were instrumental in helping me complete my research project successfully.