

UNIVERSITY HONORS PROGRAM

SENIOR PROJECT - APPROVAL

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PROJECT TITLE: POSTMORTEM POLYAMINE DEGRADATION IN MICE

I have reviewed this completed senior honors thesis with this student and certify that it is a project commensurate with honors level undergraduate research in this field.

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HONORS PROGRAM

SENIOR HONORS THESIS

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POSTMORTEM POLYAMINE DEGRADATION IN MICE

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Senior Honors Project

POSTMORTEM POLYAMINE DEGRADATION IN MICE

Abstract

The polyamines putrescine (Put), spermidine (Spd), and spermine (Spm), occur in all eukaryotic cells and play an important roles in cell growth and differentiation. Cadaverine (Cad) also appears in the human body. Polyamines and their metabolites are considered to be involved in the process of cell multiplication and its regulation. Rapidly growing tissues have higher amounts of polyamines and they have a stimulating effect on DNA, RNA and protein synthesis. Conversely, severe depletion of polyamines will reduce growth in mammalian cells.

The analysis of polyamines is carried out by an extraction from tissue to isolate polyamines. Then this extract is reacted with a pyrene-labeling reagent that forms intramolecular excimers so that it might be observed by fluorescence during HPLC assays. Through analysis of these peaks and taking into account the amount of sample and the concentrations used and injected, one is able to predict the amount of each polyamine in the sample.

These assays and reactions are carried out on mouse leg muscle over a 5-day period to see what the levels of polyamines are in them. It is not seen as conclusive as to whether there is a predictable degradation timeline for polyamines in mice however further experimentation might provide better results.

PURPOSE

The purpose of this project was to analyze the presence of polyamines in the human body postmortem to determine what relation, if any, there is to their presence and the rate of decomposition. These findings might provide insight to a timescale of decomposition on a biochemical level, rather than on a gross anatomical one. Since an opportunity to sample a body did not present itself, it is believed that an analysis in mice would provide a similar study and might provide insight into that of the human body. These findings in respect to the human body could be useful to help establish another way to determine the time since death based on the levels of polyamines. Since many pathologists disregard the body after there is a great deal of soft tissue decomposed, it is believed that this might provide a useful timeline after that.

INTRODUCTION

The polyamines putrescine (Put), spermidine (Spd), and spermine (Spm), occur in all eukaryotic cells and play an important roles in cell growth and differentiation. Cadaverine (Cad) also appears in the human body.¹ It has been found that the concentration of these polyamines together with that of their acetyl conjugates increase significantly in the biological fluids and the affected tissues of cancer patients. Polyamines and their metabolites are considered to be involved in the process of cell multiplication and its regulation.^{2,3} Rapidly growing tissues have higher amounts of polyamines and they have a stimulating effect on DNA, RNA and protein synthesis. Conversely, severe depletion of polyamines will reduce growth in mammalian cells.^{4,5} Polyamines are reported to have a role in the prevention of nerve damage^{6,7} and duodenal

mucosal repair.⁸ Spermine has been recommended for the treatment of human prostate cancer by itself and in combination with other anticancer drugs.^{9,10} Put, Cad, Spd, and Spm are polycations under physiological conditions and exhibit net charges of 2+, 2+, 3+, and 4+, respectively. As polycations, they could interact electrostatically with negatively charged moieties such as DNA, RNA, proteins, and phospholipids and stabilize their structure. Spd and Spm are particularly flexible and have charge distributions along the whole molecule, this facilitating greater interaction with the negatively charged backbone of DNA.^{11,12} Free and acetylated Spd, Spd and their precursor Put have been reported in mammalian tissue, serum, urine, and the central nervous system.¹³ Cad is produced by the decarboxylation of lysine and has been considered to have a role in prokaryotic cells.

The names and structures are as follows:

Putrescine

(Put)



1,4

Diaminobutane



Cadaverine

(Cad)



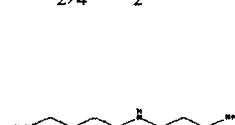
1,5-

Diaminobutane



Spermidine

(Spd)



Spermine

(Spm)



All eukaryotic cells synthesize Put, Spd, and Spm and contain a low level of Put and higher levels of Spd and Spm.¹⁴ The principal precursors of the polyamines are amino acids L-ornithine and L-methionine. The initial hydrolysis of L-arginine to L-ornithine and urea, catalyzed by arginase may also be considered. Put is formed by direct decarboxylation of L-ornithine. The reaction is catalyzed by ornithine decarboxylase (ODC) and may be a rate-determining step in polyamine synthesis in nonproliferating

cells. However, in rapidly dividing cells (e.g. embryonal cells, cells of gut mucosa, and tumor cells) ODC activity is higher and is not considered a rate-limiting parameter.^{15,16} ODC activity is dependent on pridoxial 5'-phosphate for its catalytic activity with a short half-life of 20 minutes.

Spd and Spm are synthesized from Put by the action of spermidine synthase and spermine synthase, where the reaction involves the addition of aminopropyl to Put or Spd. The aminopropyl groups are derived ultimately from L-methionine. Polyamine is catabolized and degraded back to Put by the action of polyamine oxidase and spermidine/spermine N'-acetyltransferase. The activity of acetyl transferase is low under normal conditions and might be attributed to being a rate-limiting factor in the interconversion.. The activity of the acetyltransferase can be induced by introducing exogenous polyamines.^{4,14,17}

The analysis of polyamines is carried out by an extraction from tissue to isolate polyamines. Then this extract is reacted with a pyrene-labeling reagent that forms intramolecular excimers so that it might be observed by fluorescence during HPLC assays. Through analysis of these peaks and taking into account the amount of sample and the concentrations used and injected, one is able to predict the amount of each polyamine in the sample.

PROCEDURE

Before any analysis is performed it is important to familiarize oneself with the techniques and protocols required to perform the assays. To do this, one should perform the derivations and reactions on standards of each of the polyamines. The pyrene-

labeling reagent used is 4-(1-Pyrene)butyric acid N-hydroxysuccinimide ester (PSE), obtained from Molecular Probes Inc. (Eugene, OR). Distilled water is used for all aqueous solutions and any organic solvents used were of HPLC grade. Stock solutions (10mM) of the amines are prepared in a mixture of tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), and water (1:2:1, v/v) and are stored at -80°C . These are further diluted with the solvent mixture before use to obtain the appropriate concentrations. A 5mM solution of PSE is prepared in acetonitrile and is stored at -80°C where it is useable for at least a week.

Once all reagents are made, one can then perform the pyrene labeling. This is done in Reacti-vials where 60 μL of 5mM PSE is placed in the vial, followed by 60 μL of each stock solution (10mM) of polyamine. To this, add 3.5 μL of 1M sodium carbonate so the reaction can run at the desired pH. The reaction is to be done in a block heater at 100°C for 30 minutes. The vials are then placed in an ice water bath and can be stored in a -20°C freezer until HPLC is performed on them.¹⁸

HPLC was performed on an Agilent HPLC system with gradient mixer and C_{18} reverse-phase column rather than the prescribed C_8 . The column is washed before the run so that it will provide a clean run. For each HPLC run, 10 μL of the polyamine standard was injected. An elution gradient of 50 %– 100% Acetonitrile is used and a flow rate of 1 mL/min is established. Elution is monitored at 220 nm, 254 nm, 375 nm, and 475 nm and fluorescence was monitored with an excitation and emission wavelength of 345 nm. Each run takes approximately 30 minutes or longer and this is performed on each of the standards, along with a reaction blank and a monoamine standard.

Once all standards are run with good results obtained, tissue sampling commenced. The original choice for tissue samples was postmortem human tissue from a fresh body (within 24 hours of death). When this did not work out, mice became the second option. For the purposes of this project, it was established that a 5-day timescale for death would be appropriate and it was also determined that 2 replicates would be sufficient. Once this was established, 6 mice were killed and then the four for days 2 and 5 were set aside and placed in a desiccators jar. The 2 for day 1 were dissected and the rear leg muscles for each were removed and placed in their respective marked tubes. We also removed the livers and placed them in their respective tubes. Once samples were collected, they were placed on dry ice to quickly freeze them in their state and then these were placed in the -80°C freezer until all samples are collected. This was repeated for days 2 and 5 and with all dissection being done in a hood with vertical laminar flow so as to prevent contamination and to keep down the smell. All mice used were approved for scientific use and were already scheduled to be killed.

Once all samples were collected, we started extracting the polyamines from each sample through a relatively extensive process. It was determined that the most reasonable extraction would involve the use of 80% methanol solution that would allow the solubility of the polyamines. To accomplish this, eppendorf tubes with micropestels seemed to be the best solution to grind up the tissue samples. A portion of each sample was taken from each tube and placed in the eppendorf tube and then each was ground to a pulp and set on dry ice and then ground again. Then, .5 mL of 80% methanol was added, ground again and then the micropestel is rinsed over the tube with another .5 mL of 80% methanol solution. This is then vortexed for approximately 30 seconds and then the

sample is centrifuged for 5 min. at 14000 min^{-1} . The supernatant is then poured off into a labeled tube and a small pinhole poked into the top and the samples are placed into SpeedVac to evaporate for approximately 45 min. until solution is brought to 100x. Once this is done, the pyrene labeling reaction is performed on each sample under the conditions previously noted and then these samples are run through the HPLC system with each run taking approximately 1 hour. Once all samples are run, the data is collected and the excitation peaks and fractional peaks are analyzed, it is determined what polyamines came out at what time based on the standards, which were run earlier.

RESULTS

The following chromatograms serve as a summary of the results obtained through the analyses performed by HPLC. The rest of the data is taken from the other chromatograms. All polyamine standards were run at the same time and then all tissue samples were run at the same time.

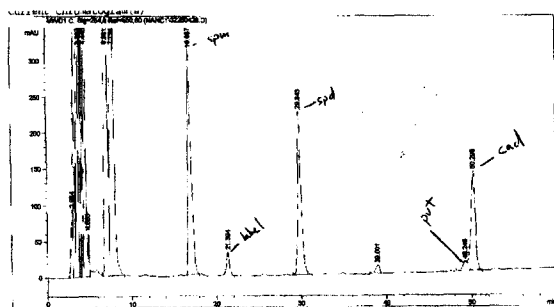


Figure 1. Chromatogram at 254 nm illustrating the collection time for each fraction of the standards polyamines on a C_{18} column.

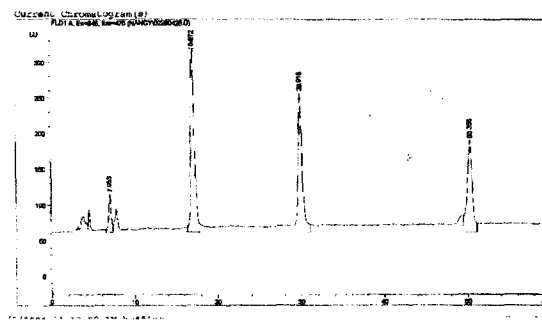


Figure 2. Chromatogram at 345 nm illustrating fluorescence for the polyamine standard

Sample	MW(g/mol)	10 mM grams/L	10 ul inj grams injected	Area of peak	Grams per unit area	Micrograms/unit area
Spermine	348.2	3.482	0.00003482	11020.7	3.15951E-09	0.00315951
Spermidine	254.63	2.5463	0.000025463	9802.17	2.59769E-09	0.00259769
Cadaverine	175.1	1.751	0.00001751	7648.64	2.2893E-09	0.0022893
Putrescine	161.08	1.6108	0.000016108	1239.23	1.29984E-08	0.01299839

Figure 3. Calculations for determining the amount of standard polyamine representing each unit of area determined by the amount injected of each standard.

Polyamine obs.	Int. area	ug per 20ul of sample	ug per mg tissue
m1d1 calculations			
spd	9388.6	29.66336548	3.115899736
spm			0
put			0
cad	5571.5	12.75481197	1.339791173
m2d1 calculations			
spd	3327.7	10.51389785	1.104401034
spm			0
put			0
cad	1035.7	2.371023738	0.249057115
m3d2 calculations			
spd	6178.2	19.52007803	2.050428365
spm			0
put	1107.3	14.39312186	1.511882548
cad	603.3	1.381132201	0.145076912
m4d2 calculations			
spd	1.00E+04	3.16E+01	3.318811895
spm			0
put			0
cad	1073.2	2.456872333	0.258074825
m5d5 and m6d5			
No measurable polyamines detected			

Figure 4. Calculations from chromatograms to quantify the amount of each polyamine in each tissue sample based on the polyamine standards and a 20 µL sample injection.

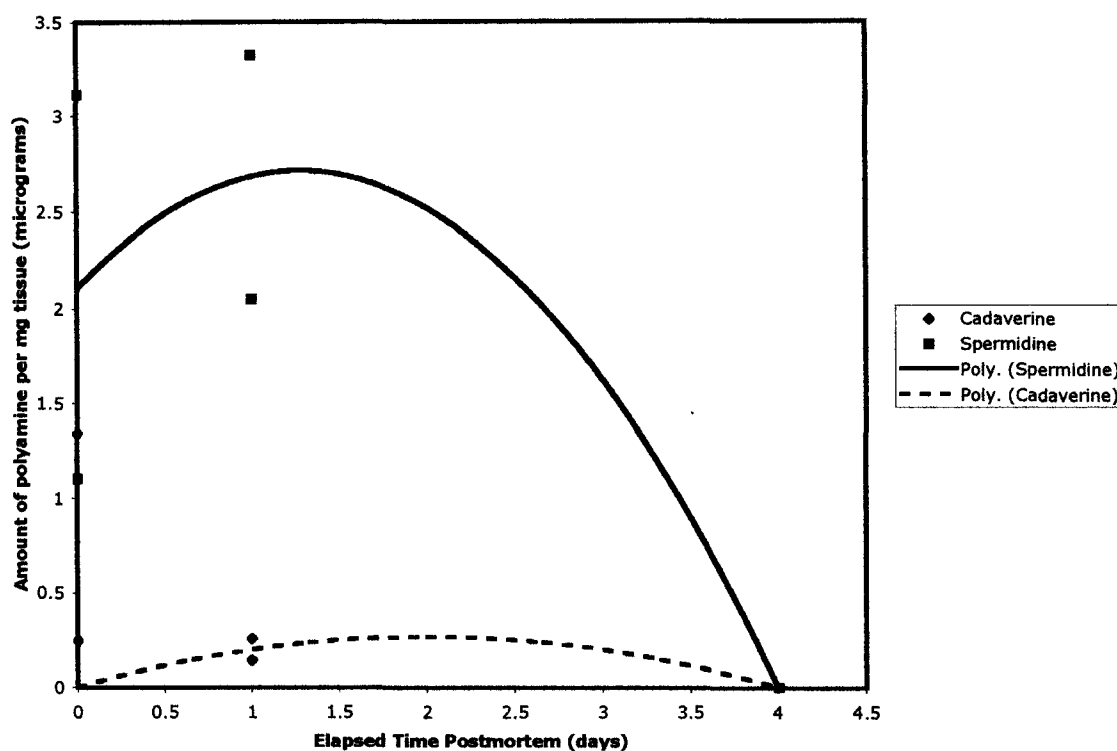


Figure 5. Amount of cadaverine and spermidine present in tissue sample over 4 days of postmortem analysis measured by HPLC.

Other chromatograms performed on the mouse muscle samples are attached at the end to refer to see where the information presented in Figures 4 & 5 was taken. These chromatograms are at 254 nm and can be compared to the standards in Figure 1.

DISCUSSION

According to Fig. 1 and Fig. 2, it is apparent that the pyrene-labeling reagent worked to fluoresce the polyamines through HPLC analysis showing that the fraction of each of the polyamine standards matched with the fluorescence showing superimposability. This would suggest that HPLC of polyamines is possible and that it

can accurately detect them in solution as the peaks of the mixture match up with those of the individual runs of each standard.

Fig. 3 helps by integrating the peaks of each of the polyamine standards to give a baseline to compare the findings from the tissue samples from each of the mice. With these findings, it is possible to calculate as illustrated in Fig. 4 the amount of each polyamine that is represented in each peak of the chromatogram at each time point. From this, one can create a graph to predict the degradation of polyamines in tissue samples of mice as is illustrated in Fig. 5.

The results of this experiment turned out to be somewhat inconclusive. There are many possible explanations for these findings. The findings for the tissue samples revealed that the amount of polyamines in each sample varied even between replicates and that there is not enough evidence to point that one is more correct than the other. More replicates would help to achieve this better. Another key aspect to observe is that in any given chromatogram of the tissue samples, not all polyamines are observed in any noticeable amount. It would seem that all four polyamines would show up, but this does not seem to be the case. There was also found to be an error in the extraction process in that it is necessary to deproteinate the sample before carrying out the reaction, which was not done here.¹⁹ More information regarding the baseline levels of polyamines in mice would have also served as indicators of the results obtained. Ideally, collection would have produced better results had all samples been taken from the same corpse. This was not possible due to the size of the mouse and the amount of tissue needed to carry out the extraction. Had this research been done on a human cadaver, it might have been possible to sample the same tissue over a longer period of time to cut out certain variables, which

were experienced here as the mice varied in muscle composition and overall size. Another variable that might come into play is that of variability due to cancer as polyamines with their acetyl conjugates increase significantly in biological fluids and affected tissues of cancer patients.²⁰ This might be more of an issue when researching humans.

The next step in research would be to start with a broader sampling group using mice of similar size and health and increasing the number of replicates to allow for a more accurate average. Extraction should be done with a de-proteinating step as well. It might also work well to sample the livers after killing the microbes to see if they exhibit a different mix of polyamines. The ideal step would be to carry out these assays on the human body tissue perimortem and post mortem over a timescale using several replicates to see if the levels from the assays are indicative of a trend in degradation that might lead to a new technique in establishing time since death on a biochemical level.

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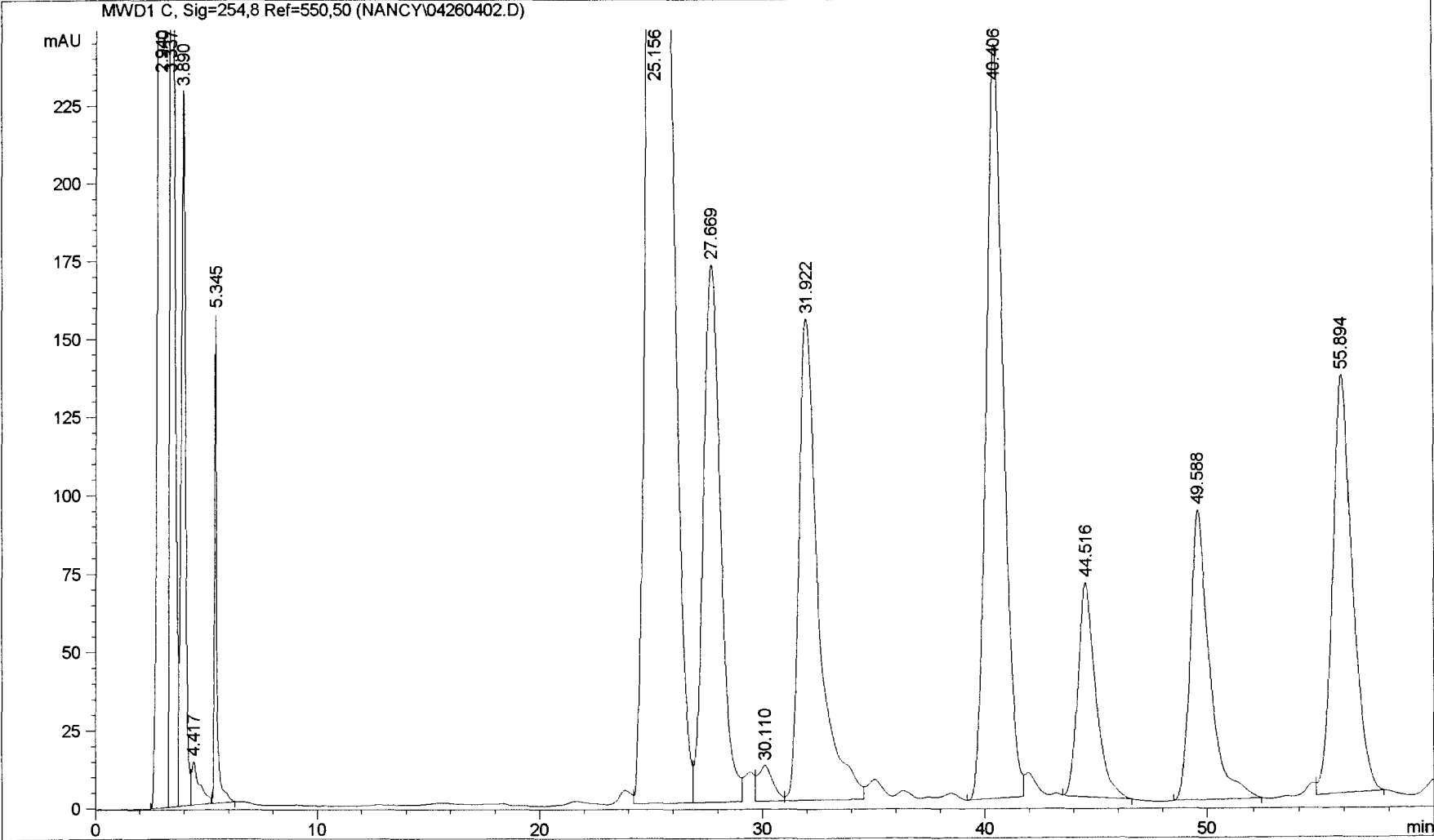
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Acq. Method	: C:\HPCHEM\1\METHODS\POLYAL.M		
Last changed	: 3/3/2004 11:54:35 AM by huffing		
Analysis Method	: C:\HPCHEM\1\METHODS\POLYAL.M		
Last changed	: 4/27/2004 8:56:10 AM by huffing		

(modified after loading)

polyamine

Current Chromatogram(s)

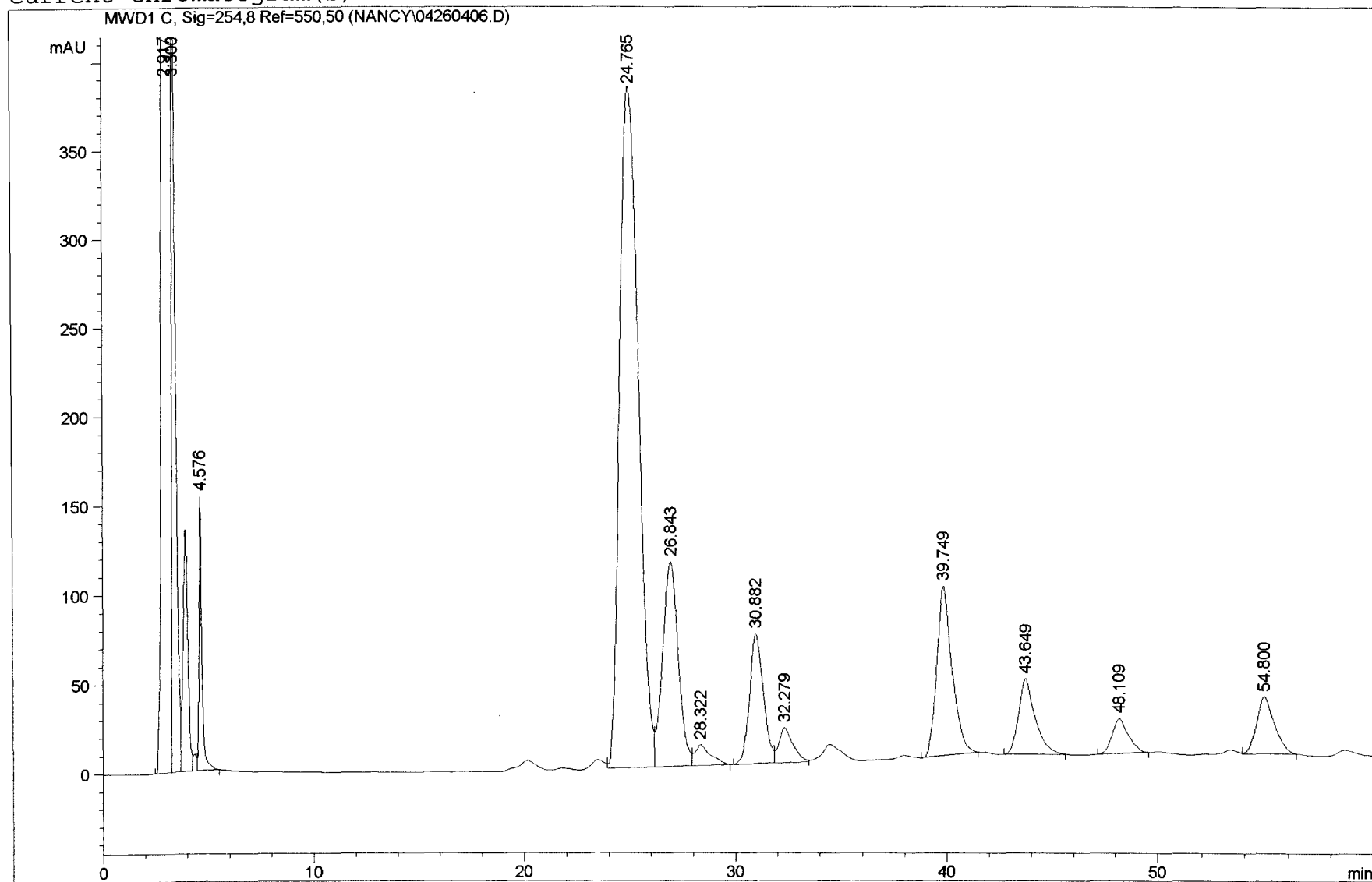


31.922 = SPD
49.588
cad

=====

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polyamine

Current Chromatogram(s)

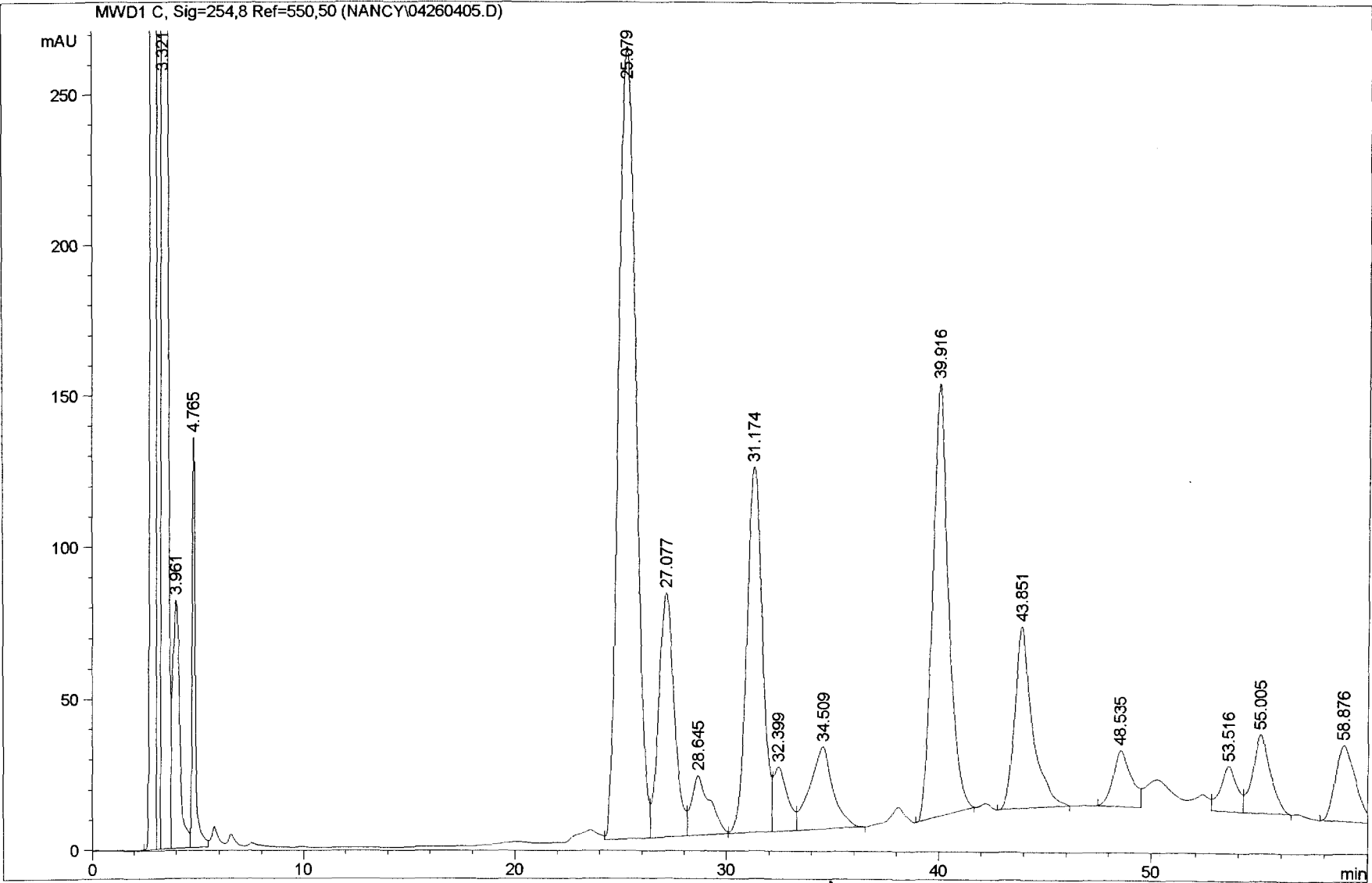


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polyamine

Current Chromatogram(s)



31.174 = spd

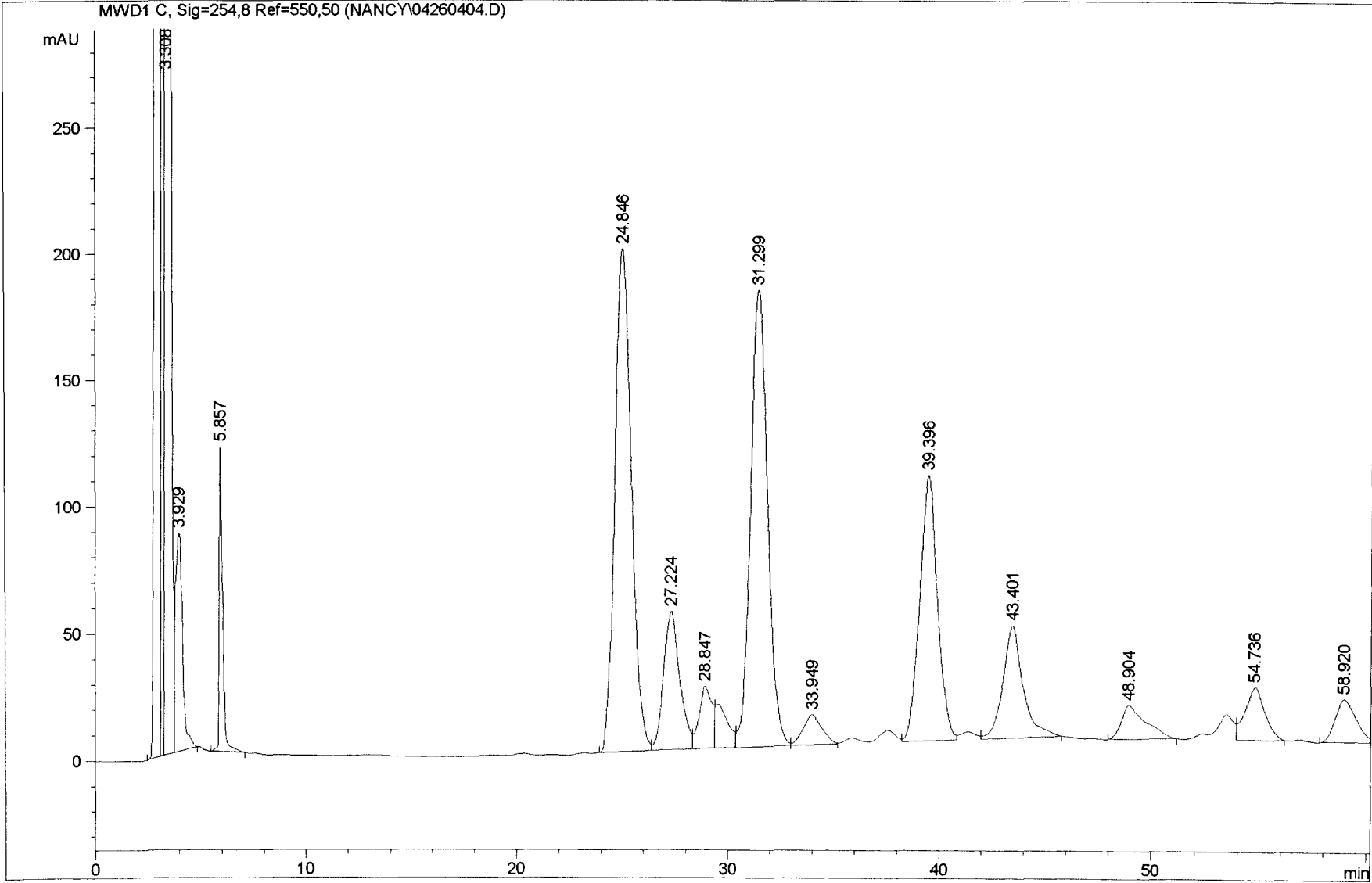
put = 48 936
cad = next

Print of window 38: Current Chromatogram(s)
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polyamine

Current Chromatogram(s)



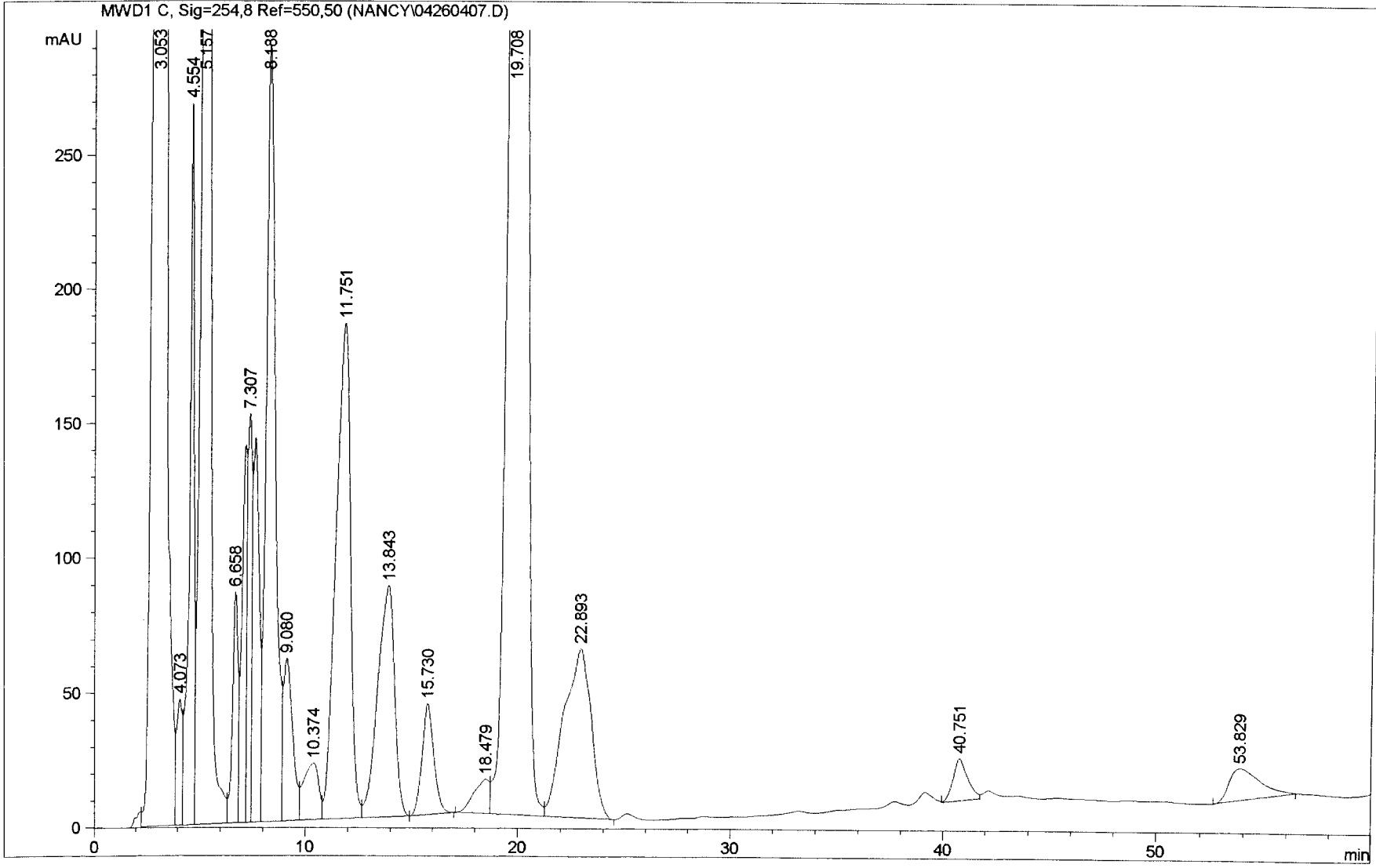
31.299 = SP1 ?
48.904 = CAD

=====

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polyamine

Current Chromatogram(s)



no recog pa-

Injection Date : 4/27/2004 11:33:10 AM
Sample Name : m6d5 Vial : -
Acq. Operator : huffing
Method : C:\HPCHEM\1\METHODS\POLYAL.M
Last changed : 4/27/2004 8:56:10 AM by huffing
(modified after loading)

polyamine

Current Chromatogram(s)

