# The role of p-glycoprotein and peptides in attention deficit disorder with hyperactivity (ADHD)

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# Index

# Abbreviations

| | |
|---|---|
| **A** | |
| Å | Angström |
| ABC | ATP binding cassette domain |
| ABCB1 | ATP-binding cassette, subfamily B, member 1 |
| ADD | Attention deficit disorder |
| ADHD | Attentiondeficit disorder with hyperactivity |
| ADP | Adenine dinucleotide diphosphate |
| ATP | Adenine dinucleotide triphosphate |
| **B** | |
| BBB | Blood-brain barrier |
| β-CM | Beta-casomorphin |
| β-CM-5 | Beta-casomorphin, fragment 1-5 |
| **C** | |
| CFTR | Cystic fibrosis transmembrane conductance regulator |
| CNS | Central nervous system |
| CSF | Cerebrospinal fluid |
| CYP 3A | Cytochrome P450 3A |
| **D** | |
| Da | Dalton |
| kD | Kilo dalton |
| DA | Dopamine |
| DC | Dendritic cell |
| DOPEG | 3, 4-dihydroxyphenyl glycol |
| DSM | Diagnostic and statistical manual of mental disorders |
| **E** | |
| EFAs | Essential fatty acids |
| ER | Endoplasmic reticulum |

**G**

| | |
|---|---|
| GABA | γ-aminobutyric acid |
| GIT | Gastro intestinal tract |

**H**

| | |
|---|---|
| 5-HIAA | 5-hydroxyindole acetic acid |
| 5-HT | Serotonin |
| HIV PI | Human immunodeficiency virus protease inhibitor |
| HPLC | High-performance liquid chromatography |
| HVA | Homovanillic acid |
| HDL | High-density lipoprotein |

**M**

| | |
|---|---|
| MDR/mdr | Multidrug resistance |
| MRP | Multidrug resistance associated protein |

**N**

| | |
|---|---|
| NA | Noradrenalin |
| NBD | Nucleotide binding domain |

**O**

| | |
|---|---|
| OCD | Obsessive compulsive disorder |

**P**

| | |
|---|---|
| PBP | Periplasmic binding protein |
| PDD | Pervasive developmental disorder |
| P-gp | P-glycoprotein |
| Pi | Inorganic phosphate |
| PST | Phenyl sulphur transferase |
| pro | Proline |

**R**

| | |
|---|---|
| mRNA | Messenger ribonucleic acid |

T

| | |
|---|---|
| TFA | Trifluoroactic acid |
| TMD | Transmembrane domain |
| tyr | Tyrosine |

V

| | |
|---|---|
| VLCFA | Very long chain fatty acid |

# Glossary

A

**Absorption:** Movement of materials across an epithelial layer from body cavity or component towards the blood.

**Affinity:** An attractive force between substances or particles that causes them to enter into and remain in chemical combination.

**Agonist:** A chemical substance such as a drug, capable of combining with a receptor on a cell and initiating the same reaction or activity typically produced by the binding of an endogenous substance.

**Antagonists:** A chemical that acts within the body to reduce the physiological activity of another chemical substance such as an opiate.

**Aphasia:** Loss or impairment of the power to use or comprehend words usually resulting from brain damage.

**Apical:** Portion of plasma-membrane facing the lumen.

**Attention deficit disorder with hyperactivity:** A lifelong developmental disorder that with symptoms such as inattention, hyperactivity, impulsivity and learning difficulties.

**Auxiliary:** Serving to supplement or assist.

B

**Blood-brain barrier:** Group of cells that form a special, impermeable lining in the blood vessels of the brain. The blood-brain barrier is made up of astrocytes and prevents toxic substances in the blood form entering the brain.

C

**Catecholamine:** Any of various amines such as adrenalin, noradrenalin and dopamine, that contain a dihydroxy benzene ring, that are derived from tyrosine, and that function as hormones, neurotransmitters or both.

**Cytoplasmic:** The organized complex of inorganic and organic substances external to the nuclear membrane of a cell, including the cytosol and membrane-bound organelles such as mitochondria or chloroplasts.

D

**Deficiencies:** A shortage of substances (as vitamins) necessary to health.

**Digestion:** The process of making food absorbable by dissolving it and breaking it down into simpler chemical compounds that occur in the living body mainly through the action of enzymes secreted into the alimentary canal.

**Domains:** Any of the three-dimensional subunits of a protein that together make up its tertiary structure, that are formed by folding its linear peptide chain, and that are variously considered to be the basic units of protein structure, function, and evolution.

E

**Efflux:** The action or process of flowing or seeming to flow out.

**Endogenous:** Relating to or produced by metabolic synthesis in the body.

**Endorphins:** Any of a group of endogenous peptides (as enkephalin and dynorphin) found especially in the brain that bind chiefly to opiate receptors and produce some of the same pharmacological effects such as pain relief, as those of opiates.

**Exogenous:** Originating or produced outside the body.

**Extracellular:** Outside of cell.

F

**Filtration:** The process of passing through or as if through a filter also known as diffusion.

H

**Hepatobiliary:** Of, relating to, situated in or near, produced in, or affecting the liver and bile, bile ducts, and gallbladder.

**Homodimer:** A protein composed of two polypeptide chains that are identical in the order, number, and kind of their amino acid residues.

**Hydrophilic:** Of, relating to, or having a strong affinity for water.

I

**Inhibitor:** An agent that slows or interferes with a chemical reaction or a substance that reduces the activity of another substance such as an enzyme.

**Intestine:** The tubular portion of the alimentary canal that lies posterior to the stomach from which it is separated by the pyloric sphincter and consists of a slender but long anterior part made up of the duodenum, jejunum, and ileum which function in digestion and assimilation of nutrients and a broader shorter posterior part made up of the cecum,

colon, and rectum which serve chiefly to extract moisture from the by-products of digestion and evaporate them into faeces.

**Intracellular:** Inside of the cell.

L

**Leaky gut syndrome:** is a term used to describe a phenomenon where there is increased intestinal permeability resulting from chronic irritation to the gut wall. A leaky gut wall can lead to a variety of systemic problems, including gluten and casein sensitivity and food allergies.

**Ligands:** A group, ion, or molecule coordinated to a central atom or molecule in a complex.

**Lipophilicity:** Having an affinity for lipids.

N

**Nephron:** A single excretory unit especially of the vertebrate kidney typically consisting of a Malpighian corpuscle, proximal convoluted tubule, loop of Henle, distal convoluted tubule, collecting tubule, and vascular and supporting tissues and discharging by way of a renal papilla into the renal pelvis.

**Neurotransmitter:** Any one of numerous chemicals that modify or result in the transmission of nerve impulses between synapses.

O

**Opioid peptide:** Any of a group of endogenous neural polypeptides such as an endorphin or enkephalin that bind especially to opiate receptors and mimic some of the pharmacological properties of opiates, also called opioid.

**Opioid:** Possessing some properties characteristic of opiate narcotics but not derived from opium of, involving, or induced by an opioid substance or an opioid peptide.

P

**Peptides:** Any of various amides that are derived from two or more amino acids by combination of the amino group of one acid with the carboxyl group of another and are usually obtained by partial hydrolysis of proteins.

**Permeable:** Having pores or openings that permit liquids or gases to pass through.

**Pinocytotic:** The uptake of fluid by a cell by invagination and pinching off of the plasma membrane.

**Proximal tubule:** The convoluted portion of the vertebrate nephron that lies between Bowman's capsule and the loop of Henle, is made up of a single layer of cuboidal cells with striated borders, and functions especially in the resorption of sugar, sodium and chloride ions, and water from the glomerular filtrate.

**R**

**Reabsorption:** The act, process, or condition of absorbing again or of being absorbed again.

**S**

**Secretion:** The process of segregating, elaborating, and releasing some material either functionally specialized (as saliva) or isolated for excretion (as urine).

**Specificity:** The condition of participating in or catalyzing only one or a few chemical reactions.

**Substrates:** A substance acted upon (as by an enzyme).

**T**

**Toxin:** Any poisonous substance that can cause a disease.

**X**

**Xenobiotic:** A chemical which is not a natural component of the organism exposed to it. Synonyms include drug, foreign substance or compound, exogenous substance or compound.

# Uittreksel

'n Kenmerkende peptiedprofiel is waargeneem in die urien van sekere pasiënte wat aan ADHD lei. Die doel van hierdie studie was om te bepaal of defektiewe p-glikoproteïene verantwoordelik is vir die voorkoms van hierdie peptiedprofiel in kinders met ADHD.

Die urienanalises van kinders met ADHD is uitgevoer deur middel van HPLC met UV-deteksie by 215 en 280 nm. Gradiënteluering, bestaande uit ses stappe, is verkry met 2 mobiele fases: buffer, A 0.1 % trifluoroasynsuur (TFA) en buffer B, 0.1 % TFA in asetonitriel op 'n Vydac $C_{18}$ kolom. 'n Rotmodel is gebruik om die resultate wat in die mense gekry is te bevestig. Die rotte is in 4 groepe verdeel. Die eerste groep het kaseïen saam met hulle gewone dieet gekry en het gedien as kontrole vir groep 2 wat die p-gp inhibeerder, siklosporien, tesame met die kaseïen gekry het. Groep 3 was op 'n normale dieet geplaas en was die kontrole vir groep 4 wat siklosporien ontvang het. Die urien is versamel terwyl die rotte in metaboliese hokke gehuisves is. Die urien is geanaliseer met die HPLC-metode wat bo beskryf is.

Die meerderheid van die ADHD-kinders het nie enige merkwaardige urinêre peptiedprofiele getoon nie en is vir 8 maande of langer met metielfenidaat behandel. Twee pasiënte het wel die urinêre peptiedprofiel getoon. Die een pasiënt is vir 2 maande met metielfenidaat behandel en die ander pasiënt was nie op enige medikasie nie. Ons spekuleer dat metielfenidaat, moontlik deur p-gp aktivering, betrokke mag wees by die terugvervoer van die peptiede na die maag toe.

Die rotmodel het nie enige merkwaardige urinêre peptiedprofiele in enige van die groepe gelewer nie. Daar is kontrasterende resultate in studies oor die inhiberende/induserende effekte van siklosporien op p-gp wat dit moeilik maak om die netto effek van siklosporien op p-gp in die onderskeie weefsels te bepaal. Dit is ook moontlik dat defektiewe p-gp alleen nie verantwoordelik is vir die voorkoms van die peptiede in die urien nie, maar dat 'n deurlaatbare maagwand, tesame met defektiewe p-gp die oorsaak is.

# Abstract

A characteristic peptide profile was detected in the urine of certain patients suffering from ADHD. The purpose of this study was to determine whether defective p-glycoprotein may be responsible for the occurrence of this peptide profile in children with ADHD.

Urine analysis of children with ADHD was performed using HPLC with UV detection at 215 and 280 nm. A six step gradient elution was attained by using two mobile phases: buffer A was 0.1 per cent trifluoroacetic acid (TFA) and buffer B 0.1 per cent TFA in acetonitrile on a Vydac $C_{18}$ column. To verify the results obtained in humans, the following procedure was adopted using a rat model: The rats were divided into four groups. The first group received casein and their normal diet and served as control for group 2 which received the p-glycoprotein inhibitor, cyclosporine in addition to the casein. Group three was placed on a normal diet and served as control for group 4 who was given cyclosporine. Urine was collected from metabolic cages housing the test animals. The urine was analysed using the same HPLC procedure as above.

A large group of the ADHD children did not display any significant urinary peptide profiles and were on methylphenidate for 8 months or longer. Two ADHD patients presented with the urinary peptide profile. One patient had been on methylphenidate for 2 months and the other patient had not received any medication. We speculate that methylphenidate may be involved in the transport of these peptides back into the gut, possibly by activating p-gp.

The rat model did not reveal any significant urinary peptide profiles in any of the various groups. There are conflicting reports about the inhibiting/inducing effects of cyclosporine on p-gp which caused difficulties in predicting the net effect of cyclosporine on the p-gp in the various tissues. It is also possible that defective p-gp alone may not be responsible for the occurrence of peptides in the urine, but that both defective p-gp as well as a leaky gut may be responsible.

# Introduction

Attention-deficit hyperactivity disorder (ADHD) is a clinically heterogeneous condition, also known as hyperkinetic or hyperactive syndrome. It is the most common heritable and behavioural disorder of childhood and epidemiological studies suggest that the syndrome is three to four times higher in males than in females, and occurs in approximately 3–5 % of school-age children in Western countries or 5–10 % worldwide (Shastry, 2004). Although the aetiology of ADHD is unknown, a cohesive body of evidence has accumulated in recent years, suggesting that ADHD may be a manifestation of nutritional deficiency (Ottoboni & Ottoboni, 2003).

Prominent findings in children with developmental and behavioural disorders are maldigestion and immune dysregulation often related to dietary gluten, casein and intestinal dysbiosis. Researchers, Reichelt *et al.* (1986) and Hole *et al.* (1988) found breakdown products of food-derived polypeptides in the urine of patients with a variety of behavioural conditions such as ADHD and autism. The same food derived polypeptides are also found in schizophrenia (Reichelt *et al.*, 1981). The presence of these polypeptides is attributed to a reduction in the efficiency of peptidases in the bowel lumen and an increase in the transport of the peptides across the intestinal membrane. Some of these peptides are biologically active with actions similar to endorphins and are described as opioid peptides. One of these peptides, casomorphin, is derived from casein and another, gliadomorphin, from gluten (AAL reference laboratories, 2000). Shattock & Lowdon (1991) used high-performance liquid chromatography (HPLC) to isolate and measure these peptides which are excreted in the urine.

Potential treatment for ADHD includes removal of the source of these substances that affect the brain function, and these strategies form the basis for elimination diets. Strict removal of casein/dairy can often result in noticeable improvement in ADHD symptoms, usually within a few weeks. Other behavioural symptoms, such as temper outbursts and mood swings, also often respond well to casein removal (Compart, 2003).

P-glycoprotein (p-gp) is a cell membrane-associated protein that transports a variety of drug substrates (Matheny *et al.*, 2001), including morphine and small hydrophobic peptides (van Tellingen, 2001). P-gp can influence drug absorption, distribution to the

site of action, and elimination in many organ systems and several tissues, such as the intestine, central nervous system (CNS), liver and kidney. P-gp is one of the xenobiotic transport proteins and it functions as a drug export pump that decreases intracellular concentrations of unwanted substances and thus acts as a protective mechanism against potentially toxic substances (Matheny *et al.*, 2001). Absence of p-gp in the blood-brain barrier (BBB) leads to highly increased brain penetration of a number of important drugs and this can result in severely increased neurotoxicity (Schinkel, 1999). Disorders such as ADHD may be worsened by exposure to toxic environmental impingements (Anon, 2000).

Peptides derived from the diet and excreted in the urine in certain disorders, led us to postulate that there may be defective p-glycoproteins in the intestines preventing these peptides to be transported back into the intestine, giving rise to specific urinary profiles.

# Chapter 1

# Attention deficit disorder with hyperactivity (ADHD)

## 1.1 Introduction

Attention deficit disorder with hyperactivity is the most commonly diagnosed behavioural disorder of childhood, and is estimated to affect between 3 % and 5 % of school-aged children (Glickman-Simon *et al.,* 2001).

The core symptoms of ADHD include inattention, hyperactivity, impulsivity and learning difficulties (Glickman-Simon *et al.,* 2001; Kirley *et al.,* 2002). Although many people occasionally have difficulty sitting still, paying attention or controlling impulsive behaviour, these behaviours are so persistent in people with ADHD that they interfere with daily life and cause significant functional problems at home, in school and various social settings (Glickman-Simon *et al.,* 2001).

It is not generally realized, but persons with ADHD seem to have an overall abnormal physical health profile. These abnormalities include gastrointestinal abnormality, compromised immunity, detoxification weakness and abnormal nutritional profiles such as various vitamin and mineral deficiencies (McGinnis, 1999).

## 1.2 Origin

The exact cause of ADHD remains uncertain, but the prevailing theories include genetic and hereditary factors, neurobiological conditions and pathologies, prenatal influences, nutritional factors and deficiencies, environmental/toxin influences and gut immunology mechanisms that directly affect the central nervous system (Anon, 2000; Mehl-Madrona, 2003). Those with the disorder usually present symptoms before the age of 7 years and continue to exhibit symptoms throughout their lifespan (Glickman-Simon *et al.,* 2001; Kirley *et al.,* 2002; Fisher, 1998:2).

### 1.2.1 Neurobiological conditions and pathologies

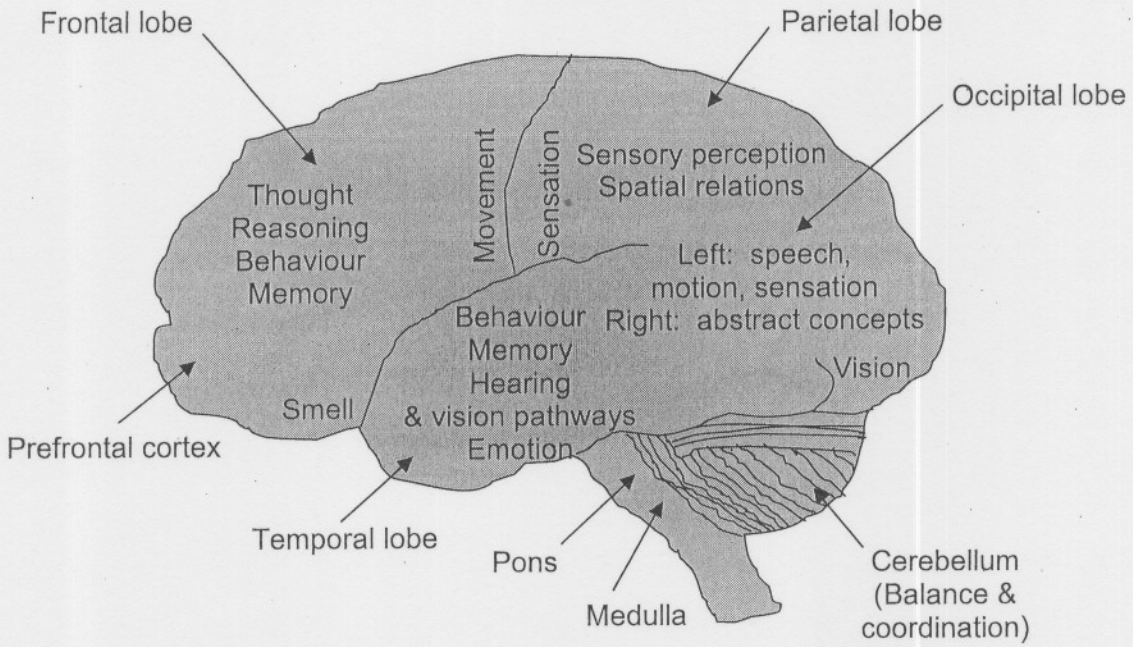


**Figure 1.1:** Parts of the brain and their function (BBC news, 1999).

Brain scans have revealed a number of differences in the brains of ADHD children compared to those of non-ADHD children (figure 1.1). For example, many children with ADHD tend to have altered brain activity in the prefrontal cortex, a region thought to be the brain's command centre. Irregularities in these areas may impair an individual's ability to control impulsive and hyperactive behaviours (Glickman-Simon *et al.*, 2001). Abnormalities in different brain regions such as an abnormal frontal lobe, reduced volume throughout the brain and hyperfusion in the sensorimotor area have been identified (Shastry, 2004).

In another study conducted, ADHD subjects had reduced regional temporal areas, as well as significant increases in the grey matter in large regions of the posterior temporal and inferior parietal cortices. The findings were not only in brain regions controlling attention, but also impulse control, which is often the most clinically debilitating symptom in children with ADHD. Although measures of the severity of ADHD symptoms subtypes generally did not correlate significantly with these morphological measures, grey matter

in the occipital lobe was inversely correlated with measures of inattention (Sowell *et al.*, 2003).

A number of studies have also found that brain volume is 3 to 4 percent smaller in individuals with ADHD (Castellanos *et al.*, 1996; Mostofsky *et al.*, 1998). In addition to shrinkage in total brain volume, it was found that shrinkage also occurred in brain components, such as the cerebrum and cerebellum. This observation persisted across all ages and sexes in both the medicated and unmedicated children with ADHD. It was suggested that genetic and/or early environmental influences on brain development in ADHD is permanent, not progressive, and not linked to the drugs commonly used for ADHD treatment (Castellanos *et al.*, 2002; Ottoboni & Ottoboni, 2003).

Other possibilities for hyperactive behaviour in children are that it may be the result from of excessive slow wave activity in certain regions of the brain, or that ADHD may be caused by abnormally low levels of dopamine, a neurotransmitter involved with mental and emotional functioning (Glickman-Simon *et al.*, 2001).

### 1.2.2 Environmental factors and toxins

Environmental factors associated with ADHD include low birth weight, hypoxia at birth, and exposure in utero to a number of toxins including alcohol, cocaine, and nicotine. Studies have found correlations between certain toxic agents/nutrient deficiencies and learning disabilities. Iron deficiency can cause irritability and attention deficits. Magnesium deficiency is characterized by fidgeting, anxiousness, restlessness, psychomotor inability, and learning difficulties (Mehl-Madrona, 2003).

### 1.2.3 Related mental disorders and comorbidity

Over 50 % of persons diagnosed with ADHD also have another psychiatric disorder which may mask or complicate their diagnosis and treatment. Depressive disorders, learning disorders, anxiety disorders, substance abuse, aggression and behaviour disorders and sleep disorders have all been reported to occur in persons with ADHD. These disorders appear significantly more in people with ADHD than in people without ADHD. Close biological relatives of children with ADHD are far more likely to have ADHD, major depressive disorder, multiple anxiety disorders, conduct disorder, anti-social personality disorder, and/or suffer from substance abuse than are relatives of

children without ADHD. All of these disorders tend to run in families and may be inherited in various combinations by some, though not all, family members (Mehl-Madrona, 2003).

## 1.3 Risk factors

- Heredity: Children with ADHD usually have at least one first-degree relative who also has ADHD and one-third of all fathers who had ADHD in their youth have children with ADHD.
- Gender: ADHD is four to nine times more common in boys than girls; however some believe that the disorder is under diagnosed in girls.
- Prenatal and early postnatal health: Maternal drug, alcohol, and cigarette use; exposure of the foetus to toxins; nutritional deficiencies and imbalances.
- Learning disabilities, communication disorders, and tic disorders such as Tourette's syndrome.
- Other behavioural disorders, particularly those that involve excessive aggression such as oppositional defiant or conduct disorder.
- Nutritional factors, allergies or intolerances to food, food colouring, or additives (Glickman-Simon *et al.*, 2001).

## 1.4 Diagnosis and classification

The names and symptoms for ADHD have changed frequently since the turn of the century. What is now referred to as ADHD has been described in the past as Minimal Brain Dysfunction, Hyperkinetic Reaction of Childhood and Attention Deficit Disorder (ADD) With or With out Hyperactivity. The name ADHD was adopted in 1987 by the third revision of the Diagnostic and Statistical Manual of Mental Disorders (DSM-III-R) (Glickman-Simon *et al.*, 2001).

Diagnosis is difficult but essential, as early treatment can substantially alter the course of a child's educational and social development. It is largely dependent on specific observed behaviours. The first step in establishing the diagnosis of ADHD is to determine whether the individual meets the diagnostic criteria as defined in the DSM-IV (Glickman-Simon *et al.*, 2001).

Since most of the characteristic behaviours of childhood ADHD occur at home and in the school setting, parents and teachers play an important role in providing information to establish the diagnosis (Glickman-Simon *et al.,* 2001).

The condition has been classified into 3 sub-types depending on the predominance of symptoms according to the DSM-IV. It could be the

1. predominantly inattentive type, previously known as ADD (Mehl-Madrona, 2003),
2. hyperactive-impulsive type or
3. combined inattentive and hyperactive type. This type is the most common in children and adults (Glickman-Simon *et al.,* 2001).

## 1.5 Role of neurotransmitters

The brain has a limited quantity of any neurotransmitter available at any given time. After having been released for use and having completed its task, the neurotransmitter is rapidly destroyed or recycled and stored for later use. If neurotransmission were not limited in this way, the brain might race out of control, virtually burning itself out. Neurotransmitters must be made ongoing because the brain must continually renew its supply of raw materials, such as amino acids, vitamins and minerals. The brain needs these raw materials to manufacture more neurotransmitters as well as glucose and oxygen to function properly. The antioxidants are needed for protection of the brain (Child Wisdom, 2003).

If neurotransmitter precursors are in short supply, problems in perception, behaviour, cognition and mood will result. Amino acids, the building blocks of protein, are the most important of the neurotransmitter precursors. The brain uses some of the unaltered amino acids as neurotransmitters, directly. Glutamate, aspartate, and glycine are three such amino acids. It builds other neurotransmitters by altering the amino acids slightly and/or combining them with other substances (Child Wisdom, 2003).

ADHD is seen as a biochemical disorder involving neurotransmitters, primarily dopamine (DA) and noradrenalin (NA). These neurotransmitters are responsible for arousal and alertness in the brain. The biochemical imbalance in ADHD results in a lack of the neurotransmitter substance. The lack of arousal and alertness does not allow the brain to function properly in its respective areas nor communicate effectively with other parts of the brain for maximized functioning to occur (Fisher, 1998:2). Studies suggest that

dysregulation of these catecholamines is central to the pathophysiology of ADHD (Paule *et al.,* 2000).

### 1.5.1 Dopamine

Performance and memory are facilitated by dopamine. Its action has been described as connected with appetite and reward-seeking behaviours. It is involved with the sensory, motor, and neurohormonal activities processing the motivated approach behaviours. DA also plays a role in fine movement, muscle tone, emotionality, and internal motivation states. It is thought that disruption of mesocortical dopaminergic systems results in attentional dysfunction and impedes normal development of the corticolimbic circuitry (Fisher, 1998:49).

### 1.5.2 Noradrenalin

Noradrenalin plays a role in the regulation of selective attention and attention to significant external stimuli. The noradrenergic transmitter produces an alerting, attention focusing, orienting response. NA facilitates activities like learning, memory and awareness (Fisher, 1998:49). A hypoactive NA system may have severe functional consequences that could explain the inattentiveness of ADHD children (Oades, 2002).

The urinary catecholamine excretion in boys with ADHD was measured by Hanna *et al.* (1996). Dihydroxyphenylalanine, DA, NA, adrenalin, 3,4-dihydroxyphanylacetic acid, and 3,4-dihydroxyphenylglycol (DOPEG) concentrations were assayed by high-pressure liquid chromatography (HPLC) with electrochemical detection. The urinary concentration of DOPEG, a NA metabolite, and adrenalin was significantly lower in the ADHD subjects than in the normal controls. The results were consistent with previous reports of abnormal metabolism of NA and adrenalin in ADHD. The neurochemical findings may be due to differences between ADHD and normal boys in neuronal (central or peripheral) or nonneuronal (adrenal or renal) activity.

### 1.5.3 Serotonin

Altered function of the serotoninergic (5-HT) system may have a functional role in causing motor and cognitive impulsivity (Adriani *et al.,* 2003). Some symptoms in ADHD have been related to an altered 5-HT metabolism. The peripheral levels of the 5-HT

metabolite, 5-hydroxyindole acetic acid (5-HIAA) are found to be increased (rather than decreased) in ADHD children and the lower homovanillic acid (HVA)/5-HIAA ratio is also suggestive of 5-HT hyperactivity relative to DA (Oades, 2002; Castellanos *et al.*, 1994).

### 1.5.4 Relationship between the catecholamines

It is thought that mesocortical or frontal catecholaminergic changes reflect the more cognitive manifestations of ADHD such as attentional control and working memory while mesolimbic changes may reflect motor activity and drinking. It is the relationship of the level of activity of one monoamine to the other that proves psychopathologically significant (Oades, 2002). Oades (2002) concludes that DA is hyperactive with respect to NA activity, but often hypoactive with respect to 5-HT activity in certain manifestations of ADHD.

## 1.6 Nutrition

### 1.6.1 Eating habits of children

Our brain's biochemistry is fuelled by the food we eat, far more than in most organs in the body. Metabolism is central to brain function, particularly in growing children. Children's brains are hungrier, more metabolically active, and proportionally larger than adults' brains. Per pound of body weight, children eat more food, drink more fluids, and breathe more air than adults, thereby increasing their exposure to environmental toxins. Unfortunately, young children's immature intestinal linings and blood-brain barriers are not as protective as those of most adults. Because of this increased exposure and reduced protection, children are more likely than adults to have acute brain and behavioural dysfunctions related to toxins, allergens, and metabolic by-products. Additionally, because of their greater nutritional needs and generally poorer eating habits (less health-building foods, more fast food), children are more likely than most adults to have nutrient deficiencies. All of these factors contribute to children's heightened susceptibility to dietary imbalances, including increased exposures to neurotoxins, contributing to neurobehavioral problems (Child Wisdom, 2003).

### 1.6.2 Deficiencies and the effect of supplementation

The aetiology of ADHD is unknown, yet a cohesive body of evidence has accumulated in recent years suggesting that ADHD may well be a manifestation of nutritional deficiency (Ottoboni & Ottoboni, 2003).

#### 1.6.2.1 Vitamin B

Adequate levels of vitamin B6 (pyridoxine) are required for normal brain development and are essential for the synthesis of essential brain chemicals including serotonin, dopamine and noradrenalin. A preliminary study found that pyridoxine was slightly more effective than methylphenidate in improving behaviour among hyperactive children. The results, however, were not significant and no other studies have been able to confirm these findings. Therefore, supplementation with B6 is not considered a standard treatment for ADHD (Glickman-Simon *et al.,* 2001).

Supplementation with B-complex vitamins can be paradoxical. Some children became more hyperactive with pyridoxine (vitamin B6) but became calmer when thiamine (vitamin B1) was administered. Some children whose behaviour improved with pyridoxine supplementation, deteriorated when thiamine was administered. These differences appeared to be stable over time (Anon, 2000; Galland, 2003).

#### 1.6.2.2 Essential fatty acid deficiency

Many of ADHD children have a deficiency of essential fatty acids (EFAs) either because they cannot metabolise linoleic acid normally, or because they cannot absorb EFAs normally from the gut, or because their EFA requirements are higher than normal (Colquhoun & Bunday, 1981).

Significantly lower levels of EFAs have been found in hyperactive children compared to controls without ADHD. Excessive thirst along with dry skin and hair are symptoms characteristic of essential fatty acid deficiency and are frequently seen among hyperactive children with learning and behaviour problems. Evening primrose and fish oils give good behavioural responses with children who have essential fatty acid deficiency symptoms such as thirst, dry skin and hair (Anon, 2000; Stevens *et al.,* 1995; Galland, 2003; Mitchell *et al.,* 1987). Burgess *et al.* (2000) states that omega 3 fatty

acids are present in large quantities in the retina of the eye and in certain regions of the brain, thus depletion of omega 3 from these regions may compromise sensory and brain function. He states that subclinical omega 3 deficiency may be responsible for the abnormal behaviour and outward symptoms of ADHD children. Mitchell *et al.* (1987) observed that children deficient of these essential fatty acids had auditory, visual, language, reading and learning difficulties and lower birth weight than the controls. The hyperactive children also were more likely to suffer coughs, colds and serious illness.

Many ADHD children are deficient in zinc, which is required for the conversion of essential fatty acids (EFAs) to prostaglandins. Some ADHD children are badly affected by wheat and milk, which give rise to exorphins in the gut which can also block this conversion (Colquhoun & Bunday, 1981).

Studies designed to determine whether ADHD can be helped by dietary supplementation with essential fatty acids have shown mixed results. For example, Voight *et al.* (2001) showed no benefit, but Richardson & Puri (2002) reported a significant reduction in ADHD symptoms in the treated group.

#### 1.6.2.3 Magnesium deficiency

Magnesium deficiency in children with ADHD occurs more frequently than in healthy children. Low magnesium results in a syndrome of abnormalities including irritability, restless sleep, muscle tensions with spasms, and poor exercise tolerance. Children receiving magnesium supplementation show a significant decrease in hyperactivity (Anon, 2000; Simmons, 2002; Kozielec & Starobrat-Hermelin, 1997; Starobrat-Hermelin & Kozielec, 1997).

#### 1.6.2.4 Zinc deficiency

Zinc regulates the activity of neurotransmitters, fatty acids, and melatonin, all of which are related to the biology of behaviour. Two separate studies found that children with ADHD have significantly lower blood zinc levels than children without ADHD. Another study indicated that ADHD children with mild zinc deficiency may be less likely to improve from a commonly prescribed stimulant than children with adequate zinc levels. To date, however, no studies have been conducted to evaluate whether zinc

supplementation improves behaviour in children with ADHD who are deficient in this mineral (Glickman-Simon *et al.*, 2001).

### 1.6.3 Wheat and dairy

The offending substances in these foods are casein, a milk protein found in dairy and dairy products, and gluten found in wheat and other grains. Strict removal of casein/dairy can often result in noticeable improvement in ADHD symptoms, usually within a few weeks. Other behavioural symptoms, such as temper outbursts and mood swings, also often respond well to casein removal (Compart, 2003). A complete discussion is given in chapter 2.

### 1.6.4 Evidence of dietary intervention

Controversy exists over whether the diet can influence the symptoms of ADHD. Many studies have been done with various results.

Zametkin (1998) stated that there was minimal support of a relationship existing between diet and hyperactivity and in further studies declared that sugar and food additives causing ADHD, was a myth (Zametkin, 1995).

It was Feingold who, in the early seventies first proposed that artificial colours and flavours might affect children's behaviour. Further studies revealed that a certain tartrazine dye inhibited the uptake of all the neurotransmitters and their precursors which they tested (TePas, 1996). Rowe & Rowe (1994) observed that behavioural changes in irritability, restlessness and sleep disturbance were associated with the ingestion of tartrazine in some children.

Atopic children with ADHD had a significantly more beneficial response to an elimination diet than nonatopic children according to a study done by Boris and Mandel (1994). Carter *et al.* (1993) placed hyperactive children on a 'few foods' elimination diet and it showed improved behaviour during the trail. The majority of studies designed to look at sugar consumption in ADHD children have failed to show a significant or causal relationship (Kanarek, 1994; Krummel *et al.*, 1996) and it has been shown that children with ADHD tend to eat no more sugar than average (Kaplan *et al., 1989a;* Kaplan *et al., 1989b* ). Sugar alone does not cause hyperactivity, but it reduces the nutritional quality

of the diet and may aggravate other food intolerances (Galland, 2003). A detailed review of the controlled scientific literature regarding the role of the diet and behaviour in childhood has shown that diet definitely affects some children (Breakey, 1997).

## 1.7 Treatment

Stimulant medications are most widely researched and commonly prescribed treatments for ADHD. Although researchers do not fully understand how these medications improve ADHD symptoms, studies indicate that methylphenidate (Ritalin®), the most commonly prescribed stimulant, significantly increases dopamine levels in the brain. People with ADHD are believed to have abnormally low levels of dopamine in the brain. Approximately 70 % of people with ADHD benefit from the first stimulant prescribed, usually methylphenidate, and an additional 20 % may respond to one or the other two drugs in this class if the first did not work (Glickman-Simon *et al.*, 2001).

Stimulant medications prescribed for ADHD include:

- Methylphenidate: Most commonly used medication for ADHD. Effective in 75 % to 80 % of patients. Not recommended for children under 6 years old.
- Dextroamphetamine: Effective in 70 % to 75 % of patients. Not recommended for children under 3 years old.
- Pemoline: Effective in 65 % to 70 % of children. Not recommended for children under 6 years of age. Should be used as a second line drug for ADHD, because it has been associated with liver failure (Glickman-Simon *et al.*, 2001).

The following medications are recommended for those who do not improve from stimulants:

- Alpha$_2$ agonists (such as clonidine, guanfacine): helpful in individuals who are particularly aggressive or oppositional. May cause low blood pressure in some individuals.
- Antidepressants: bupropion for children who also have mood disorders such as depression; tricyclics (such as imipramine) for individuals who also have tic disorders or significant symptoms of anxiety and depression (Glickman-Simon *et al.*, 2001).

# Chapter 2

# Peptides

## 2.1 Introduction

Peptides with opioid activity are produced during the digestion of gluten and casein, and are absorbed from the gut into the bloodstream. The majority is excreted in the urine, but some cross the blood-brain barrier and enters the CNS, where they can have direct opioid activity or form irreversible complexes with the peptidase enzymes that break down natural endorphins. The result is increased opioid activity in the CNS, a phenomenon found particularly in people with autism. These peptides act as neuroregulators within the CNS and effectively cut down transmission in all neurological systems. The precise mechanism is unclear but stimulation of presynaptic receptors is a possibility (Shattock & Whiteley, 2001). The same urinary profiles are observed in certain children with ADHD (Hole *et al.*, 1988).

## 2.2 The excessive opioid theory

Dohan, who died in 1992, proposed that schizophrenia could be caused by a dietary overload of peptides formed from gluten and possibly milk proteins, due to genetically determined insufficient peptide metabolism. This presupposes that:

1. peptides are formed in the gut,
2. intact peptides and/or proteins are taken up from the gut,
3. some peptides have access to the CNS across the blood to brain barrier,
4. removing the offending proteins will have a clinical effect, and
5. peptides found to be increased in the body fluids display bioactivities relevant to schizophrenia (Reichelt *et al.*, 1996).

A model for a possible cause of autism was based on a similar excessive opioid theory initiated by Panksepp (1979) and extended by Reichelt *et al.* (1981) and Shattock & Lowdon (1991). The hypothesis is that autism could be the result of peptides of exogenous origin affecting neurotransmission within the CNS. The effects of these peptides are opioid in nature and they may have direct opioid activity or form ligands for

the enzymes which break down the opioid peptides that occur naturally within the CNS. The CNS neuroregulatory role, which is normally performed by the natural opioid peptides such as the enkephalins and endorphins, is intensified to such an extent that normal processes within the CNS is severely disrupted. The presence of this intense opioid activity can result in a large number of the systems of the CNS being disrupted to varying extents (Shattock & Savery, 1996).

## 2.3 The absorption of peptides in the central nervous system and gastrointestinal tract

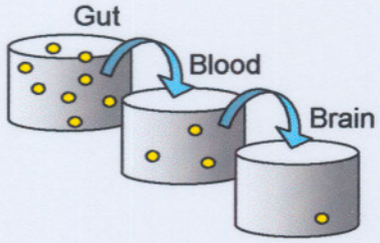


**Figure 2.1:** Normal peptide transfer across the intestinal wall and blood-brain barrier (Shattock & Whitely, 2001).

Figure 2.1 represents normal circumstances, where there are low levels of peptides in the intestine. Each yellow dot represents one peptide molecule with biological, in this case opioid, activity. Proteins are broken down in the gut and peptides occur as intermediate compounds, which will then be broken down further into their amino acid components. A small proportion transfers across the intestinal wall and blood-brain barrier to the CNS, but at such low level that they have little effect. For example, 10 % of the peptides may cross through the normal intact gut wall and appear in the blood stream. If 10 % of this complement crosses the blood-brain barrier, then 1 % of the total peptides present in the gut will have reached the CNS. Once there, they may directly regulate transmission in all of the main neurotransmission systems or, alternatively, may form ligands for the enzymes which would normally break down the opioid peptides which occur naturally in the CNS. The consequence would be an increase in opioid activity. In this normal situation, the levels of peptides in the gut are comparatively small and the quantities reaching the brain are minimal so the net effects are negligible (Shattock & Savery, 1996; Shattock & Whitely, 2001).

There are however, a number of situations in which the peptide levels can rise:

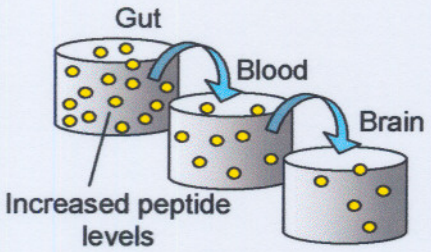


**Figure 2.2:** Inefficient enzyme functioning in the intestine give rise to higher peptide levels in the CNS and urine (Shattock & Whitely, 2001).

Given the same order of leakiness of the gut wall and BBB as before (figure 2.1), greatly increased levels of peptides in the gut will result in an increased quantity of peptides reaching the CNS. The reason for the increase may be the result of inadequate enzyme systems, which are responsible for their breakdown, for example, genetically determined deficiencies of the required endopeptidase enzymes. There could be shortages of cofactors, such as vitamins and minerals required for the enzymes to function properly. Alternatively, the pH in the relevant areas of the gut may be inappropriate for the specific enzymes to act as they should (figure 2.2) (Shattock & Savery, 1996).

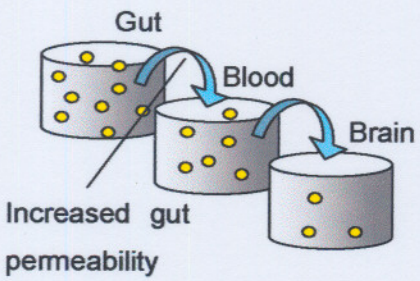


**Figure 2.3:** Increases in intestinal permeability results in greater leakage of peptides into the urine and CNS (Shattock & Whitely, 2001).

Figure 2.3 represents the situation in which the levels of peptides in the gut are normal, but for some reason, the gut wall is excessively leaky so that vastly increased quantities

of the peptide will cross the gut wall and enter the blood stream. Thus, there will be an increased level of peptides in the CNS with possible clinical consequences. There are a number of factors, which could result in increased leakiness of the gut. There may be damage caused by purely physical action such as a surgical operation or some natural flaw. Deficiencies in the phenyl sulphur transferase (PST) systems can lead to increased permeability of the gut wall. Normally the proteins lining the gut wall are sulphated and, in this state, form a continuous protective layer over the surface of the gut wall. When there is insufficient sulphation, the proteins clump together and the layer becomes irregular. The net result is an increased permeability of the gut wall. In this case, the passage of peptides across the gut wall is greatly enhanced (Shattock & Savery, 1996).

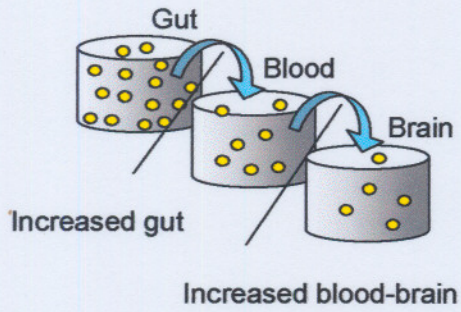


**Figure 2.4:** Infections and physical damage lead to increased permeability (Shattock & Whitely, 2001).

In figure 2.4, the blood-brain barrier is less effective than normal so that any opioid peptides in the blood stream would easily pass into the CNS and exert their full range of actions. The blood-brain barrier is a complex system, which is partly physical and partly biochemical. The biochemical element consists, in part, of enzymes, which should destroy potentially harmful substances such as exogenously derived peptides. According to these hypotheses, the peptidase activity may be depressed and the barrier could be somewhat more permeable than normal. There may be other environmental factors which could exacerbate the process either slightly or dramatically (Shattock & Savery, 1996). Meningitis, other infections and physical damage can all greatly increase the permeability of the blood-brain barrier and increase peptide levels in the CNS (figure 2.4) (Shattock & Whitely, 2001).

If peptides are present in the blood, they tend to be collected by the kidneys and dumped in the urine. Thus, the peptide content of the urine will, to some extent, be reflective of the content of the blood (Shattock & Savery, 1996).

Another possible mechanism for the occurance of these peptides may be through defective p-glycoprotein. Several studies have demonstrated that peptides and opioids are substrates for p-gp (Chen & Pollock, 1998; Sharma *et al.*, 1991; Thompson *et al.*, 2000; Letrent *et al.*, 1999; Matheny *et al.*, 2001).

### 2.3.1 Symptoms of high urinary opiates

Clinical signs that may attend high urinary opiates are aphasia or poor language development, constipation or constipation mixed with wet stools, strong growth and gain or excess weight for stature, marked perseveration and rigidity, marked lack of social connectedness (Whiteley *et al.*, 1998).

## 2.4 Possible behaviour of peptides in CNS

The pepides responsible are not endogenous in nature, and are likely to come from an exogenous source since the quantities found are significantly higher than those from endogenous sources. It is likely though, that once these peptides have entered the bloodstream, they may partition through the blood-brain barrier, and therefore be able to act in the CNS. Enkephalins have been shown to easily cross the blood-brain barrier in significant amounts and could do one of several things:

1. act in an agonistic way on any of the opioid receptors in the CNS,
2. act as a partial agonist at any of the opioid receptors in the CNS thus stimulating the receptor, yet remaining bound like an antagonist,
3. be full antagonists for the opioid receptors, hence preventing endogenous endorphins from binding,
4. inhibit the natural peptidase system for endorphins namely endorphinases. This would have the effect of increasing the amounts of naturally occurring endogenous endorphins
5. act in a non-opioid manner utilising an unknown receptor population in a similar manner to noradrenaline-ATP co-transmission in sympathetic nervous systems (Braithwaite, 1995).

It is possible, that a partial agonistic effect is being observed and that these peptides have such a high affinity for the opioid receptors that they bind with sufficient strength so that there is no competitive agonism between the endogenous endorphins, and the exogenous peptides. The peptides themselves may also bring forth some form of response at the receptor site too, although this may not be of sufficient value for a full response, that is, a partial agonistic effect (Braithwaite, 1995).

## 2.5 Peptides involved

Peptides with activity similar to that of morphine and other opioids have been isolated from the brain and other sources such as the pituitary. These peptides, the endorphins and enkephalins, are synthesized *in vivo* and may function both as hormones and neurotransmitters. An alternative source of peptides, some of which may have biological activities, is dietary protein or exorphins (Zioudrou *et al.*, 1979).

In order for these exorphins to function as opioid peptides in the central nervous system *in vivo* they must:

1. be produced in the gastrointestinal tract,
2. survive degradation by intestinal proteases,
3. be absorbed, without degradation, into the bloodstream,
4. cross the blood-brain barrier and thereby reach central opiate receptors,
5. interact as opiates with these receptors (Zioudrou *et al.*, 1979).

The peptides, resulting from the incomplete breakdown of certain foods are the proteins gluten from wheat, and casein from milk and dairy related proteins (Reichelt *et al.*, 1981).

### 2.5.1 Peptides derived from dairy products

In various studies, milk has been screened for the presence of free or precursor-bound opioids. In fact, various opioid receptor ligands with agonistic or even antagonistic activity were found. Besides the alkaloid morphine, peptides derived from alpha-casein (alpha-casein exorphins), beta-casein (beta-casomorphins; beta-casorphin), alpha-lactalbumin (alpha-lactorphins) and beta-lactoglobulin (beta-lactorphin) were among the agonists. In addition, certain peptides derived from k-casein (casoxins) or from lactoferrin (lactoferroxins) were found to behave like opioid antagonists. Although a

functional role in the mammalian organism for all of these compounds appears to be possible, evidence has only been presented for the functional significance of beta-casomorphins, so far. Opioids related to milk, might represent essential exogenous extensions of the endogenous opiodergic systems (Teschemacher & Koch, 1991).

#### 2.5.1.1 Beta-casomorphin 7

Studies have established that beta-casomorphin 7 (β-CM7) is capable of readily crossing the blood-brain barrier and activating brain cells mediated by opioid receptors. Some of the brain areas affected is originators or components of dopaminergic, serotinergic and GABA (γ-aminobutyric acid)-ergic pathways, suggesting that β-CM7 can affect the function of all of these systems (Sun *et al.*, 1999). Further research involving the injection of β-CM7 into rats in order to observe behavioural responses, has yielded astonishing results. It was reported that roughly seven minutes after the injection of β-CM7, the rats became inactive, distancing themselves from the other rats in the same cage, while showing no social interaction and very little reaction to sound (Sun & Cade., 1999). These β-CM7 induced behaviours, exhibit a distinct resemblance to those observed in humans with autism.

Damage to the tight junctions linking intestinal epithelial cells causes increased absorption of harmful exorphins such as β-CM7 through the mucosal barrier of the gut into the bloodstream (figure 2.5) (Ali, 2004).

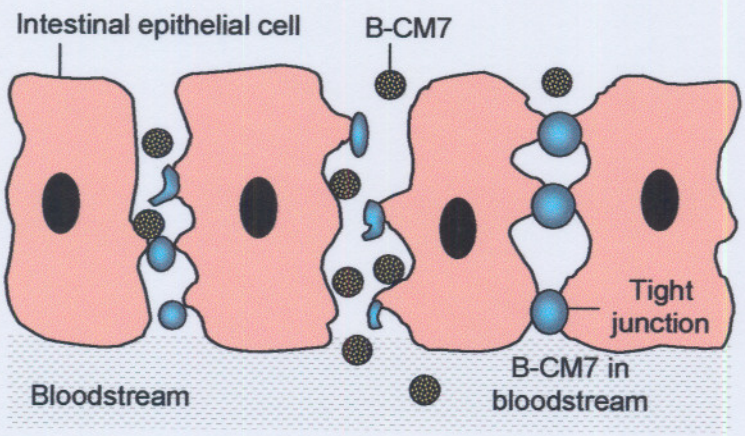


**Figure 2.5:** Absorption of β-Casomorphin 7 into the bloodstream (Ali, 2004).

### 2.5.2 Peptides derived from wheat products

The opioids formed from gluten and gliadin are gluteomorphines (Fukudome & Yoshikawa, 1992) and gliadinmorphines (Graf *et al.*, 1987). Other related grains such as rye, barley and oats, also contain the sequence of amino acids found in gluten (Great plains laboratory, 2001).

Gliadorphin is very similar to casomorphin. Casomorphin and gliadorphin are composed of seven amino acids. Both casomorphin and gliadorphin start with the beginning N-terminal sequence tyr-pro (for tyrosine and proline) and the additional pro (proline) in positions 4 and 6 of both peptides (Great plains laboratory, 2001).

### 2.5.3 Peptides found in other disorders

High concentrations of the peptide casomorphin were found in the urine samples of people with autism, celiac disease, pervasive developmental disorder (PDD) and schizophrenia. It is suspected that these peptides may also be elevated in other disorders such as chronic fatigue, fibromalgia and depression based on anecdotal reports of symptom remission after exclusion of wheat and dairy from the diet (Great plains laboratory, 2001).

Other disorders that seem to show similar abnormalities in the breakdown of peptides are ADHD, ADD, dyslexia and obsessive compulsive disorder (OCD) (Shattock & Savery, 1996; Center for autism and related disorders, 2001).

#### 2.5.3.1 ADHD

In a study done on behavioural disorders, abnormal amounts of peptide and protein-associated peptide complexes excreted in the urine were observed. The symptoms of all the patients who participated in the study fit the criteria for diagnosis of attention deficit disorder with hyperactivity (ADHD) (Hole *et al.*, 1988). Parents of these children seem to have a higher difficulty with digestion of these peptides as well (Center for autism and related disorders, 2001). Although the focus of these urinary peptides was mainly on autism for years, the profiles from subjects with ADD and ADHD are now being studied in a more systematic way (Shattock & Savery, 1996).

# Chapter 3

# P-glycoprotein

## 3.1 Introduction

ABC (ATP Binding Cassette) transporters form one of the largest protein families and execute a diversity of physiological functions (Higgins, 2001). P-gp (p-glycoprotein), a member of this family, is a transmembrane protein expressed by multiple mammalian cell types, including the endothelial cells that comprise the BBB. P-gp functions to actively pump a diverse selection of xenobiotics out of the cells in which it is expressed (Thompson *et al.*, 2000). P-gp thus acts as protective mechanism against a wide variety of potentially toxic substances, serving to limit distribution and accelerate elimination of p-gp substrates (Matheny *et al.*, 2001).

## 3.2 Classification

Currently 49 human ATP-Binding Cassette transporters have been identified. These transporters are classified into 7 families (Table 3.1) according to sequence similarity (Müller, 2003). P-gp is the 170-kD protein product of the MDR1 (multidrug resistance) gene. On the basis of the homology of the p-gp ATP-binding domains with those of other transport proteins, p-gp is classified as a member of the ATP-binding cassette super family of transport proteins. This family includes other membrane-associated proteins that transport drugs and endogenous substances, for example the multidrug resistance associated protein (MRP1), and proteins with ion channel function, for example the cystic fibrosis transmembrane conductance regulator (CFTR), the product of the cystic fibrosis gene. Rodents express two isoforms of the gene encoding p-gp, designated *mdr1a* and *mdr1b*, which together serve a similar function as the single human MDR1 gene. A new nomenclature system was proposed for the ATP-binding cassette genes where the MDR1 (p-gp) gene is referred to as ABCB1, for **A**TP-**B**inding **C**assette, subfamily **B**, member **1** (Matheny *et al.*, 2001).

**Table 3.1:** Classification of ATP-Binding Cassette Transporters and their functions (Müller, 2003) (Dean *et al.*, 2001).

| Subfamily | Name | Number of members | Functions of the various genes |
|---|---|---|---|
| ABCA | ABC1 | 12 | ABCA1: Cholesterol efflux into high-density lipoprotein (HDL)<br>ABCA2: Drug resistance<br>ABCA4: N-retinylidene-phosphatidylethanolamine efflux |
| ABCB | MDR | 11 | ABCB1: Multidrug resistance<br>ABCB2 & ABCB3: Peptide transport<br>ABCB4: Phosphatidylcholine transport<br>ABCB6: Iron transport<br>ABCB11: Bile salt transport |
| ABCC | MRP | 13 | ABCC1 & ABCC3: Drug resistance<br>ABCC2: Organic anion efflux<br>ABCC4 & ABCC5: Nucleoside transport<br>ABCC7 (CFTR): Chloride ion channel function<br>ABCC8: Sulfonylurea receptor |
| ABCD | ALD | 4 | ABCD1: Very long chain fatty acid (VLCFA) transport regulation |
| ABCE | OABP | 1 | ABCE1: Oligoadenylate binding protein |
| ABCF | GCN20 | 3 | No membrane transport functions |
| ABCG | White | 6 | ABCG1: Possibly cholesterol transport<br>ABCG2: Toxin efflux, drug resistance<br>ABCG5 & ABCG8: Sterol transport |

## 3.3 Organisation and structure

The basic unit of an ABC transporter consists of four core domains. Each of the four core domains is encoded as a separate polypeptide. In other transporters the domains can be fused in any one of a number of ways into multidomain polypeptides as shown in figure 3.1. In cases in which one of the four domains appears to be absent, one of the remaining domains functions as a homodimer to maintain the full complement (Higgins, 2001).

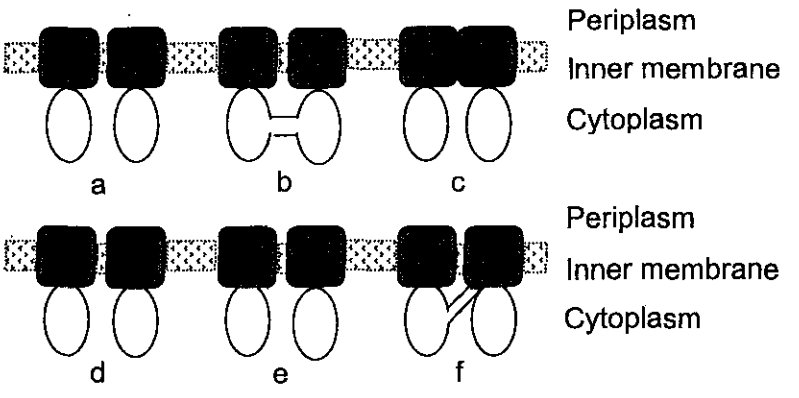


**Figure 3.1:** The organisation of ABC transporters. The ABC transporter consists of four domains. The two transmembrane-associated domains (TMDs) can be homo- or heterodimeric and are represented by shaded squares. The two nucleotide or ATP-binding cassette domains (NBDs or ABCs) can also be either homo- or heterodimeric and are represented by ovals at the cytoplasmic face of the membrane. The domains can be fused in various ways: (a) four separate polypeptides, (b) fused NBDs with heterodimeric TMDs, (c) fused TMDs with homodimeric NBDs, (d) one NBD fused to one TMD, (e) one TMD fused to one NBD, with the other TMD and NBD as separate polypeptides (f) all four domains fused into a single polypeptide, often found in eukaryotic ABC transporters (Linton & Higgins, 1998).

According to Higgins (2001), the two transmembrane domains (TMDs) span the membrane multiple times via putative α-helices. Typically, there are six predicted membrane-spanning α-helices per domain and a total of twelve per transporter, although there is some variation on this formula. Some of the predicted membrane-spanning α-

helices may not be crucial to the core function of the transporter but may serve auxiliary functions such as membrane insertion or regulation. The TMDs form the pathway through which solutes cross the membrane and determine the specificity of the transporter through substrate-binding sites.

The other two domains, the ATP or nucleotide-binding domains (NBDs), are hydrophilic and peripherally associated with the cytoplasmic face of the membrane. These domains consist of the core 215 amino acids of the ABC domain by which these transporters are defined. It is important to emphasise that it is the conservation of this entire domain which is important in defining and delimiting the family. Other ATP-binding proteins which are not ABC transporters can include the Walker A and Walker B motifs. The various structures differ significantly in the dimer interface and even though it seems likely that the ABC domains do interact, the residues or faces of the domains involved in such interactions are unknown. Similarly, it remains unclear which faces of the NBDs interact with the transmembrane domains (Higgins, 2001).

In many ABC transporters, auxiliary domains have been recruited for specific functions. The periplasmic binding proteins (PBPs) bind substrate external to the cell and deliver it to the membrane-associated transport complex (Higgins, 2001). The PBPs have two diverse, but related functions. The first is to impart high affinity and specificity. An initial distinction between PBP-dependent and other transporters was that the PBP transporter showed remarkably high affinity. Similarly, most ABC transporters that lack a PBP, for example drug transporters, have rather broad specificity while those with a PBP can be highly specific. The second is to confer directionality. There is a 100 % correlation between the presence of a PBP and solute uptake, and between the absence of a PBP and solute export. Although this does not prove that the PBP determines directionality, the fact that interaction of the PBP with the transporter at the outside of the cell can trigger ATP hydrolysis at the cytoplasmic face of the membrane strongly implies such a role (Higgins, 2001).

The structure of the mammalian multidrug resistance p-gp has been determined to 25 Å by single particle imaging and to 10 Å by 2-D cryoelectron microscopy. A large ring-like chamber (figure 3.2) is formed by the TMDs in the membrane, with an opening to the extracellular milieu and is closed at the cytoplasmic face of the membrane. The NBDs are located at the cytoplasmic face of the membrane in tight apposition to the membrane

domains. The structure is consistent with biochemical and genetic data for other ABC transporters and it seems likely that it reflects a general architecture for ABC transporters (Higgins, 2001).

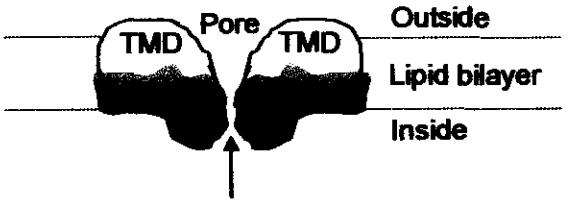


**Figure 3.2:** Structure of ABC transporters. The protein consists of an aqueous pore, formed by the TMDs, with a large opening at the extracellular face of the membrane. The NBDs (shaded) are at the cytoplasmic face of the membrane in close apposition to the TMDs and possibly partly buried in the lipid bilayer though not spanning the membrane (Higgins, 2001).

## 3.4 Mechanism

### 3.4.1 The transport cycle of p-gp

Based on data, obtained from ABCB1, trapped at different stages of the ATPase cycle, a model for the transport process has been put forward, involving the rotation of transmembrane helices. The conformational changes during the transport cycle of P-gp are shown in figure 3.3. For clarity, the ATPase cycle for only one NBD is shown, although in p-gp both NBDs are required, and operate in an alternating catalytic cycle. P-gp binds drug substrate from the inner leaflet of the lipid bilayer, at the intracellular face of the membrane. Subsequently, ATP is bound by the NBD(s), inducing a conformational change that results in a reduction in the affinity of drug binding and reorientation of the site, such that it is exposed to the extracellular milieu, which is probably an aqueous chamber formed by the TMDs of p-gp. The post-ATP hydrolysis transition state shows a third conformation that retains low drug binding. Following release of ADP and/or $P_i$ the protein returns to its starting configuration and regains high-affinity drug binding (Rosenberg *et al.*, 2001).

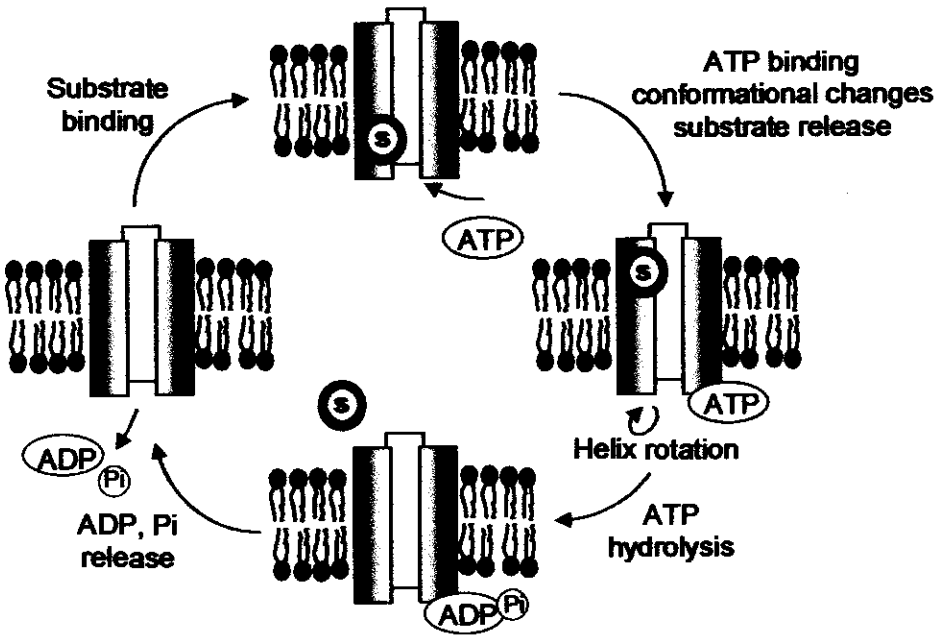


**Figure 3.3:** Conformational changes during the transport cycle of P-gp (Pohl, 2003).

### 3.4.2 Mechanism in the intestine

The presence of p-gp in the intestine is an important factor in the handling of many substances because p-gp either mediates direct efflux through the intestinal wall and/or limits the re-uptake after hepatobiliary excretion. In line with the expectations raised by its expression in the intestine, the presence of p-gp in this tissue can also reduce the absorption of compounds, following oral administration, thus protecting the host against orally ingested toxins (van Tellingen, 2001). Figure 3.4 shows the mechanism of p-gp intestinal disposition of substrate. The p-gp substrate is absorbed from the intestinal lumen into the enterocyte where the enterocyte is absorbed into circulation and metabolism of substrate in the enterocyte takes place. Secretion of substrate back into the intestinal lumen is facilitated by p-gp for elimination in the faeces (Matheny *et al.*, 2001).

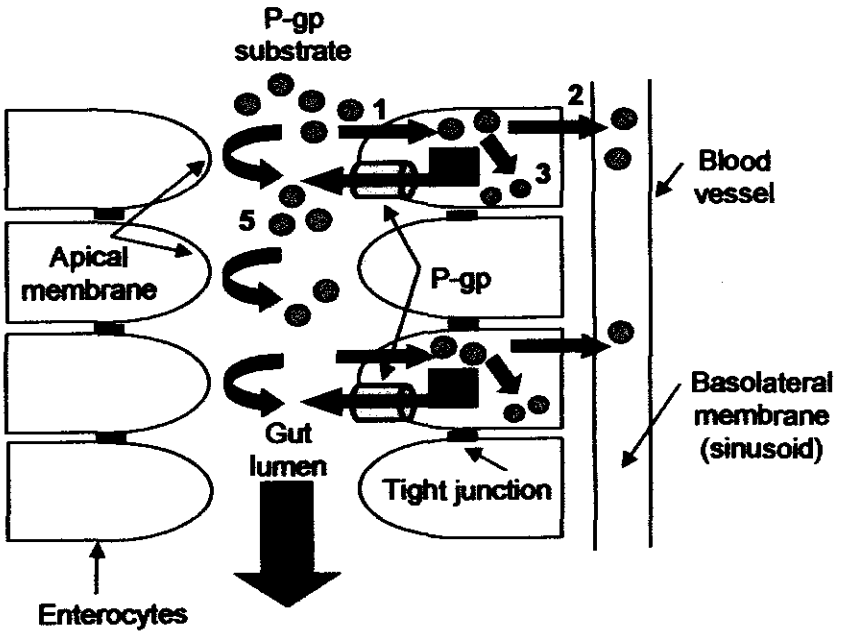


**Figure 3.4:** Mechanism of p-gp intestinal disposition of substrate. (1) Absorption of p-gp substrate from intestinal lumen into enterocyte. (2) Absorption from enterocyte into the circulation. (3) Metabolism of substrate in the enterocyte. (4) Secretion of substrate back into the intestinal lumen facilitated by p-gp. (5) Movement of substrate through the intestinal lumen for elimination in faeces (Matheny *et al.*, 2001).

### 3.4.3 Mechanism in the CNS

Many drugs exert their pharmacological effect and/or toxicity in the CNS. The blood-brain barrier (BBB), which comprises endothelial cells lining the brain capillaries, represents an important physical, biochemical, and transport barrier that serves to limit access of many xenobiotics to the CNS. Compared with vascular endothelial cells in other parts of the body, the endothelial cells of the BBB have narrow tight junctions with low paracellular transport, reduced pinocytotic activity, and specific transporters for the uptake of nutrients and extrusion of numerous compounds. Xenobiotic penetration across the BBB correlates directly with lipophilicity and affinity for transport by specific uptake carriers and correlates inversely with molecular weight, number of hydrogen bonds with water, degree of ionisation, and protein binding. Although it is generally assumed that highly lipophilic drugs will achieve high concentrations within the CNS by

passive diffusion across cell membranes, numerous lipophilic agents penetrate the CNS poorly (for example loperamide, etoposide, domperidone, colchicines) probably because most of these compounds are substrates for p-gp. The localisation pattern of p-gp within the CNS is consistent with a putative protective role by either limiting brain uptake or increasing efflux of p-gp substrates from the brain (Matheny *et al.*, 2001).

The mechanism of p-gp in the blood-brain barrier and central nervous system is shown in figure 3.5. The p-gp substrate enters the brain capillary endothelial cell through passive diffusion where it can passively diffuse to the interstitial fluid or be actively transported from the capillary endothelial cell back into the blood by p-gp. Glial cell-associated p-gp can also interact with p-gp substrate (Matheny *et al.*, 2001).

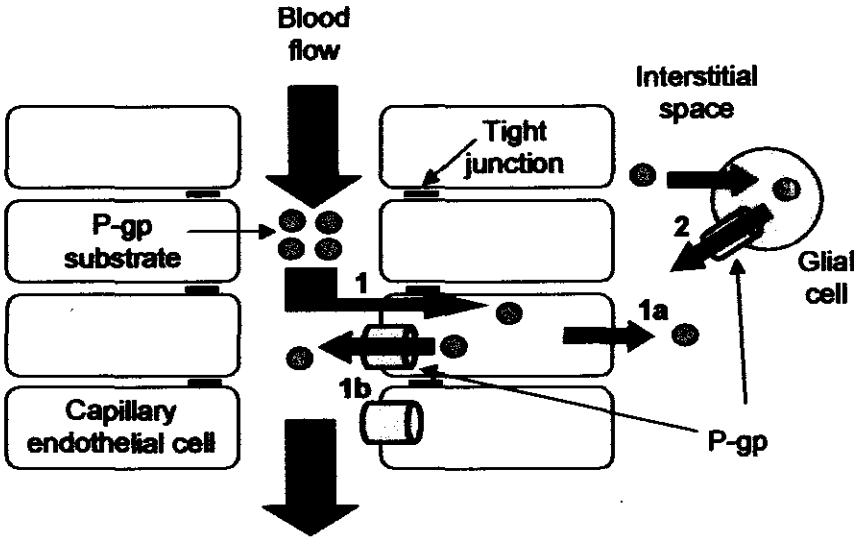


**Figure 3.5:** The mechanism of p-gp in the blood-brain barrier and central nervous system. (1) Passive diffusion of p-gp substrate from blood into brain capillary endothelial cell, which may then, (1a) passively diffuse across the endothelial cell to interstitial fluid, or, (1b) be actively transported form capillary endothelial cell back into blood by p-gp. (2) Interaction of glial cell-associated p-gp with substrate (Matheny *et al.*, 2001).

### 3.4.4 Mechanism in the kidney

Renal clearance is a major route of elimination for many drugs. Three processes govern renal clearance: filtration, secretion, and reabsorption. It has long been recognized that some xenobiotics undergo net secretion in the kidney, but the transporters involved were

poorly characterized. Recent evidence suggests that p-gp plays a key role in the renal elimination of certain substrates by means of active secretion into the urine. P-gp is expressed on the apical side of kidney proximal tubule cells and may be in other portions of the nephron, such as the loop of Henle. Nonfiltered substrates must cross the basolateral membrane, either by diffusion or carrier-mediated processes, to interact with p-gp. In addition to increasing the direct flux of drug from blood to urine, p-gp may limit reabsorption of substrates that are filtered at the glomerulus (Matheny *et al.*, 2001).

Figure 3.6 illustrates the mechanism of p-gp in renal disposition of unbound substrate. The uptake of p-gp substrate from the blood into the proximal tubule cell takes place first, followed by the metabolism or trafficking of substrate. Lastly, secretion into the tubular filtrate is facilitated by p-gp (Matheny *et al.*, 2001).

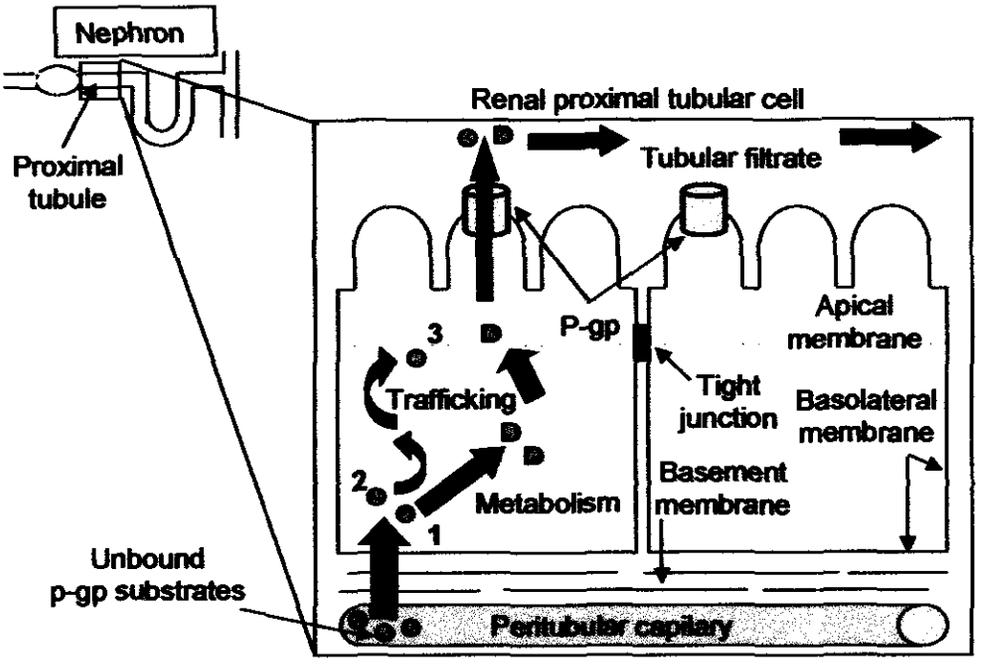


**Figure 3.6:** The mechanism of p-gp in renal disposition of unbound substrate. (1) Uptake of p-gp substrate from blood into proximal tubule cell. (2) Subsequent metabolism or trafficking of substrate. (3) Secretion into tubular filtrate by p-gp (Matheny *et al.*, 2001).

## 3.5 Location and function

In the absence of therapeutics or toxins, the normal physiological function of p-gp is uncertain. Studies done on MDR1 knock out mice, show that they have normal viability, fertility and a range of biochemical and immunological parameters (Delph, 2002). The location and function of p-gp in the body is summarized in table 3.2.

**Table 3.2:** Location and function of p-gp (Delph, 2002).

| Organ/tissue | Site | Function |
|---|---|---|
| Gut<br>• Colon<br>• Jejunum | Apical surfaces of superficial columnar epithelial cells | Intestinal excretion and reduced absorption of drugs and toxins |
| Liver and biliary system | Hepatocytes on biliary canalicular front | Hepatobiliary excretion of drugs and toxins |
| | Apical surfaces of epithelial cells of small biliary ductules | |
| | Hepatocytes | Regulation of cytochrome expression |
| Pancreas | Apical surfaces of epithelial cells of small ductules | Unknown |
| Kidney | Apical surfaces of epithelial cells of proximal tubules | Urinary excretion of drugs and toxins |
| Brain | Luminal surfaces of endothelial cells of cerebral capillaries | Contributes to the BBB, keeping drugs and toxins out of the brain |
| | Choroid plexus | Unknown |
| Peripheral nerves | Endothelial cells of nerve capillaries | Contributes the blood-nerve barrier, keeping drugs and toxins out of nerves |

| Organ/tissue | Site | Function |
|---|---|---|
| Uterus | Epithelial cells of placenta | Contributes to the materno-foetal barrier, keeping drugs and toxins out of the foetus |
| | Steroid-producing cells of endometrial glands | Unknown-possible role in steroid production |
| Testis and ovary | Capillary endothelial cells | Contributes to the blood-testis/ovary barrier, keeping drugs and toxins out of the gonads |
| Immune system | Skin dendritic cells | Migration of dendritic cells to lymph nodes |
| | Activated lymphocytes | Transport of some cytokines out of cell |
| | Natural killer and CD8+ cyctotoxic T cells | Reduces cytolytic activity |
| Bone marrow | Hematopoietic stem cells | May remove drugs and toxins from the bone marrow |
| Adrenal gland | Medulla and cortex | Unknown-possible role in steroid production |
| Large arteries | Endothelium | Unknown-possible role in accumulation of intracellular cholesterol ester in atherosclerotic lesions |

## 3.6 Result of defects

ABC transporters give rise to antibiotic and antifungal resistance in micro organisms, drug resistance of cancers occuring in man and many genetic diseases such as cystic fibrosis, Tangier disease and obstetric cholestases (Higgins, 2001).

## 3.7 Substrates

A wide variety of drugs can be transported by p-gp (Schinkel, 1999). A summary of the p-gp substrates and inhibitors is given table 3.3.

**Table 3.3:** P-glycoprotein substrates and inhibitors (Sun *et al.*, 2003).

| P-gp substrates | P-gp inhibitors |
|---|---|
| Cancer drugs:<br>• Doxorubicin • Vincristine<br>• Daunorbicin • Paclitaxel<br>• Vinblastine • Etoposide | Cyclopropylibenzosuberane:<br>• LY335979 |
| Immunosuppressive drugs:<br>• Cyclosporin A • FK506 | Immunosuppressant:<br>• Cyclosporin A • Valspodar (PSC8333) |
| Lipid-lowering agent:<br>• Lovostatin | Calcium channel blocker<br>• Verapamil |
| Steroids:<br>• Aldosterone • Corticosterone<br>• Cortisol • Dexamethasone | Progesterone antagonist:<br>• Mefipristone (RU486) |
| HIV protease inhibitors:<br>• Amprenavir • Ritonavir<br>• Indanavir • Saquinavir | Anti-arithmic agent:<br>• Quinidine |
| Cardiac drugs:<br>• Digoxin • Quinine | Antifungal agent:<br>• Ketoconazole |
| Anti-diarrheal agent:<br>• Loperamide | Acridonecarboxamide derivative:<br>• GG918 (GF120981) |
| Anti-tuberculous agent:<br>• Erythromycin | Topoisomerase<br>• Xenova (XR 5944) |
| Anti-helminthic agent:<br>• Ivermectin | Peptide chemosensitizers:<br>• Reversin 121 and 125 |
| Fluorescent dye:<br>• Rhodamine-123 | |

These substrates are a structurally diverse set of compounds with sizes ranging from about 250 Dalton (Da) to more than 1850 Da and it is uncertain how p-gp can recognize and transport them. Many compounds contain aromatic groups, but non-aromatic linear or circular molecules are also transported. Basic or uncharged compounds are most effectively transported, but zwitterionic and negatively charged compounds such as phoshatidylcholine analogues and methotrexate can also be transported. All these substrates that are transported by p-gp have thus far only one universal structural denominator, which has been identified, namely a fairly hydrophobic and amphipathic nature. These physical characteristics probably relate to the mechanism of drug translocation of the membrane lipid bilayer. One favoured, yet unproven model proposes that p-gp transports its substrates mainly by 'flipping' them from the inner to the outer leaflet of the plasma membrane, which would result in a net efflux of drug. Whatever the precise molecular mechanism of drug transport, p-gp activity can mediate very effective extrusion of drugs penetrating the plasma membrane, which results in very low intracellular drug levels (Schinkel, 1999).

It was discovered that numerous compounds with low or even absent intrinsic cytotoxicity could effectively inhibit p-gp-mediated drug transport. Many of these so-called reversal agents or p-gp blockers are in fact themselves transported substrates, which suggest that they inhibit in a competitive manner. They are as diverse in structure as the known p-gp substrates (Schinkel, 1999). See table 3.3.

## 3.8 P-glycoprotein and peptides

Several studies have demonstrated that p-gp interacts with peptides and opioids. P-gp appears to be a functional barrier to the intestinal absorption of the cyclic peptide DMP728 in rats. Mucosal-to-serosal flux was 4-fold lower than serosal-to-mucosal flux, suggesting net export from blood to the intestinal lumen. Considering the apparent role of p-gp in the disposition and action of opioids and peptides, it is not unlikely that opioid peptides are substrates for this transporter (Chen & Pollock, 1998).

Chen & Pollock (1998) suggested that the opioid peptide, [D-Penicillamine$^{2,5}$]enkephalin (DPDPE) is a substrate of p-gp, and that p-gp is responsible, in part, for the low penetration of DPDPE into the brain. Sharma *et al.* (1991) demonstrated that the

synthetic hydrophobic tripeptide, N-acetyl-leucyl-leucyl-norleucinal (ALLN) is a p-gp substrate, thus capable of transporting peptides.

According to King *et al.* (2001), functionally active neurotransmitters/neuromodulators are released directly from the brain into the blood through a saturable p-gp transport system. Downregulation of p-gp expression reduced the brain-to-blood transport of morphine, beta-endorphin and other opioids. The ability of the p-gp transport system to pump functionally active compounds from the brain to peripheric system defines a potentially important mechanism for the CNS to modulate peripheral systems.

Thompson *et al.* (2000) studied the analgesic efficacy of multiple opioids known to be p-gp substrates *in vitro*. They found that the analgesic efficacy of morphine, methadone, and fentanyl were increased in animals that lack p-gp, suggesting that p-gp plays an important role in limiting access of these drugs to the brain. In contrast, the analgesic efficacy of meperidine and morphine-6-glucuronide were not increased in animals that lacked p-gp, suggesting that p-gp does not limit the bioavailability of these opioids within the brain.

Letrent *et al.* (1999) demonstrated that morphine is transported by p-gp in brain capillary endothelium and that the BBB permeability of morphine may be altered in the presence of p-gp inhibitors such as GF120981, Cyclosporin A and verapamil. Callaghan & Riordan (1993) also found that morphine and related narcotic analgesics interact with p-gp and that p-gp may be involved in determining the distribution and side effects of these compounds.

The effect of daily morphine administration on CNS p-gp expression in rats was examined. Morphine treatment resulted in an increase in p-gp expression after 5 days. The question was raised whether long-term administration of opioids may increase p-gp expression in the CNS, therefore modulating opioid activity (Matheny *et al.*, 2001).

The peptide casomorphin, as the name implies, has opioid-like effects (Knivesberg *et al.*, 2002), in particular that of morphine (Reid & Hubbel, 1994). Therefore we postulate that casomorphin may be a substrate for p-gp, especially when taken in consideration that other opioid peptides and morphine are substrates as well.

# Chapter 4

## Hypothesis and aim of study

Some symptoms of ADHD may be caused by exogenous peptides derived from certain foods (Hole *et al.* 1988; Compart, 2003). One of the offending peptides is casomorphin, derived from milk. These peptides may cross the intestinal wall through normal absorption or a leaky gut. P-glycoprotein, located in the intestines would export the unwanted peptides back into the intestine. We hypothesize that defective p-gp in the intestine can not pump these peptides back into the intestinal gut and it is then excreted in the urine, resulting in elevated peptide levels (figure 4.1).

The aim of our study was to determine whether defective p-gp contributed to elevated peptide levels found in the urine of certain children with ADHD. To test this hypothesis, rats were treated with casein and a p-gp inhibitor.

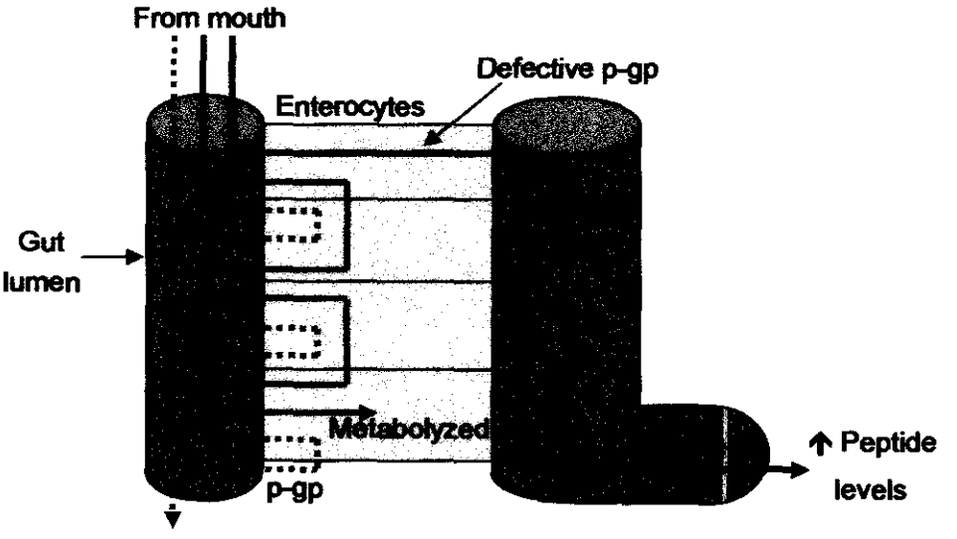


**Figure 4.1:** Effects of p-gp efflux on access of drugs in the intestines (Adapted from Benet & Cummins, 2001).

# Chapter 5

# Methods

## 5.1 Introduction

The occurrence of a characteristic urinary peptide profile and the peptides ß-casomorphin and ß-casomorphin-5 were determined in the urine samples obtained from children with ADHD as well as samples obtained form rats, using HPLC with UV detection. This method was adapted from Reichelt & Reichelt (1997).

## 5.2 Urine sample preparation for HPLC analysis

Urine samples obtained from human subjects and rats were filtered through 0.45 µ filters and their creatinine values were determined. A volume equivalent to 400 nmol of creatinine was speedvacked and diluted to 160 µl with Milli Q50 water for HPLC. A volume of 100 µl, equivalent to 250 nmol creatinine (Reichelt & Reichelt, 1997) was injected.

## 5.3 HPLC analysis

### 5.3.1 Chemicals and reagents

The chemicals and reagents used for the HPLC determination of urinary peptides are summarised in table 5.1.

**Table 5.1:** Chemicals and reagents

| Chemical or reagent | Supplier |
|---|---|
| Acetonitrile for HPLC | Acros Organics, New Jersey, USA |
| Trifluoroacetic acid | Sigma Chemical Co., St. Louis, MO |
| Hippuric acid | Sigma Chemical Co., St. Louis, MO |
| ß-casomorphin | Sigma Chemical Co., St. Louis, MO |
| ß-casomorphin, fragment 1-5 | Sigma Chemical Co., St. Louis, MO |

### 5.3.2 Instrumentation and conditions

The chromatographic conditions are summarised in table 5.2 and table 5.3.

**Table 5.2:** Instrumentation

| Instrumentation | Specifics |
|---|---|
| Software | Agilent Chemstation for LC Systems |
| HPLC system | HP (Hewlet Packard) Agilent 1100 series auto sampler |
| HPLC detector | HP Agilent 1100 series ultra violet diode array detector (UV-DAD) |
| HPLC pump | HP Agilent 1100 series quaternary pump |
| HPLC column | Vydac Protein & Peptide (C-18, 250 x 4.6 mm) |
| Pre-column | Securityguard (C-18, 4 x 3 mm) Phenomenex |

**Table 5.3:** Conditions

| Condition | Specifics |
|---|---|
| Injection volume | 100 µl |
| Flow rate | 1 ml/min |
| Mobile phase | • Buffer A: 0,1 % trifluoroactic acid (TFA)<br>• Buffer B: 0,1 % TFA in acetonitrile |
| Wavelength | 215 nm and 280 nm |

### 5.3.3 Gradient flow

The elution was performed as follows:

1. Step 1, using buffer A with 1 % B for 15 minutes to wash out most amino acids, urea, and salt,
2. Step 2, a gradient from 1-40 % B was run for 60 minutes linearly (from 15-75 minutes),
3. Step 3, from 75-80 minutes with B from 40-60 %,
4. Step 4, from 80-89 minutes isocratically at 60 % buffer B,
5. Step 5, return to 1 % B from 89-94 minutes; Step 6, re-equilibration at 1 % B from 89-115 minutes.

### 5.3.4 Preparation of standard solutions

- Hippuric acid: 100 µl of a 1mg/ml solution was injected.
- ß-casomorphin and ß-casomorphin, fragment 1-5: An amount of 5 nmol was injected.

## 5.4 Urine samples

Urine samples of children identified with ADHD, control groups and rats were analyzed using HPLC with UV detection. The Ethics committee of the North-West University approved all experiments.

## 5.5 Rat model

### 5.5.1 Preparation of materials

#### 5.5.1.1 Preparation of casein

Casein powder (BDH Chemicals Ltd., England) was mixed with sweet oil. Each rat received 0.075 g casein in 0.3 ml sweet oil twice a day which was administered with an oral tube. Dosages were calculated according to the average weight for each specific group. The dosage was adapted from Froetschel *et al.* (2001).

#### 5.5.1.2 Preparation of cyclosporin A

Cyclosporin A (Sandimmun®, Novartis South Africa (Pty) Ltd) for injection was diluted with 0.9 % sodium chloride for injection. Each rat received a volume equivalent to 50 mg cyclosporine/kg (Hendriks *et al.*, 1999).

#### 5.5.1.3 Preparation of vehicle

Each rat received a volume of vehicle equivalent to 50 mg cyclosporine/kg body weight. Cremaphor EL (650 mg/ml) was diluted with alcohol (32.9 % by volume) to volume and further diluted with 0.9 % Sodium chloride for injection.

### 5.5.2 Group division and treatment

Male *Sprague Dawley* rats weighing between 224 g – 340 g were used. 32 Rats were divided into 4 groups of 8 each (table 5.4). Group 1 and 2 received two divided oral doses of casein twice daily as part of their diet. Group 2 received cyclosporine intraperitonealy, and group 1, the control group was given a vehicle. Group 3 and 4 were placed on a normal diet. Group 4 received cyclosporine and its control group, group 3, the vehicle. The procedure was repeated for 5 days and urine samples were collected each day after injection with cyclosporine or vehicle. Urine samples were collected from metabolic cages housing the test animals and were analysed by HPLC on day 0, 1, 3 and 5, day 0 being the control before any dosage had taken place.

**Table 5.4:** Group division

| | |
|---|---|
| **Group 1** | Casein diet and vehicle (control) |
| **Group 2** | Casein diet and cyclosporine |
| **Group 3** | Normal diet and vehicle (control) |
| **Group 4** | Normal diet and cyclosporine |

# Chapter 6

# Results and discussion

## 6.1 Human subjects

We analyzed the peptide region in the urine of 12 ADHD patients and 12 healthy persons, the latter serving as control for our study, and also screened the urine samples for the occurrence of 2 peptides namely, ß-CM and ß-CM-5. These two peptides are readily available and are markers often used in peptide analyses. The peptide region, indicated with a horizontal arrow (figure 6.1), can be observed after hippuric acid which elutes at about 20 minutes. A sample was considered to be positive when it displayed a number of large peaks in the peptide region.

### 6.1.1 Positive ADHD control

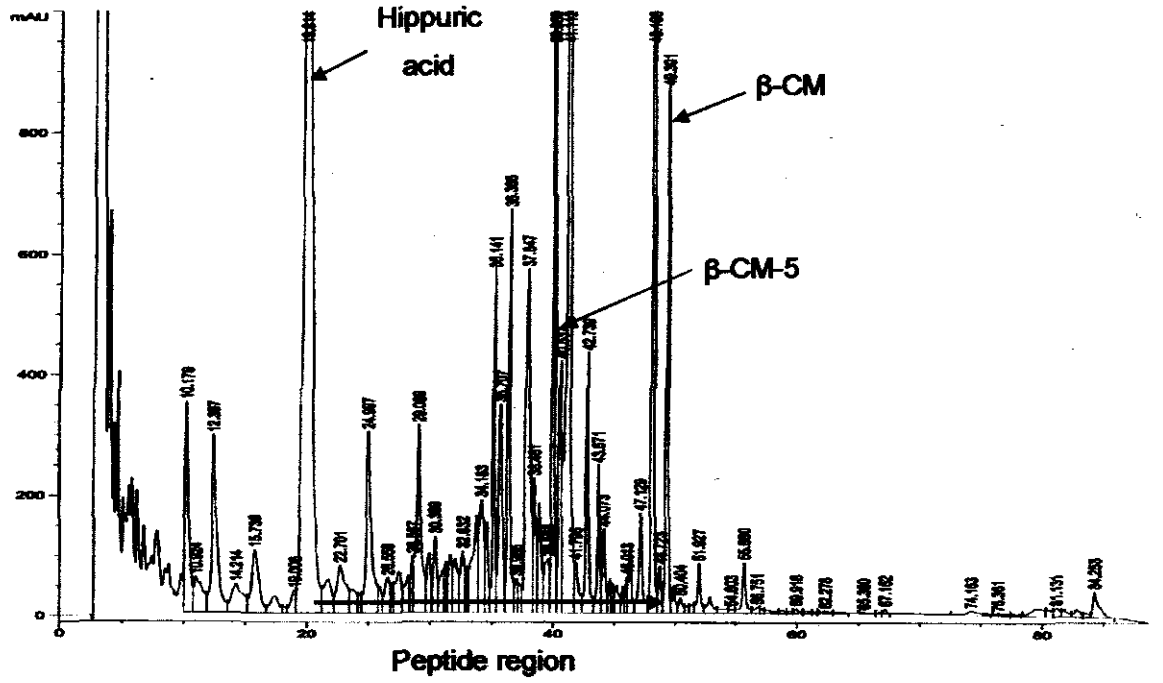


**Figure 6.1:** Urinary profile of a positive ADHD control

Figure 6.1 shows the urinary profile obtained from a child who had been diagnosed with ADHD. This patient served as positive control for our study. The area under the peak indicates the amount of each component present in the extract (Shattock & Savery, 1996). The peptides with biological activity tend to appear in the region after hippuric acid (Reichelt & Knivsberg, 2003), which elutes at about 18 -19 minutes in this particular system. Both the peptides ß-CM (40 minutes) and ß-CM-5 (49 minutes), can be seen in the profile (figure 6.1). The presence of these peptides in the chromatogram suggests that this particular HPLC method, which was also used in our study, is suitable for the analysis of the peptides in question.

### 6.1.2 ADHD subjects

Ten of the ADHD patients did not present with the expected urinary peptide profile. Two patients did however exhibit the characteristic urinary peptide profile. One of these two patients had not been on any medication and the other patient had been on Methylphenidate only for a period of 2 months when the urine sample was taken. Methylphenidate could have influenced the outcome of our results. We established that the ten ADHD patients who did not display the peptide profile had been on Methylphenidate for 8 months or longer.

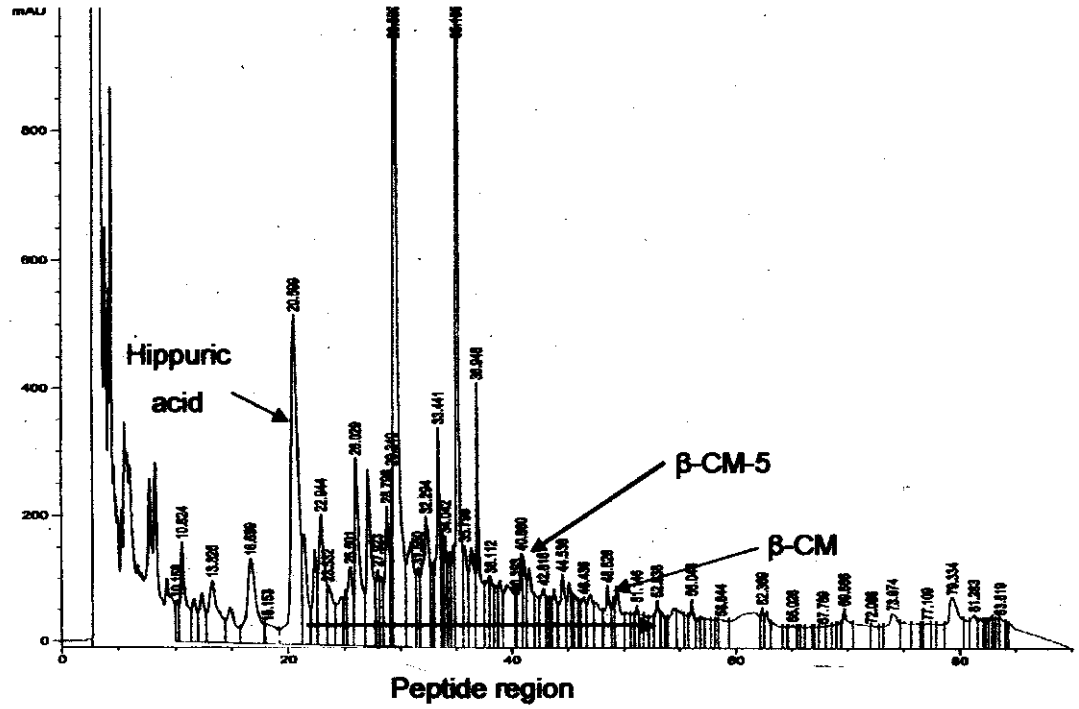


**Figure 6.2:** Urinary profile of the nonmedicated ADHD patient.

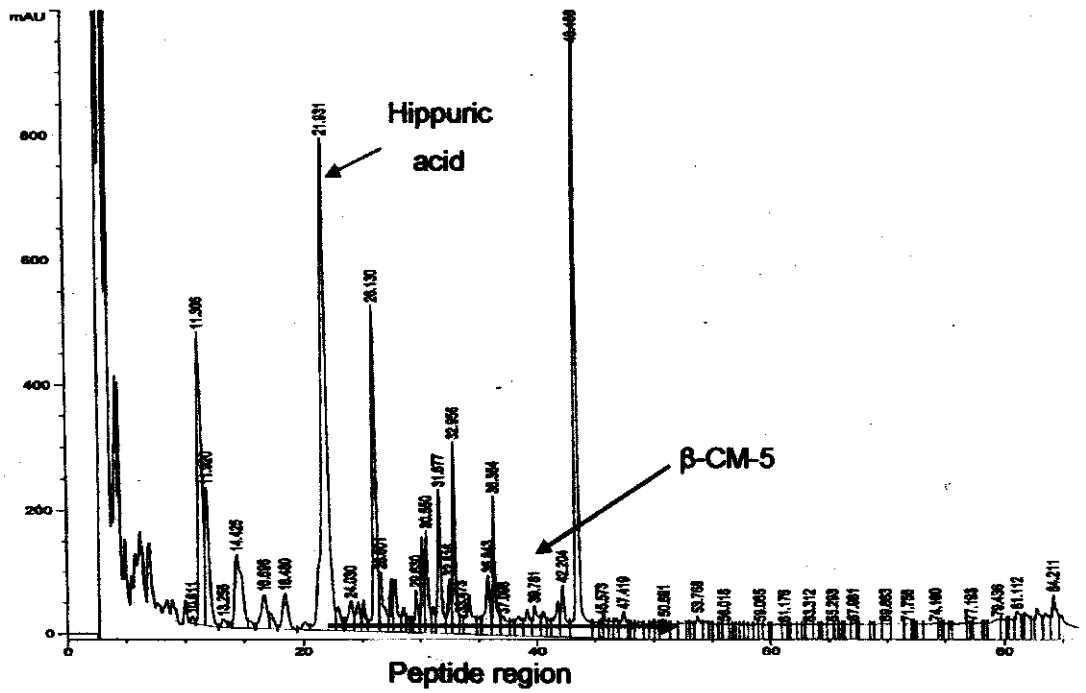


**Figure 6.3:** Urinary profile of ADHD patient who received Methylphenidate for 2 months.

Figure 6.2 and 6.3 show the urinary profiles obtained from the 2 ADHD patients who presented with the positive urinary peptide profile. The peptide profile in figure 6.2 was

obtained from the patients who had not received any medication and the peptide profile in figure 6.3 was from the patient who had been on Methylphenidate for 2 months. These chromatograms display the same characteristic peptide profile in the peptide region as can be observed in the positive control (figure 6.1). Both the B-CM (48.5 minutes in figure 6.2) and B-CM-5 (40.8 minutes in figure 6.2 and 39.7 minutes in figure 6.3) peaks, though relatively small, are present.

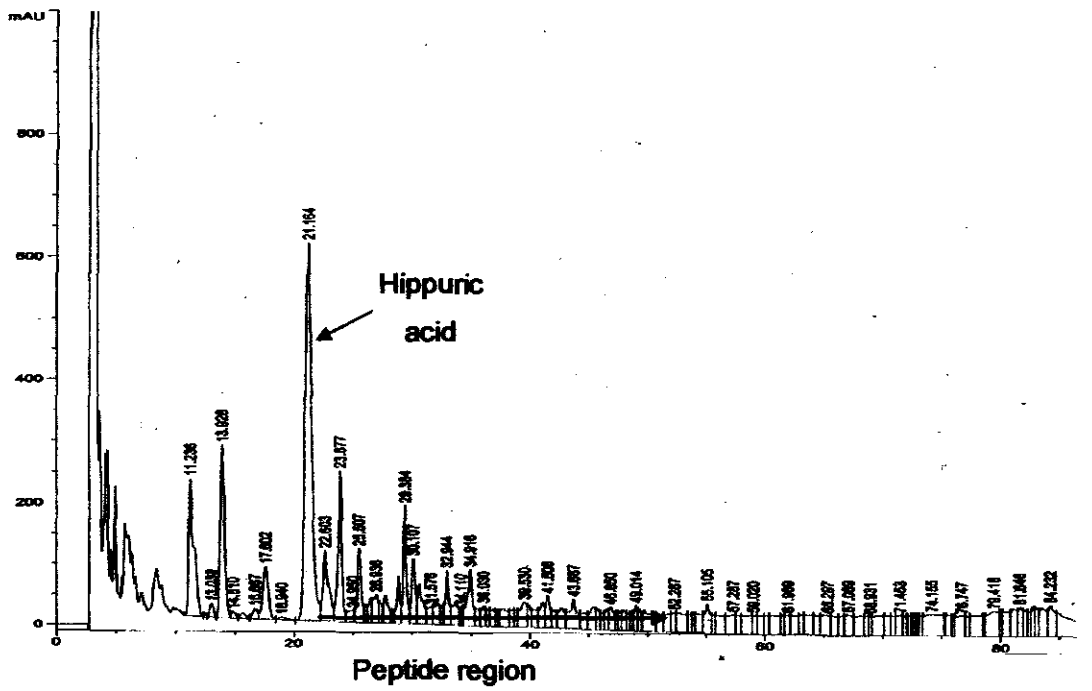


**Figure 6.4:** Urinary profile of an ADHD patient

Figure 6.4 displays the profile of one of the 10 patients who did not present with the expected peptide pattern. The profiles of the other 9 patients can be seen in Appendix A.

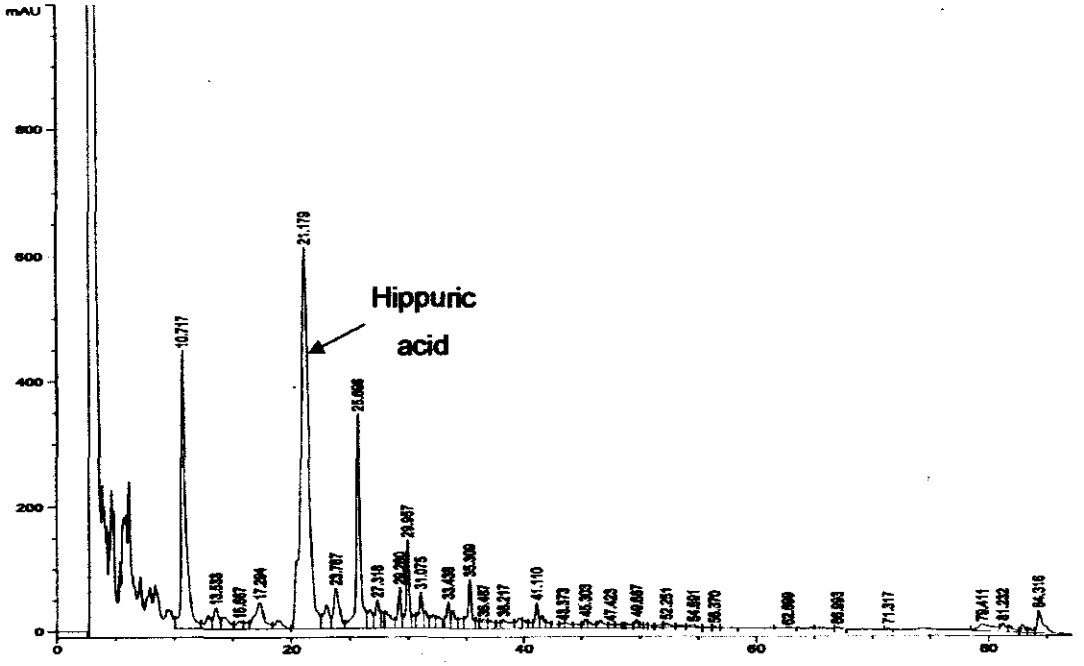


**Figure 6.5:** Urinary profile of a control subject.

Two out of the 12 subjects in the control group (patient 7 and 11, appendix A2) displayed the characteristic urinary peptide profile. The rest of the control group displayed no significant peptide patterns in the peptide region (figure 6.5 and appendix A2).

### 6.1.2.1 Patients with ß-CM and ß-CM-5 in urine

The presence of ß-CM and ß-CM-5 in the urine of the 12 healthy subjects and 12 ADHD patients was investigated. In the control group, 7 subjects exhibited the ß-CM peak and none the ß-CM-5 peak. A significant number of the ADHD group, presented with both ß-CM and ß-CM-5 in their urine. Ten out of a possible twelve of these children had ß-CM in their urine and 9 had ß-CM-5. It should be noted, however, that the area under the curve of these peaks (Appendix A1) was small compared to that of the positive control (figure 6.1).

**Discussion**

According to Shattock & Savery (1996), exogenous peptides derived from the diet are responsible for the characteristic urinary peptide profiles. An increase in urinary peptides can be observed in various conditions such as autism, schizophrenia, celiac

disease, PDD, ADHD, ADD, dyslexia and OCD (Shattock & Savery, 1996; Center for autism and related disorders, 2001).

Although ten out of the 12 ADHD patients presented with ß-CM or ß-CM-5 in their urine, only two of the 12 ADHD patients displayed the characteristic urinary peptide profile presented in figure 6.1. The presence of such peptides may be of importance in understanding ADHD and its possible causes. Although there were slight variations from day to day, which are reflective of temporary dietary or other changes, the patterns for each individual retain characteristic features.

According to Shattock & Savery (1996), there are a number of situations in which the peptide levels can rise: Given the same order of leakiness of the gut wall and BBB as in normal persons, greatly increased levels of peptides in the gut will result in an increased quantity of peptides reaching the CNS. The reason for the increase may be the result of inadequate enzyme systems which are responsible for their breakdown, for example, genetically determined deficiencies of the required endopeptidase enzymes. There could be shortages of cofactors, such as vitamins and minerals required for the enzymes to function properly. Alternatively, the pH in the relevant areas of the gut may be inappropriate for the specific enzymes to act as they should (Shattock & Savery, 1996).

The peptide levels in the gut could be normal, but for some reason, the gut wall may be excessively leaky so that vastly increased quantities of the peptides will cross the gut wall and enter the blood stream. Thus, there will be an increased level of peptides in the CNS with possible clinical consequences. There are a number of factors which could result in increased leakiness of the gut. There may be damage caused by purely physical action such as a surgical operation or some natural flaw. Deficiencies in the phenyl sulphur transferase (PST) systems can lead to increased permeability of the gut wall. Normally the proteins lining the gut wall are sulphated and, in this state, form a continuous protective layer over the surface of the gut wall. When there is insufficient sulphation, the proteins clump together and the layer becomes irregular. The net result is an increased permeability of the gut wall. In this case, the passage of peptides across the gut wall is greatly enhanced (Shattock & Savery, 1996).

Another reason could be that the blood-brain barrier may be less effective than normal so that any opioid peptides in the blood stream would easily pass into the CNS and exert

their full range of actions. The blood-brain barrier is a complex system, which is partly physical and partly biochemical. The biochemical element consists, in part, of enzymes, which should destroy potentially harmful substances such as exogenously derived peptides. According to these hypotheses, the peptidase activity may be depressed and the barrier could be somewhat more permeable than normal. There may be other environmental factors which could exacerbate the process either slightly or dramatically (Shattock & Savery, 1996). Meningitis, other infections and physical damage can all greatly increase the permeability of the blood-brain barrier and increase peptide levels in the CNS (Shattock & Whitely, 2001).

In healthy persons, proteins are broken down in the gut and peptides occur as intermediate compounds, which will then be broken down further into their amino acid components. A small proportion transfers across the intestinal wall and blood-brain barrier to the CNS, but at such low levels that they have little effect (Shattock & Savery, 1996). It is therefore expected to see some patients with these peptides occurring in the urine. Ten out the 12 ADHD patients portrayed small peaks in the peptide region and according to Shattock & Savery (1997) they are likely to be of no clinical significance due to their comparatively low levels or their structure. These 10 patients were all on Methylphenidate and it may explain why they did not present with the peptide profiles. One of the remaining patients was not on any medication while the other one had only been on Methylphenidate for 2 months. These 2 patients displayed the characteristic urinary peptide profile for ADHD. The other patients on Methylphenidate had been taking this drug for 8 months or longer. These patients showed no significant peptide pattern and it could therefore be speculated that Methylphenidate is somehow involved in the transport of these peptides. If defective p-gp is the cause of this occurrence and the use of Methylphenidate reduces the occurrence, then Methylphenidate may be a p-gp agonist or inducer. The duration of treatment may also play a role, because the patient that had been taking Methylphenidate for only 2 months did display the urinary peptide profile.

The mechanism in the gut applies to the brain as well. P-gp is present in the BBB and functions to extrude substances from the brain. Methylphenidate may also be involved in extruding these peptides from the brain.

Up to present, no studies have been conducted to study the influence of Methylphenidate on p-gp. An increase in cyclosporine blood levels was documented in a boy who received methylphenidate as well (Lewis *et al.*, 2001). Cyclosporine is also known to be a p-gp substrate (Matheny *et al.*, 2001). It could therefore be argued that methylphenidate may play a possible role in p-gp, although the precise mechanism is unknown.

Two control subjects also displayed the peptide profile. This could be attributed to an undiagnosed condition such as a leaky gut and/or ADHD.

According to our hypothesis, p-gp is responsible for the occurrence of certain peptides in the urine. If the p-gp is defective, the peptides will not be transported back into the intestinal gut and will be excreted in the urine. To verify this possible mechanism, a rat model was used.

## 6.2 Rat model

The rats were treated as discussed in 5.5.2. None of the rats in the various groups displayed the characteristic urinary peptide profile found in humans (figure 6.1). Although there were slight differences in the peptide profiles of individual rats, there seemed to be no pattern when individual rat profiles were compared over the 5 days or when the various groups were compared to each other. Group 2, which received casein and cyclosporine (p-gp inhibitor), was expected to display more peptides in their urine, but did not show any. None of the rats presented with either of the 2 peptides, ß-CM-5 or ß-CM in their urine. Figure 6.5 represents the urinary profile of a rat that received casein as well as cyclosporine.

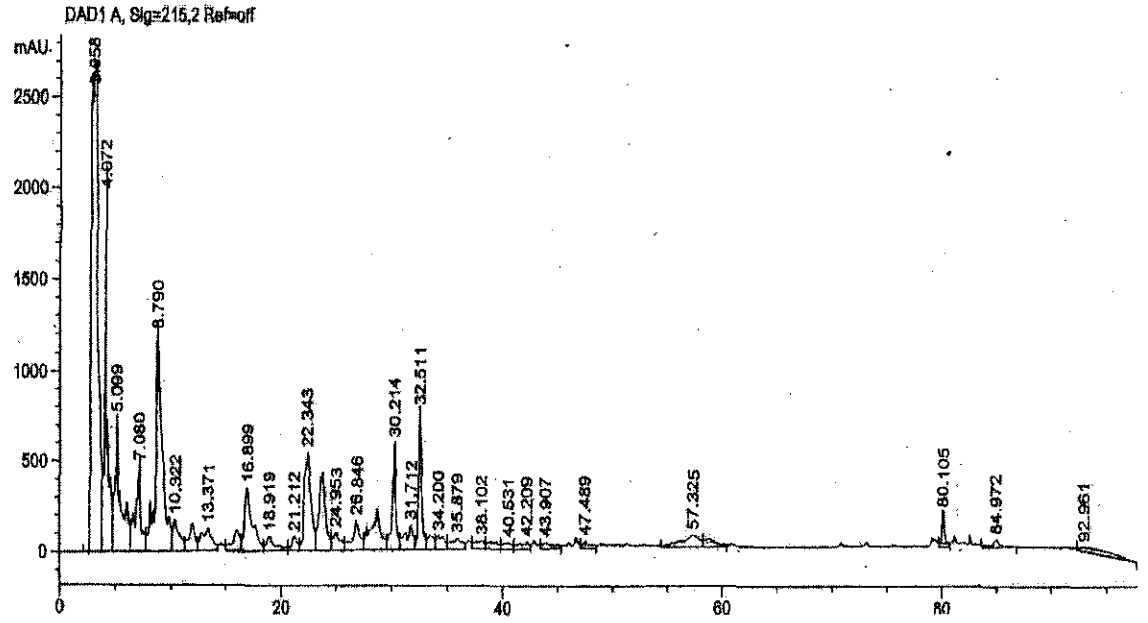


**Figure 6.5:** Urinary profile of rat that received casein and cyclosporine

**Discussion**

The results were not what we anticipated. None of the profiles showed the characteristic urinary peptide profile (figure 6.1) or significant levels of the 2 peptides, ß-CM-5 and ß-CM. According to our hypothesis, group 2, who received the casein in addition to their normal diet and cyclosporine, the p-gp inhibitor, should have shown significant levels of ß-CM-5 and ß-CM in their urine. Overall, there was no distinguishing urinary profile pattern within individuals in the groups or between the various groups. The profiles portrayed small peaks in the peptide region and according to Shattock & Savery (1997) they are likely to be of no clinical significance due to their comparatively low levels or their structure.

**P-glycoprotein**

P-gp can be found in many organ systems and several tissues, such as the intestine, CNS, liver and kidney (Matheny *et al.*, 2001). According to our hypothesis, if p-gp in the intestinal gut is defective, it will result in increased levels of peptides in the urine, for the peptides can not be transported back into the GIT. We mimicked the defective p-gp in the intestinal wall by giving a p-gp inhibitor, but did not take in account that the inhibitor may have an effect on other systems, eg. p-gp in the renal tubular.In the kidney, p-gp

participates in the excretion of xenobiotics and endogenous toxic metabolites into the urine (Liu & Brunner, 2001), thus inhibition of p-gp in the kidney will lead to increased blood levels, and the substances will not be excreted in the urine. Inhibition of p-gp in the GIT and kidney will lead to increased blood levels of orally digested substances, because the inhibition will prevent the substances from being transported back into the GIT and excreted in the urine. If we can assume that p-gp was blocked in the gut and renal tubular, we would expect to see no peptides in the urine, but elevated levels of the peptides in the blood. It is therefore possible that p-gp blood levels might have been elevated in the rats treated with the cyclosporine. According to literature, no studies to date had been done where the urine was analyzed after oral administration of a substance and a p-gp inhibitor.

**P-glycoprotein inhibitor**

Various studies on p-gp used cyclosporine as a choice for p-gp inhibition (Kabasakal *et al.*, 2000; Hendrikse *et al.*, 1999). Co administration of cyclosporine with paclitaxel (Meerum Terwogt *et al.*, 1999) and docetaxel (Malingre *et al.*, 2001) enhanced the oral bioavailability of these drugs due to p-gp inhibition. Cyclosporine markedly increased the analgesic effects of morphine in wild-type mice and the results suggest that p-gp reduces the analgesic effect of these opioids by restricting their permeability into the brain (Zhang *et al.*, 2003). Schinkel & Jonker (2003) and Matheny *et al.* (2001) also refer to cyclosporine as a p-gp inhibitor.

There are however evidence that cyclosporine may be a p-gp inducer. Lui & Brunner (2001) indicated that cyclosporine induced renal p-gp overexpression in a dose-dependant manner. Jette *et al.* (1996) revealed that the induction of p-gp expression due to cyclosporine is not only dose-dependant, but time-dependant as well. The p-gp levels in the liver, intestine, kidney and lungs were maximal after 10 days of cyclosporine treatment in rats. Chronic cyclosporine treatment reversibly induces renal p-gp expression and this induction occurs in a dose-dependant manner, and is independent of administration routes. These results implicate the potential difficulty of using cyclosporine as a modifier for reversing, thus inhibiting, multidrug resistance and drug interactions involving renal excretory mechanisms (Lui & Brunner, 2001). Johnson, (2002) and Matheny *et al.* (2001) also regarded cyclosporine as a p-gp inducer.

The implication of above mentioned for our study is that we may not have used a suitable p-gp inhibitor. Induction of p-gp in the GIT will result in the peptides being transported back into the gut for elimination in the faeces. If there is only induction of p-gp in the kidney, it will lead to increased levels of peptide in the urine.

Although the function of p-gp in the various tissues is understood, the situation in the kidney remains less clear. One expects an excretory function in the kidney, pumping substrates from the blood into the urine; however, some studies using p-gp knockout mouse models have given equivocal results (Schinkel & Jonker, 2003). In some cases the absence of p-gp in the mice resulted in increased renal excretion and even clearance of drugs, which would seem to contradict an excretory function. This however, may in part have to do with a general rerouting of excretory pathways for p-gp substrates in p-gp knockout mice, or with changes in expression of other transporters or enzymes that affect the drugs tested (Smit *et al.*, 1998).

Due to uncertainty about the tissues affected by the p-gp inhibitor and the precise mechanism of cyclosporine it may offer an explaination why none of the rat profiles revealed any significant peptide patterns.

***Sprague Dawley* rats**

*Sprague Dawley* rats were used in this study because they are readily available and handling is easier than with mice. *Sprague Dawley* rats were used in various studies investigating peptides such as gliadorphin-7 (Sun & Cade, 2003), casein (Froetschel *et* al., 2001) and β-Casomorphin (Sun *et al.*, 1999), opioids (Reid & Hubbell, 1994), as well as a variety of studies on p-glycoprotein (Letrent *et al.*, 1999; Kwan *et al.*, 2003).

There are however a few limitations one has to take in consideration when making use of an *in vivo* model: It is often difficult to get an effective amount of the inhibitor to the intended target site. If the inhibitor is given orally it may be susceptible to first pass metabolism, which can render the inhibitor inactive or increase its rate of elimination. Even when the drug is administered intravenously, the inhibitor can still be limited by factors such as protein binding and/or a lack of organ distribution. Also, when an appropriate amount of the drug reaches the intended organ, depending on the affinity of the inhibitor for the drug efflux protein, complete blockade may not be obtained. Finally, since the inhibitors modulate drug efflux function in a transient manner, the timing in

which these inhibitors are used is critical. If the inhibitor and the drug of interest are not administered appropriately, the drug efflux protein may regain function before the drug reaches its intended target (Zhang *et al.*, 2003).

To determine the impact of drug efflux transporters on drug absorption, distribution and elimination, one requires *in vivo* examination. There are transgenic and mutant animal models that can be used as an important tool for assessing drug efflux transporter activity (Zhang *et al.*, 2003).

The transgenic model involves gene knockout. Unlike humans, which have only one gene that encodes the p-gp involved in multiple drug resistance, mice have two, *mdr1a* and *mdr1b*. Consequently, one and/or both can be knocked out to evaluate p-gp function in drug absorption, distribution and excretion (Zhang *et al.*, 2003).

Mutant models are naturally deficient in the expression of a drug efflux transport protein. A subpopulation of the CF-1 mouse strain exhibits a genetic defect which results in the absence of p-gp expression and these CF-1 mutant mice can be used to investigate the effect of p-gp in drug activity (Zhang *et al.*, 2003).

In future studies, one of the transgenic or mutant models could be used, which will eliminate the problem of administering a p-gp inhibitor as well as the affinity of the inhibitor for the p-gp where complete blockade may not be obtained, and the timing in which these inhibitors are used, which is critical for desired effects to be achieved (Zhang *et al.*, 2003). The different tissues which could possibly be affected by the inhibitor will also be avoided.

**Protein loading**

The orally administered casein may host some problems as well. Froetschel *et al.* (2001) conducted a study where rats were given premeal loads consisting of casein. Each rat received the premeal equivalent to 0.25 g casein by means of a latex feeding-tube catheter. We used casein powder mixed with sweet oil, but had to lower the dosage to 0.15 g per rat due to problems with the administration of the solution. The casein solution was too dense to administer with the oral tube. Therefore, the rats might not have received an adequate dosage for the study. It is also possible that casein is fully digested by rats and that there are no peptides left after digestion for excretion.

Other studies done on opioid peptides include *in vitro* studies where Jinsmaa & Yoshikawa (1999) investigated the release of β-CM-7 from bovine β-casein by gastrointestinal proteases, and Fukudome & Yoshikawa (1992) who isolated and characterized opioid peptides from the enzymatic digest of wheat gluten. *In vivo* studies include examining the behavioural and analgesic effects of β-CM in rats (Sun & Cade, 1999), determining the brain localizaiton affected by β-CM (Sun *et al.*, 1999) and comparing the effects of gliadorpin-7 infusion in rats in comparison with β-CM-7 in various brain regions (Sun & Cade, 2003). No previous *in vivo* work has been reported that investigated the effects or the presence of peptides such as β-CM in the urine after oral administration of casein.

If these peptides are substrates of p-gp, they first need to cross the intestinal wall before being transported back into the GIT. According to Shattock & Whitely (2001), peptides cross the gut wall if it is excessively leaky, leading to higher peptide levels in the blood stream. It probably takes some time for the gut wall to become permeable, thus to investigate the effects of p-gp, one must first have a leaky gut. The rats we used were healthy with intact intestinal walls, and even if there had been adequate p-gp inhibition with the cyclosporine, the peptides might not have been able to cross the gut wall to be excreted in the urine and therefore there would be no peptides present in the urine.

# Chapter 7

## Conclusion

Attention deficit disorder with hyperactivity is the most commonly diagnosed behavioural disorder of childhood (Glickman-Simon *et al.,* 2001), with symptoms such as inattention, hyperactivity, impulsivity and learning difficulties (Glickman-Simon *et al.,* 2001; Kirley *et al.,* 2002). The exact cause of ADHD remains uncertain, but the prevailing theories include genetic and hereditary factors, neurobiological conditions and pathologies, prenatal influences, nutritional factors and deficiencies, environmental/toxin influences and gut immunology mechanisms that directly affect the central nervous system (Anon, 2000; Mehl-Madrona, 2003).

Stimulant medications are widely researched and commonly prescribed for the treatment of ADHD. Although researchers do not fully understand how these medications improve ADHD symptoms, studies indicate that methylphenidate (Ritalin®), the most commonly prescribed stimulant, significantly increases dopamine levels in the brain (Glickman-Simon *et al.,* 2001).

In a study done on several behavioural disorders, abnormal amounts of peptide and protein-associated peptide complexes were found in the urine of patients. The symptoms of these patients fitted the criteria for ADHD (Hole *et al.,* 1988). According to Shattock & Savery (1996), the presence of these peptides is the result of a leaky gut which allows the peptides to cross the gut wall and enter the blood stream.

The function of p-gp is to actively pump a diverse selection of xenobiotics out of the cells in which it is expressed (Thompson *et al.*, 2000). P-gp can be found in the intestine (Matheny *et al.*, 2001) and can limit cellular uptake of drugs from the gastrointestinal lumen into the enterocyte (Lin, 2003). According to our hypothesis, defective p-gp in the intestine can not pump these peptides back into the intestinal gut and it is then excreted in the urine, resulting in elevated peptide levels.

Only two patients used in our study showed significant levels of peptides in their urine. One patient did not receive Methylphenidate and the other patient was on Methylphenidate for 2 months. The rest of the ADHD patients did not reveal any

significant peptides in their urine and were on Methylphenidate for 8 months or longer. We speculate that the Methylphenidate influenced the outcome of the results. The exact mechanism of Methylphenidate is unknown (Glickman-Simon *et al.,* 2001), and we suspect that in addition to its known stimulant properties to significantly increase dopamine levels in the brain (Glickman-Simon *et al.,* 2001), it may activate p-gp and help with extrusion of dietary peptides back into the GIT.

The rat model we used to verify that defective p-gp is responsible for the occurrence of peptides in the urine did not give the expected results. One group of animals were on a normal diet and served as control for the second groups who received cyclosporine the p-gp inhibitor. A third group received casein in their diet as well as cyclosporine while a fourth group received only casein and served as control for the third group. There were no significant urinary peptide profiles in any of the groups. It could be speculated that cyclosporine was not the ideal inhibitor to use, because there exists some confusion regarding its effects on p-gp. Some studies refer to it as a p-gp inhibitor (Kabasakal *et al.*, 2000; Hendrikse *et al.*, 1999) and others as a p-gp inducer (Lui & Brunner, 2001; Johnson, 2002). Because of the conflicting reports about the inhibitor/inducer effects of cyclosporine on p-gp, it would be difficult to predict the net effect of cyclosporine on the p-gp in the various tissues. It may be possible that defective p-gp alone may not be responsible for the occurrence of peptides in the urine, but defective p-gp as well as a leaky gut.

Recommendations for future studies:

- Investigation of the possible role of Methylphenidate in the mechanism of p-gp using an *in vitro* model e.g. a two chamber diffusion system with rat intestine mounted between the donor and receiver compartments (Shono *et al*, 2004).

- Using a different p-gp inhibitor in the rat model to verify whether defective p-gp is responsible for the occurrence of ADHD.

- Investigating the occurrence of peptides in the urine after p-gp inhibition in rats which have a leaky gut.

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# Appendix A

## A1: Children with ADHD

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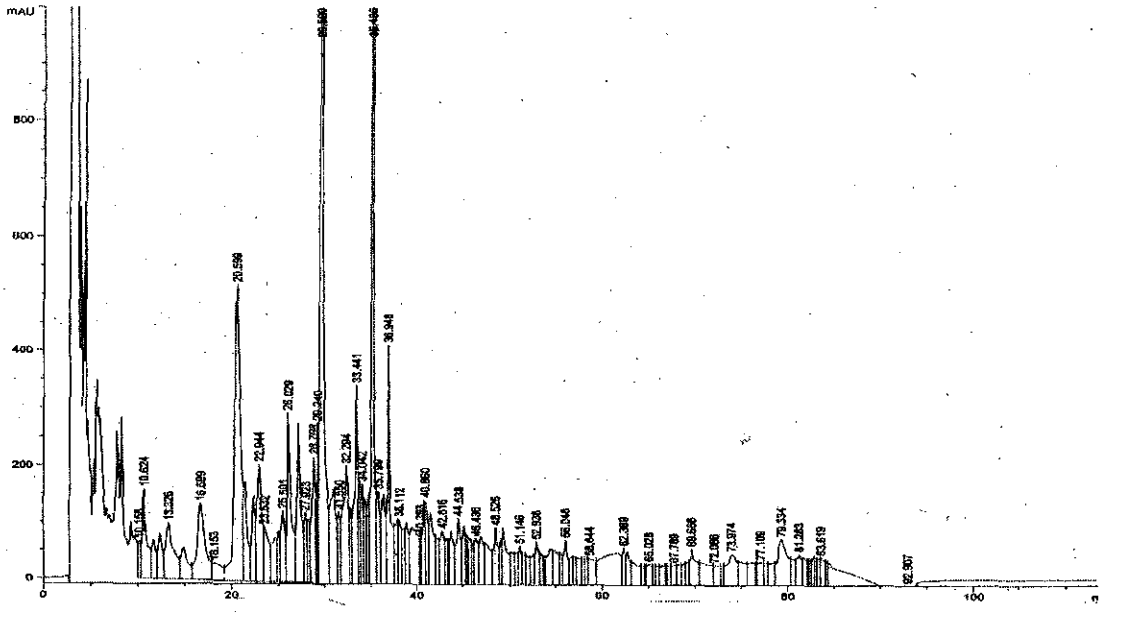


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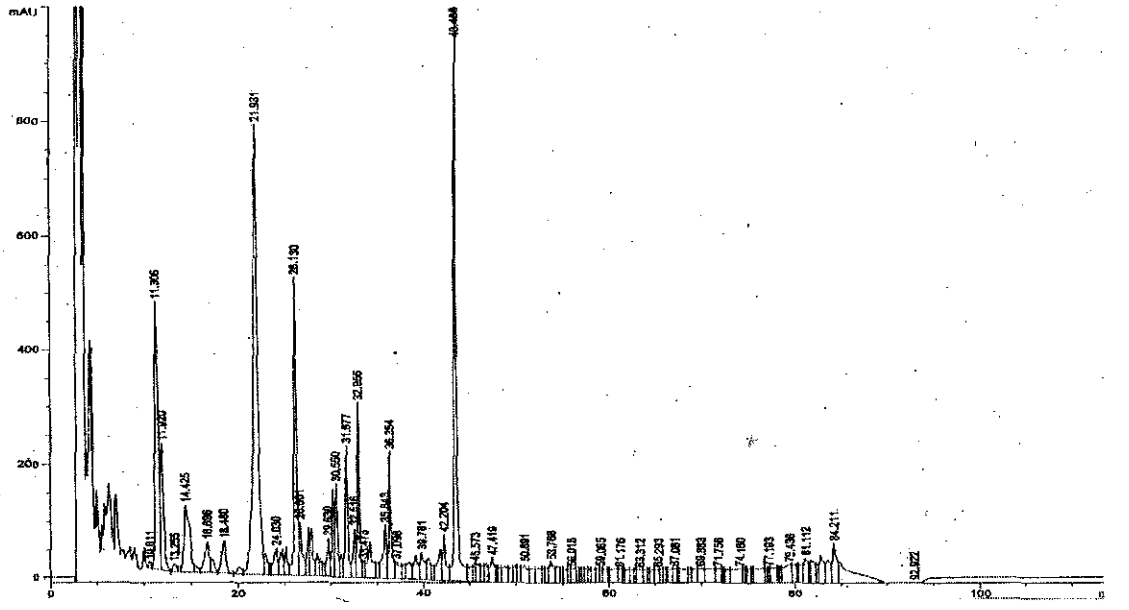

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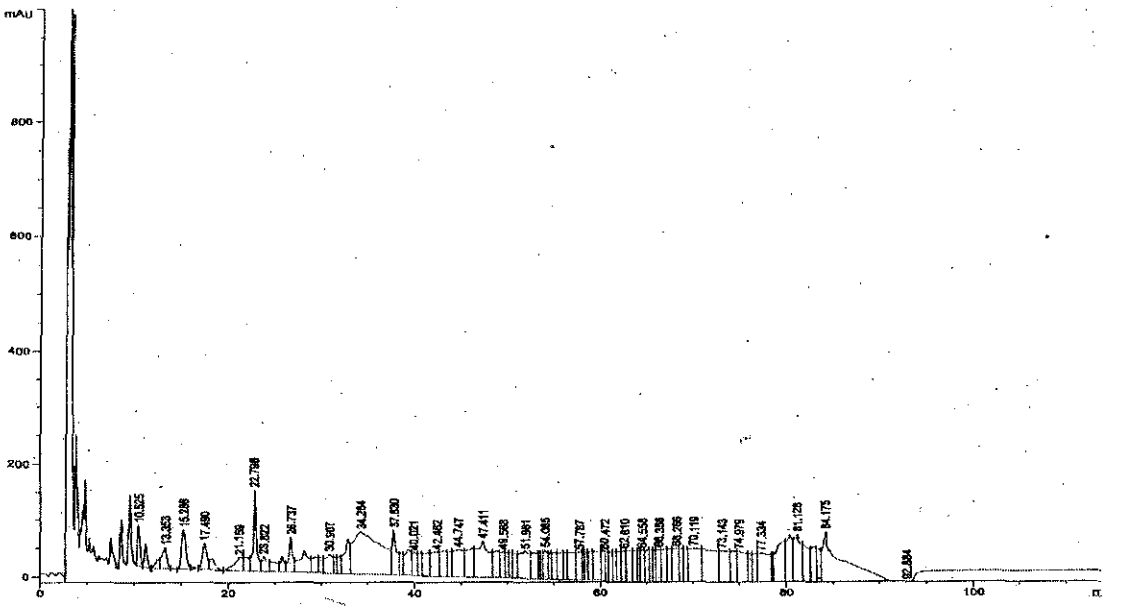


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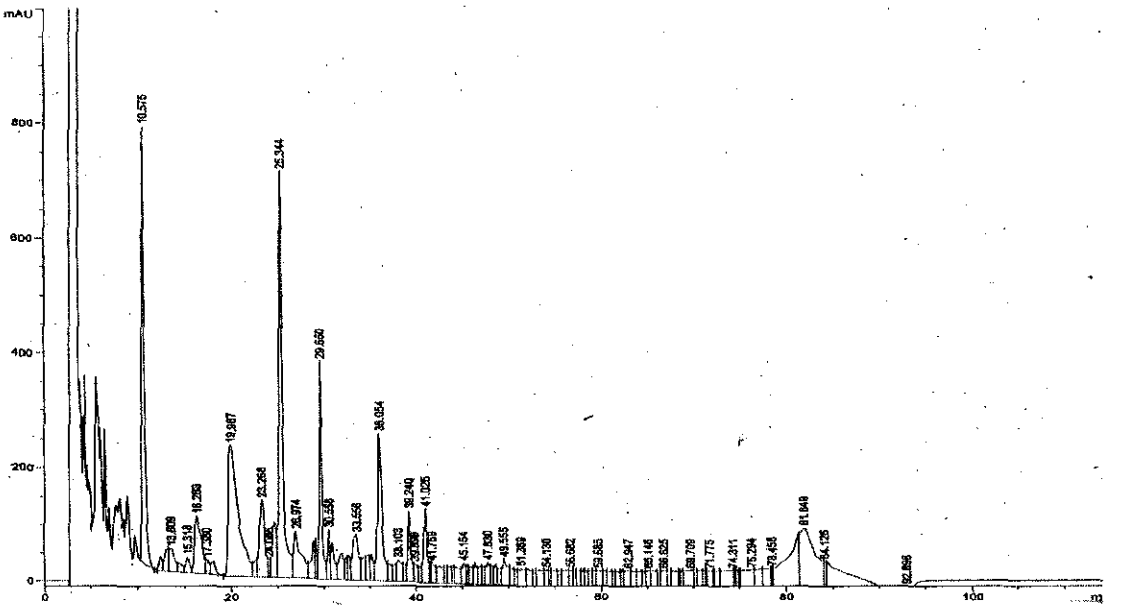

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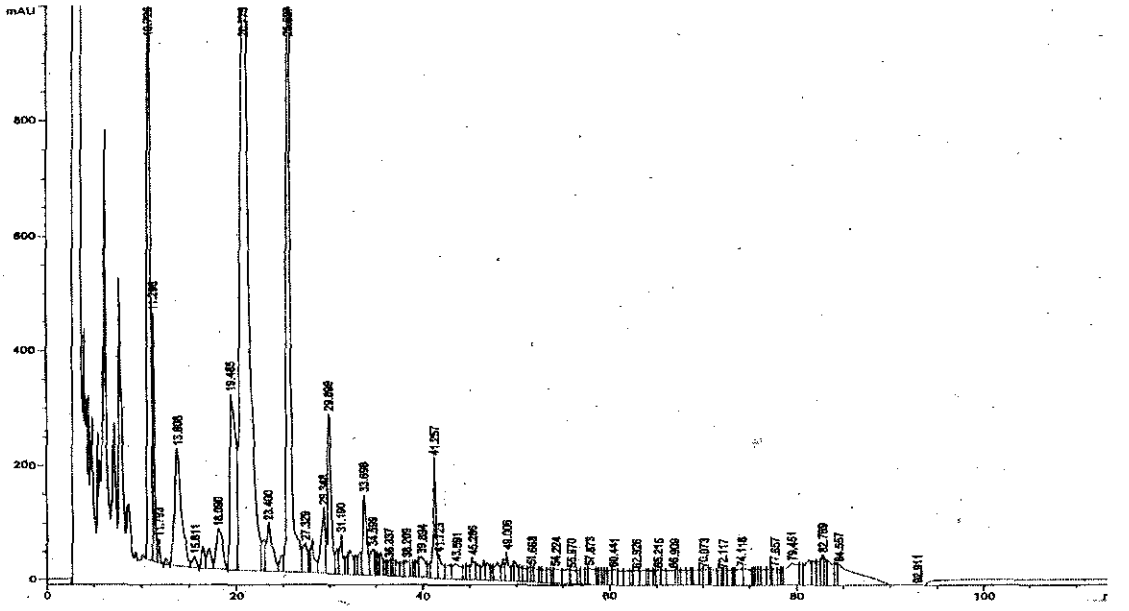


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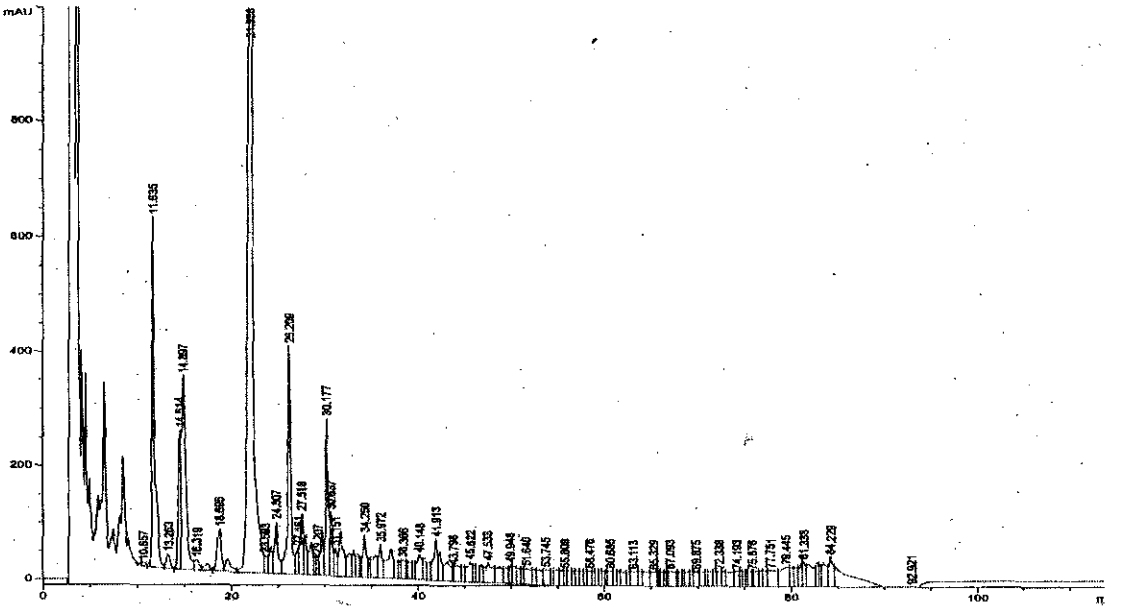

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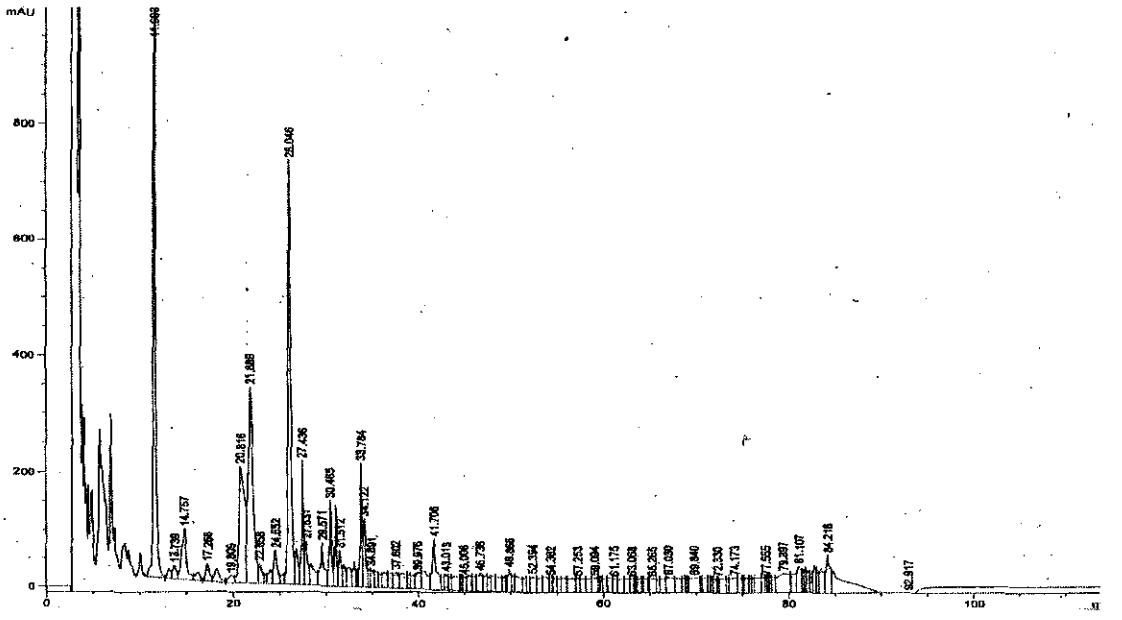


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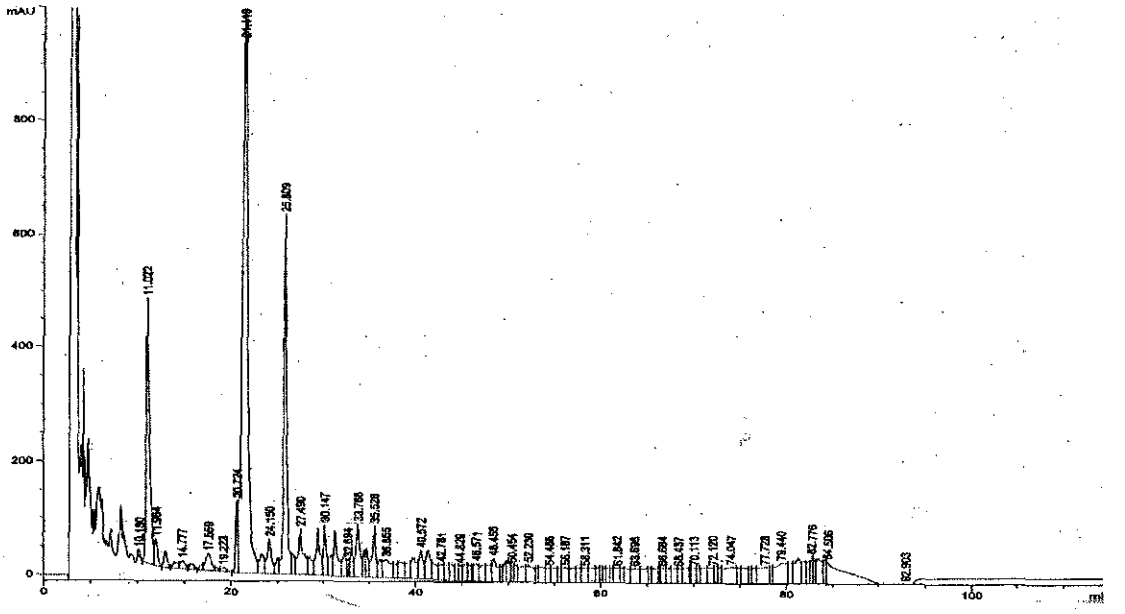

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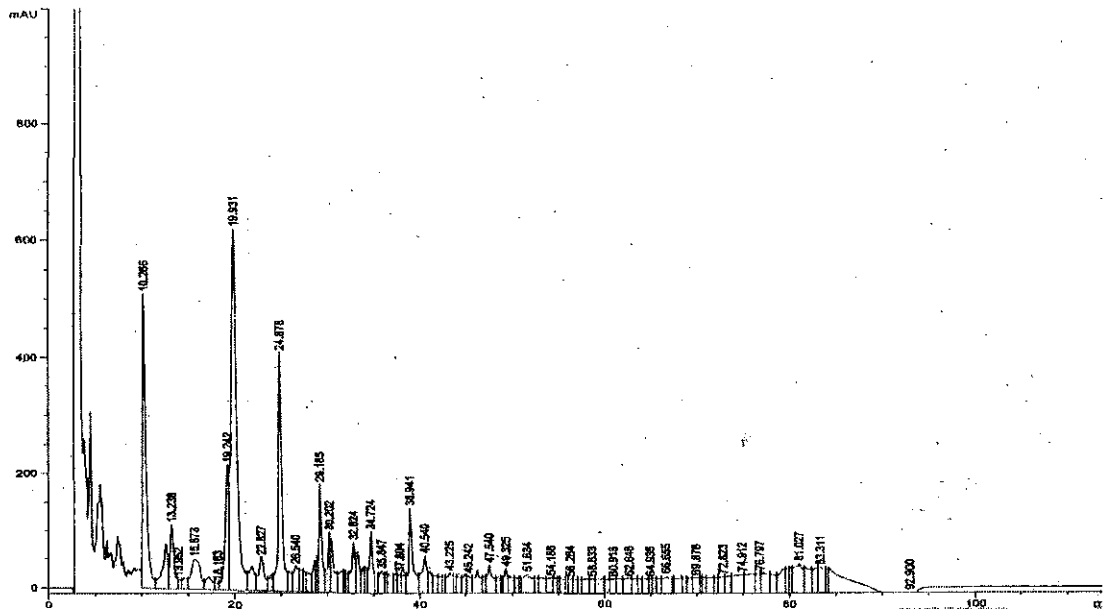


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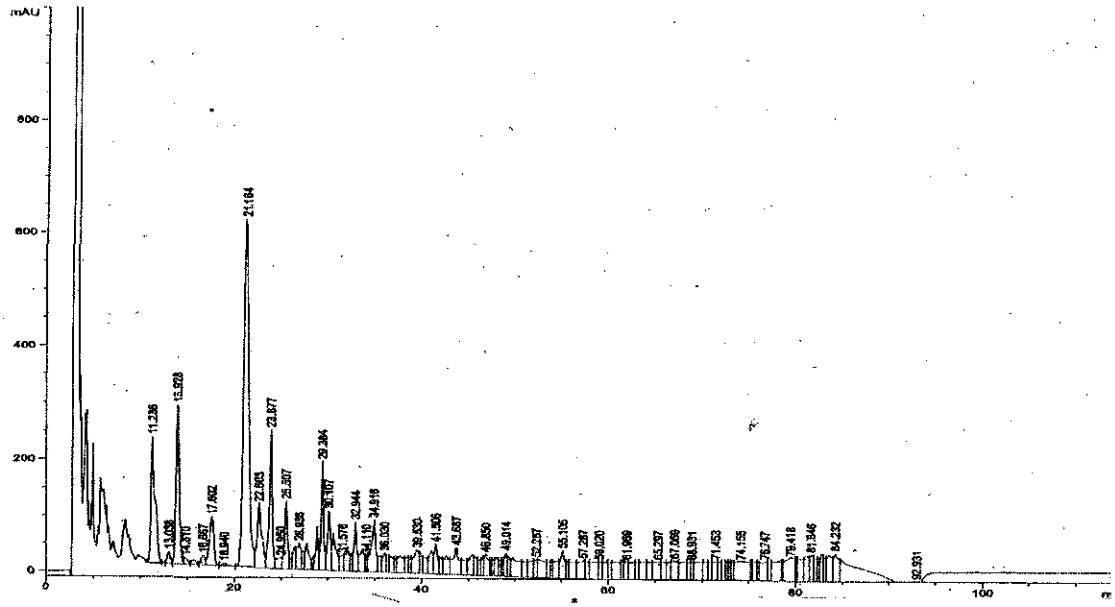

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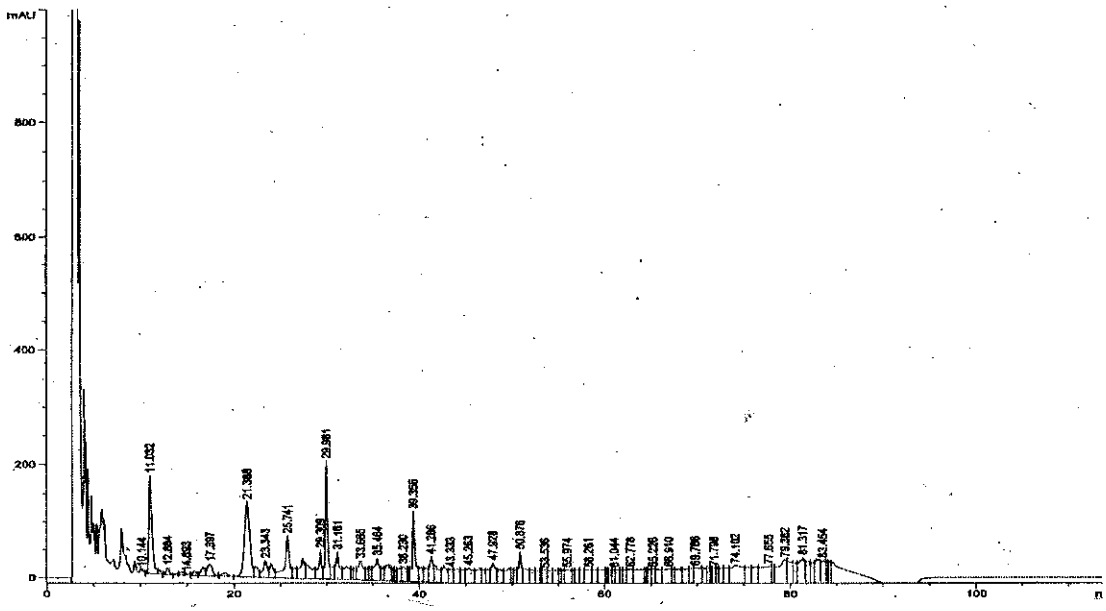


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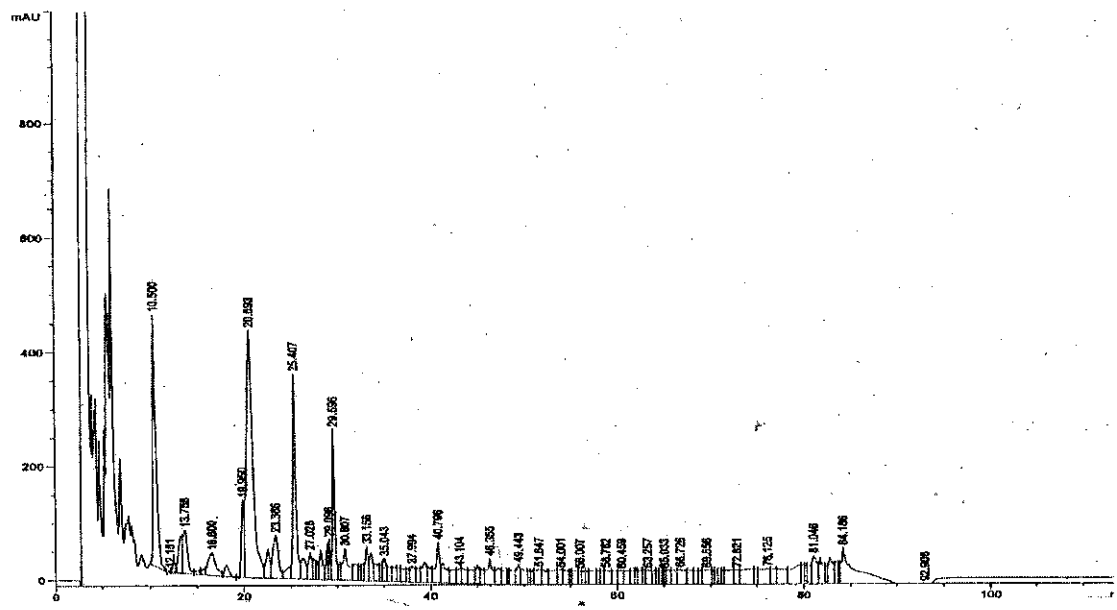

# A2: Control group

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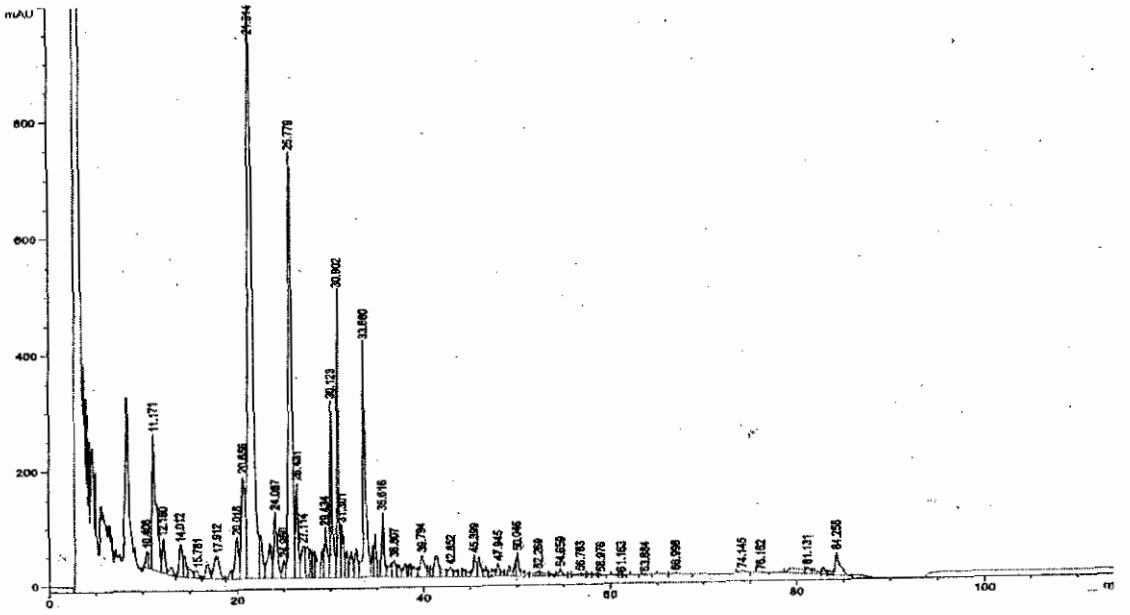


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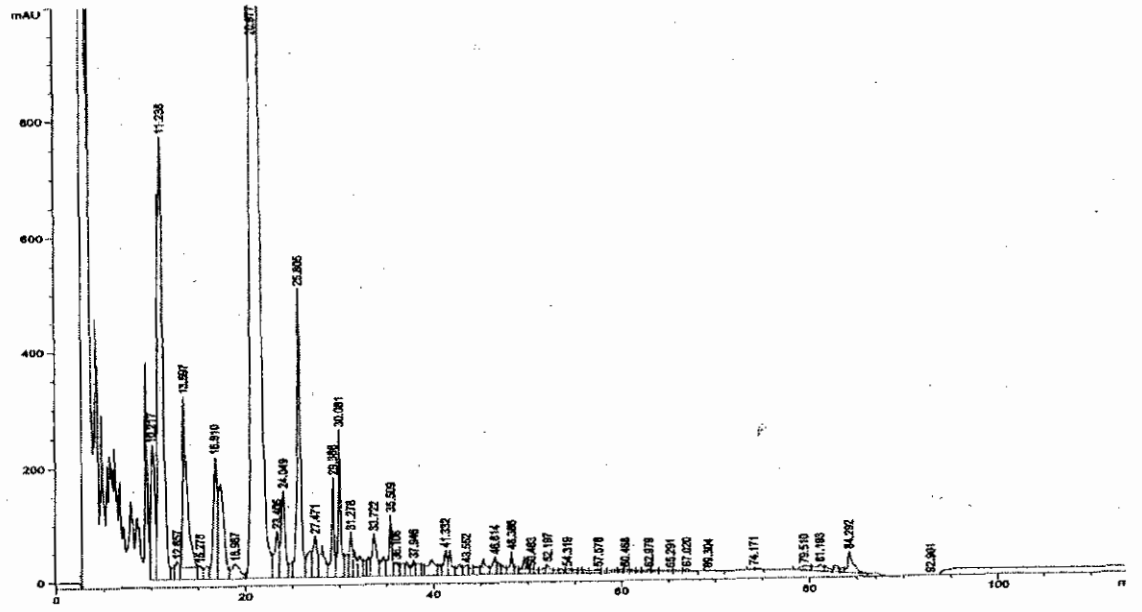

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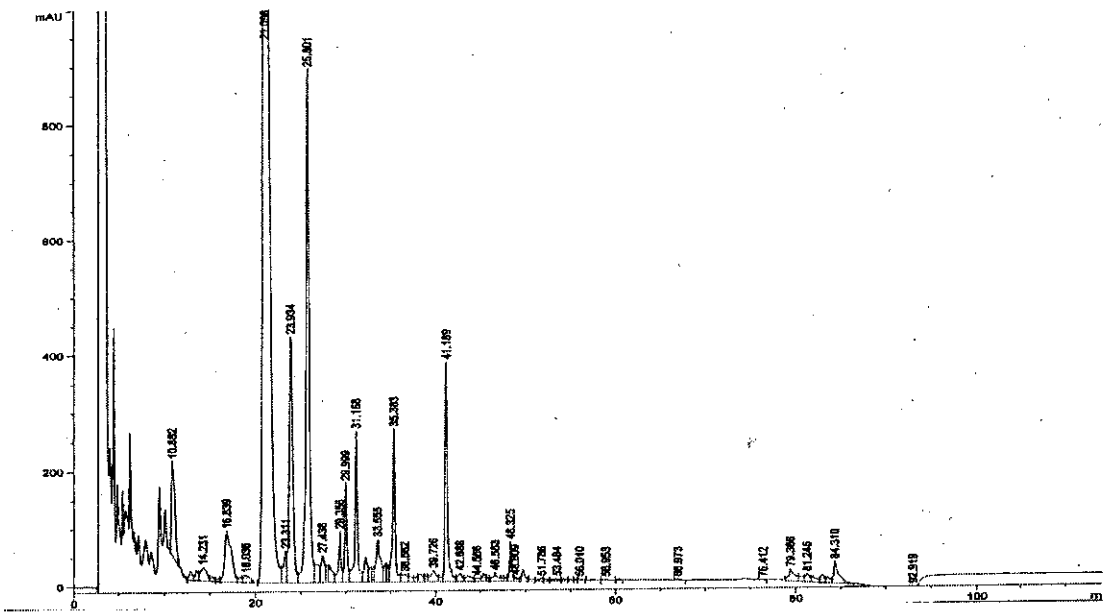


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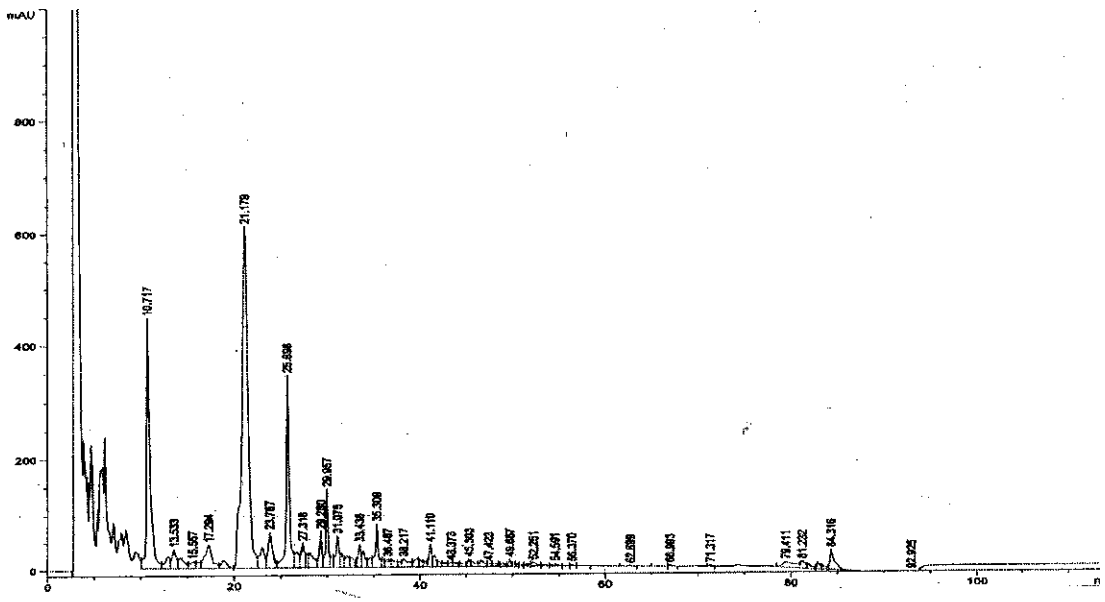

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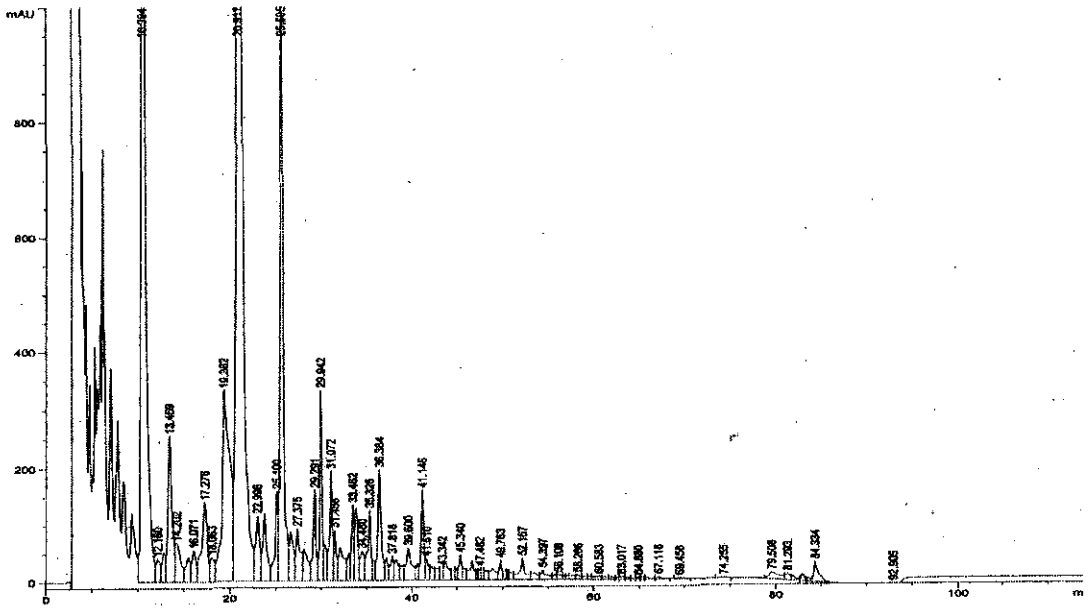


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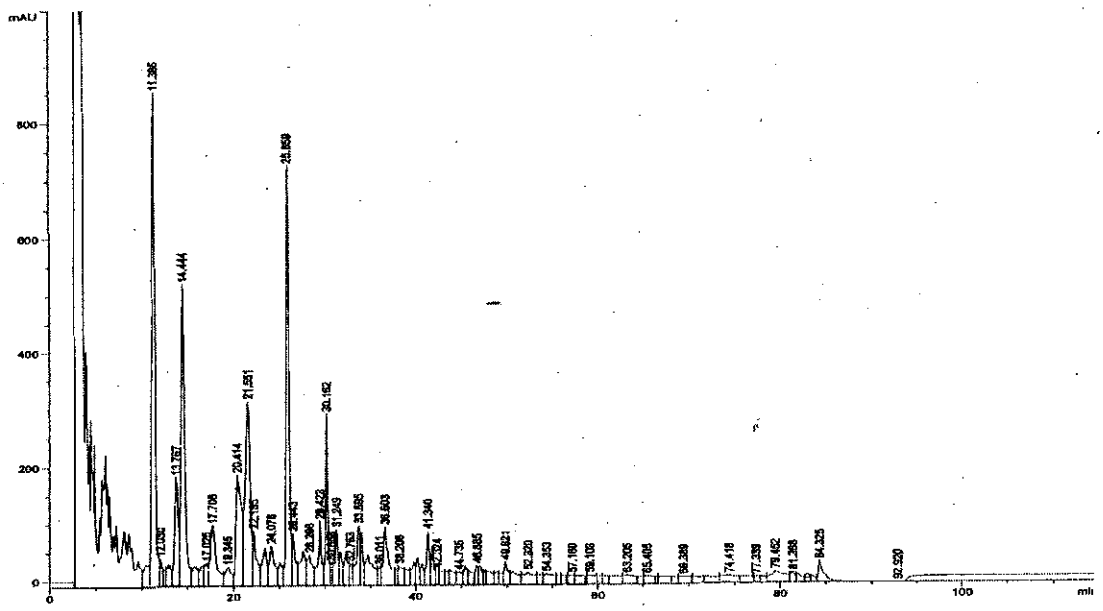

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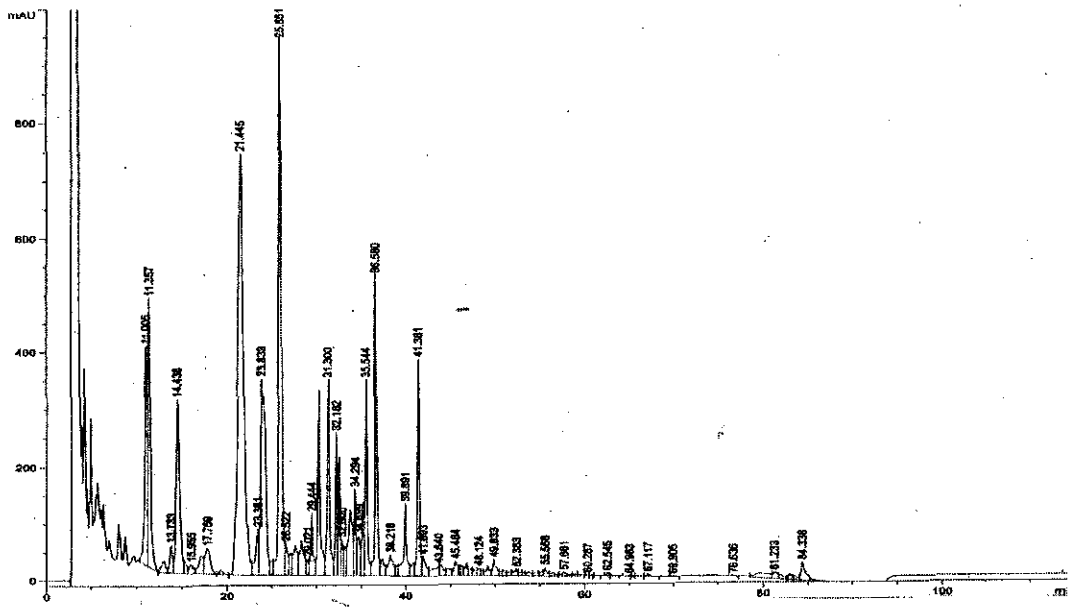


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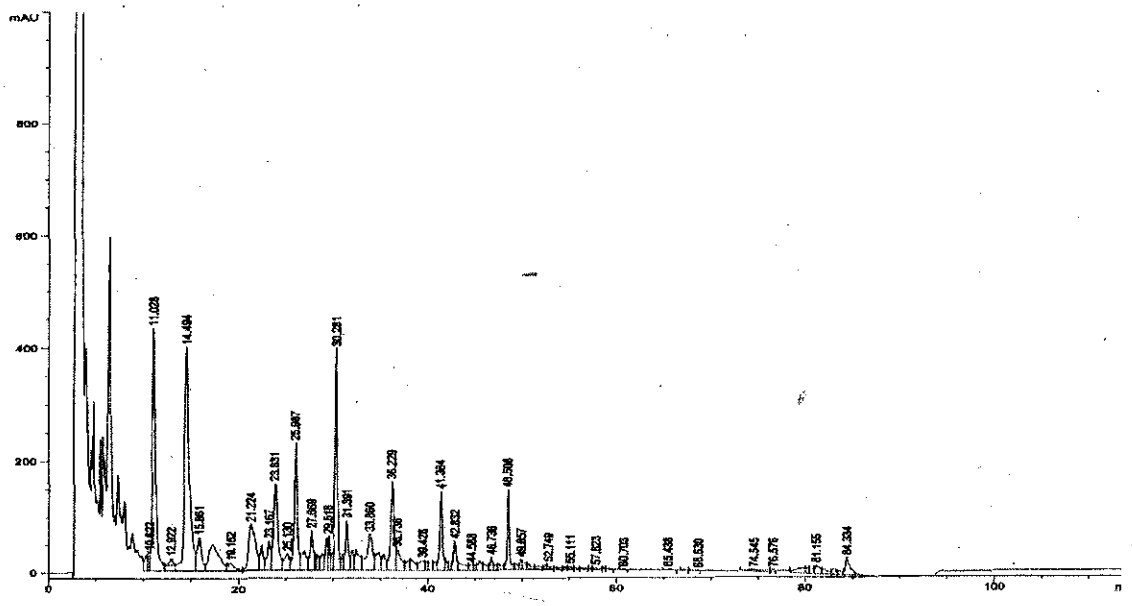

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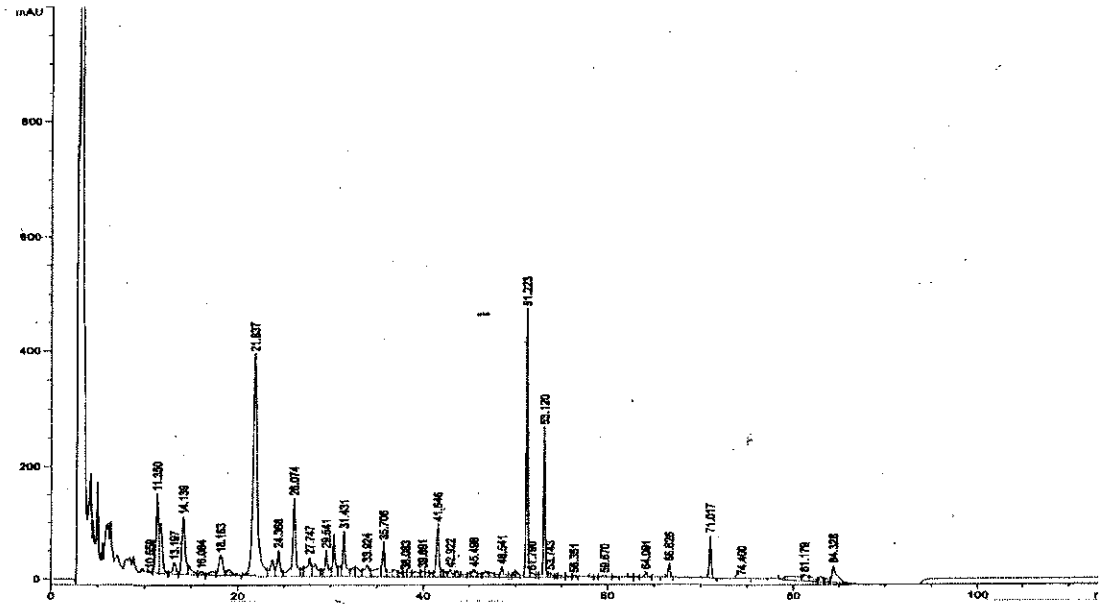


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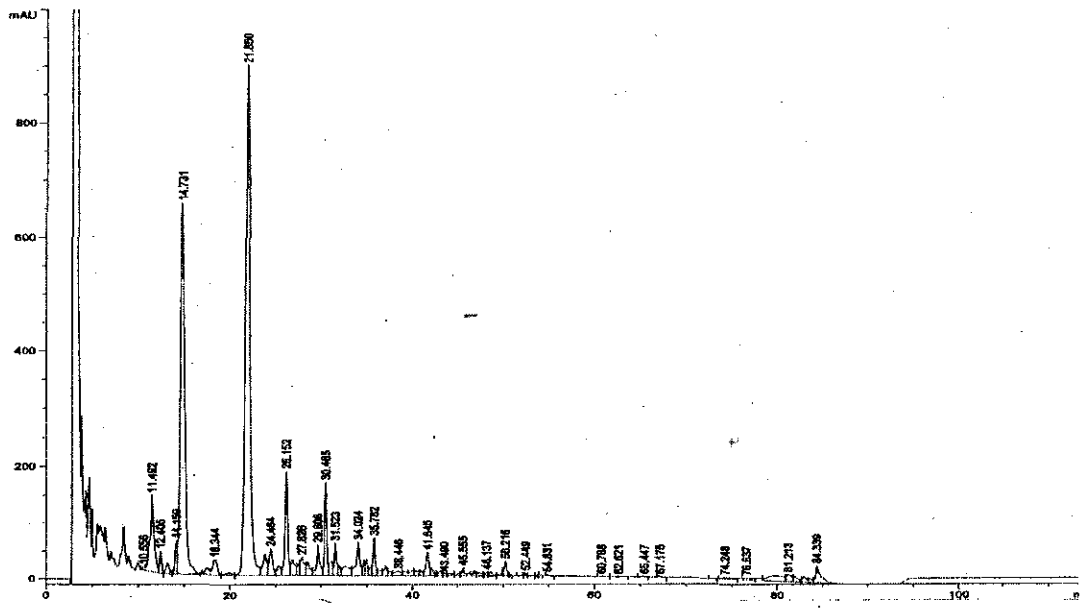

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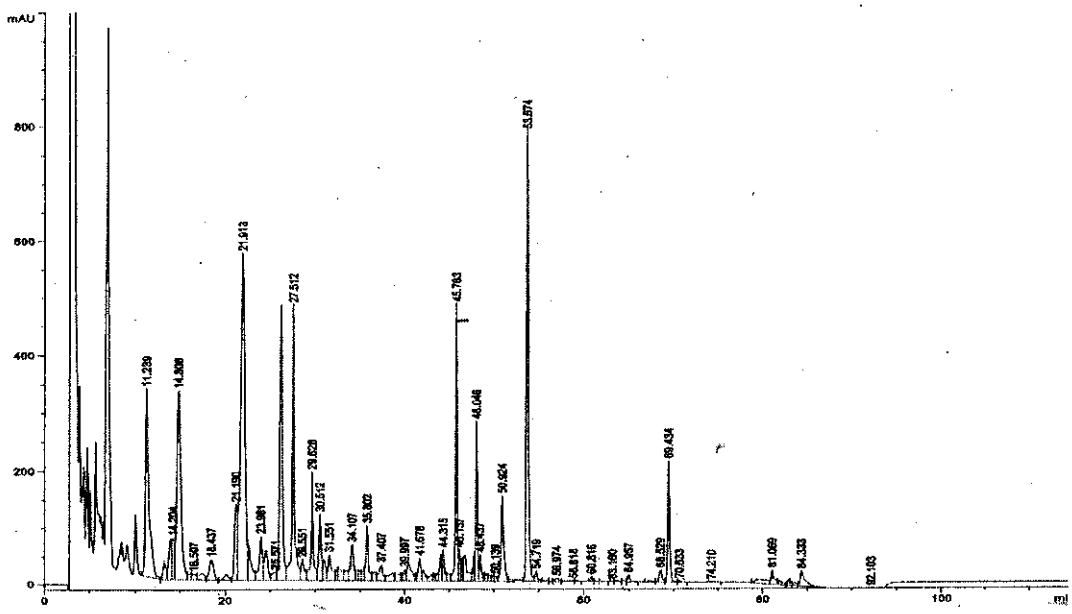


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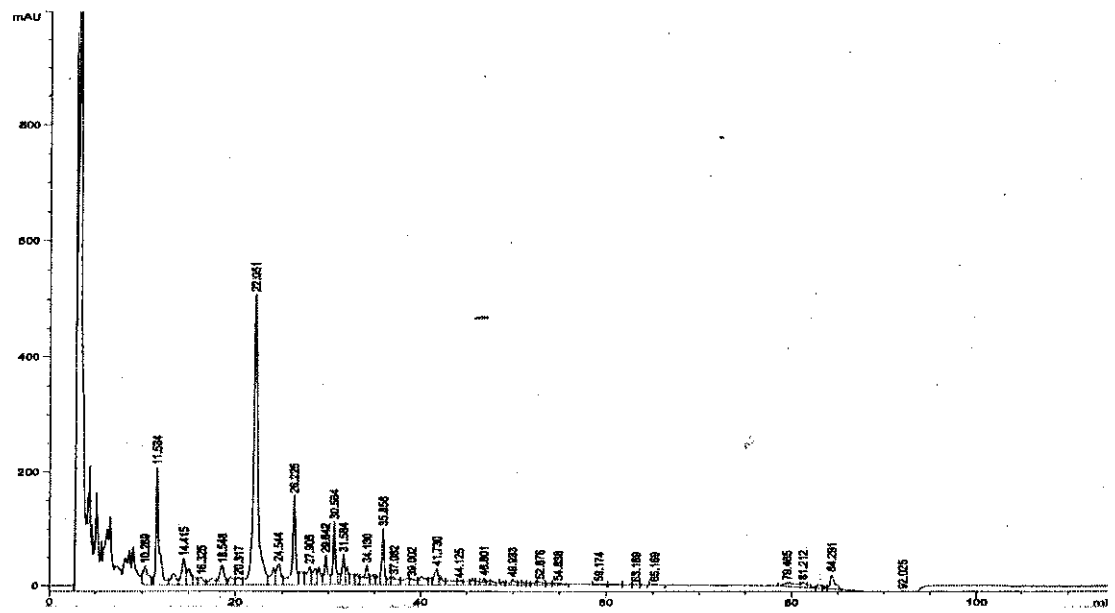

# Appendix B: Rat model

Group 1(Casein diet and vehicle (control))

Group 2 (Casein diet and cyclosporine)

Group 3 (Normal diet and vehicle (control))

Group 4 (Normal diet and cyclosporine)

## Casein diet and vehicle (control)

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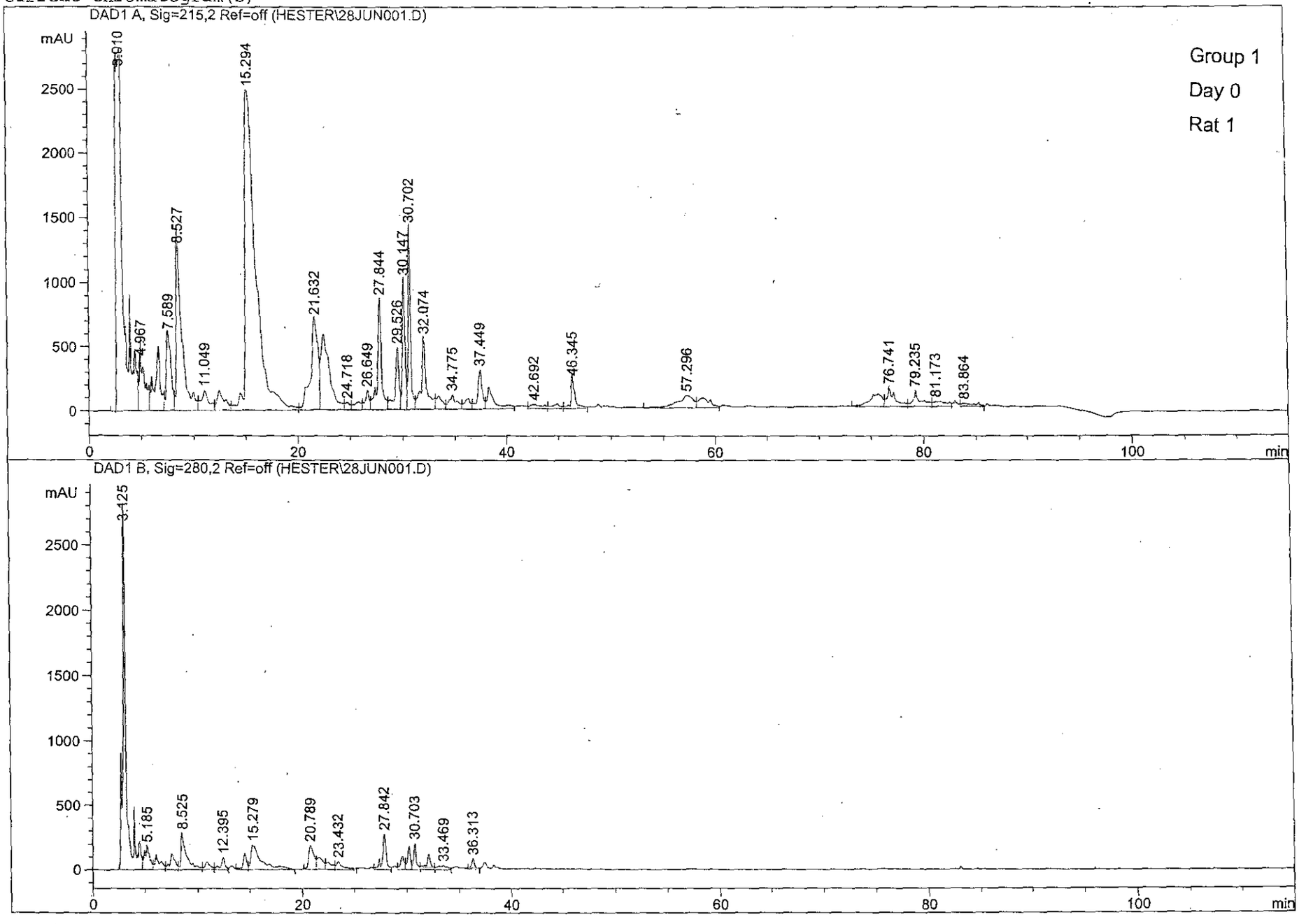

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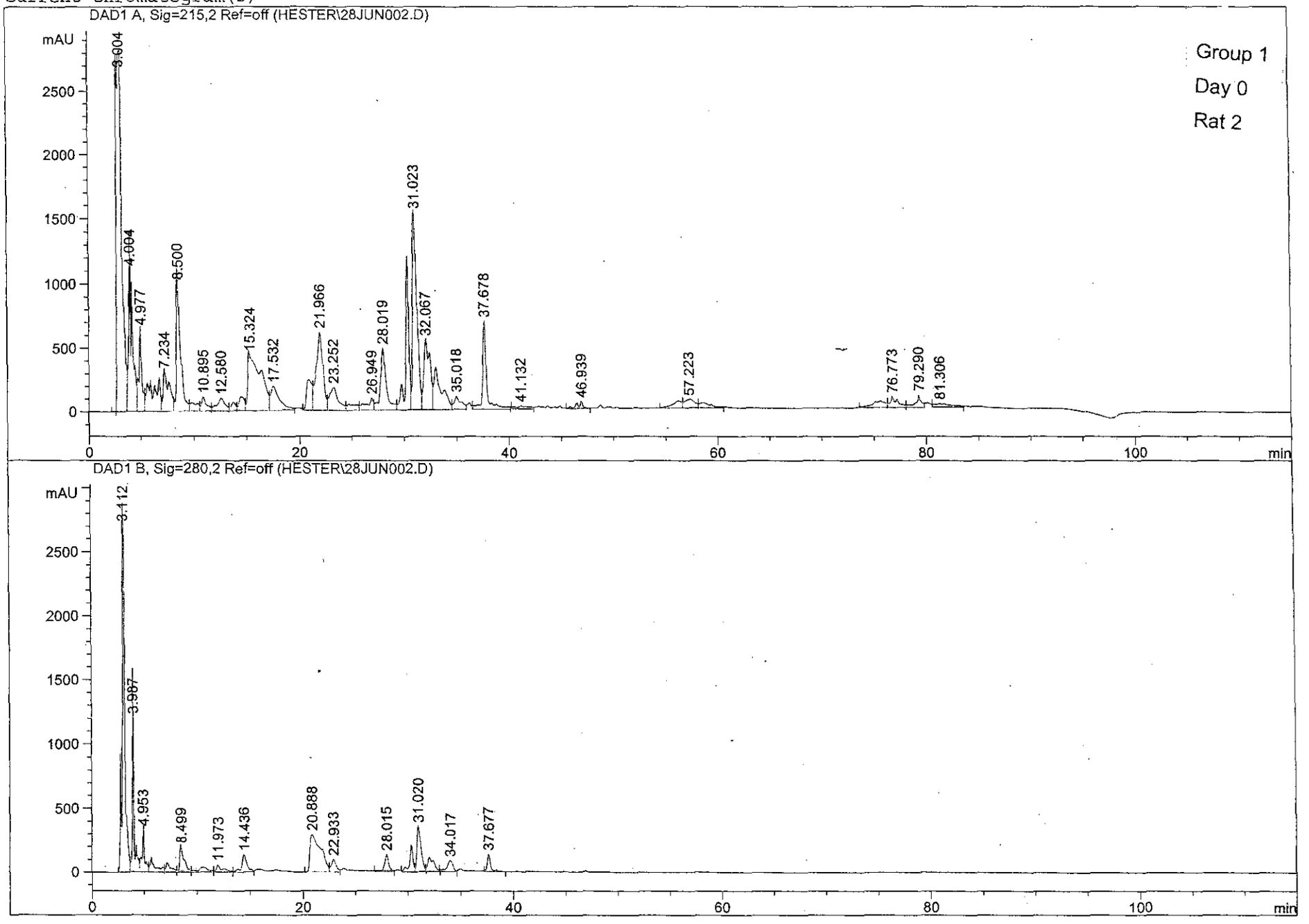

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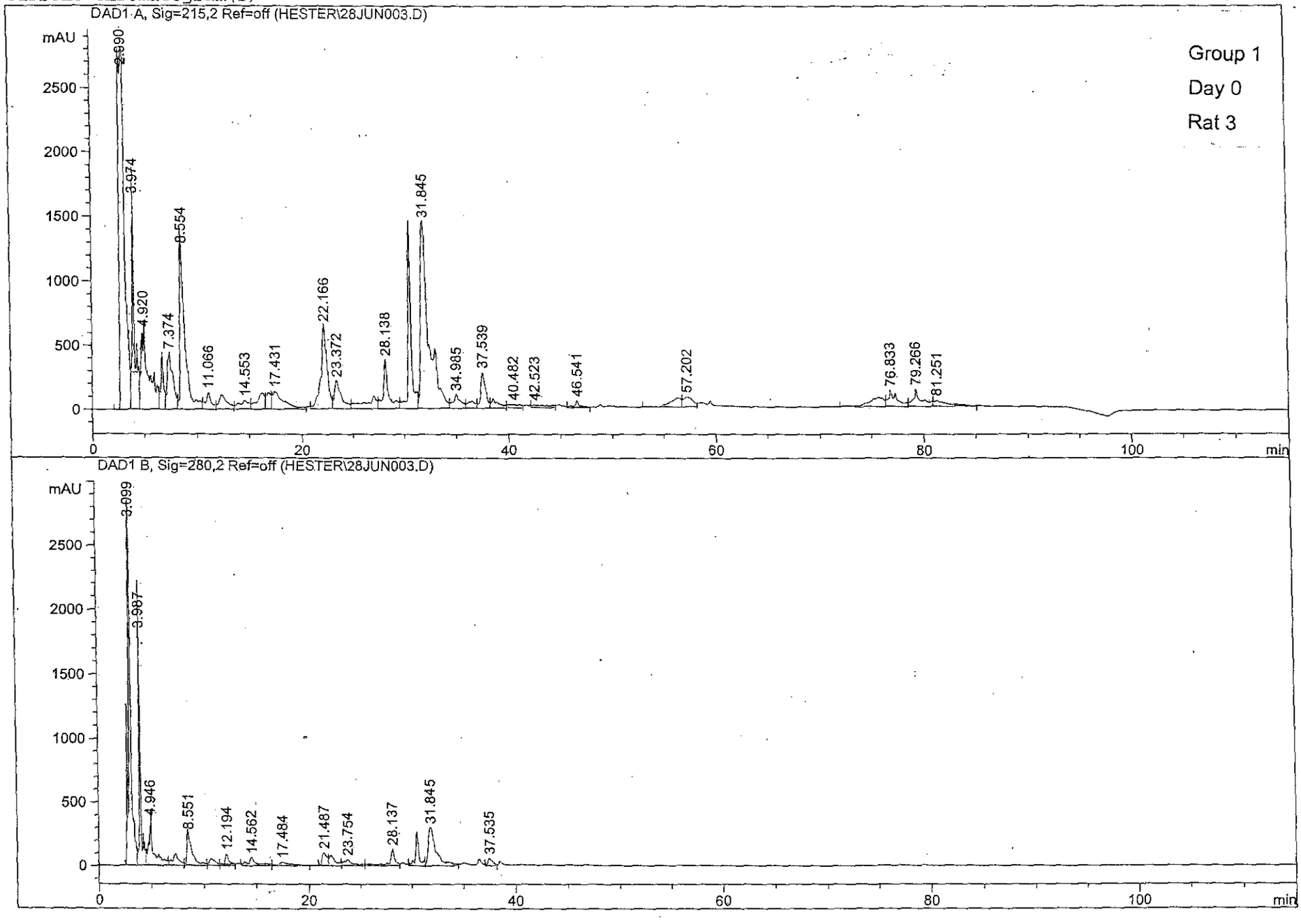

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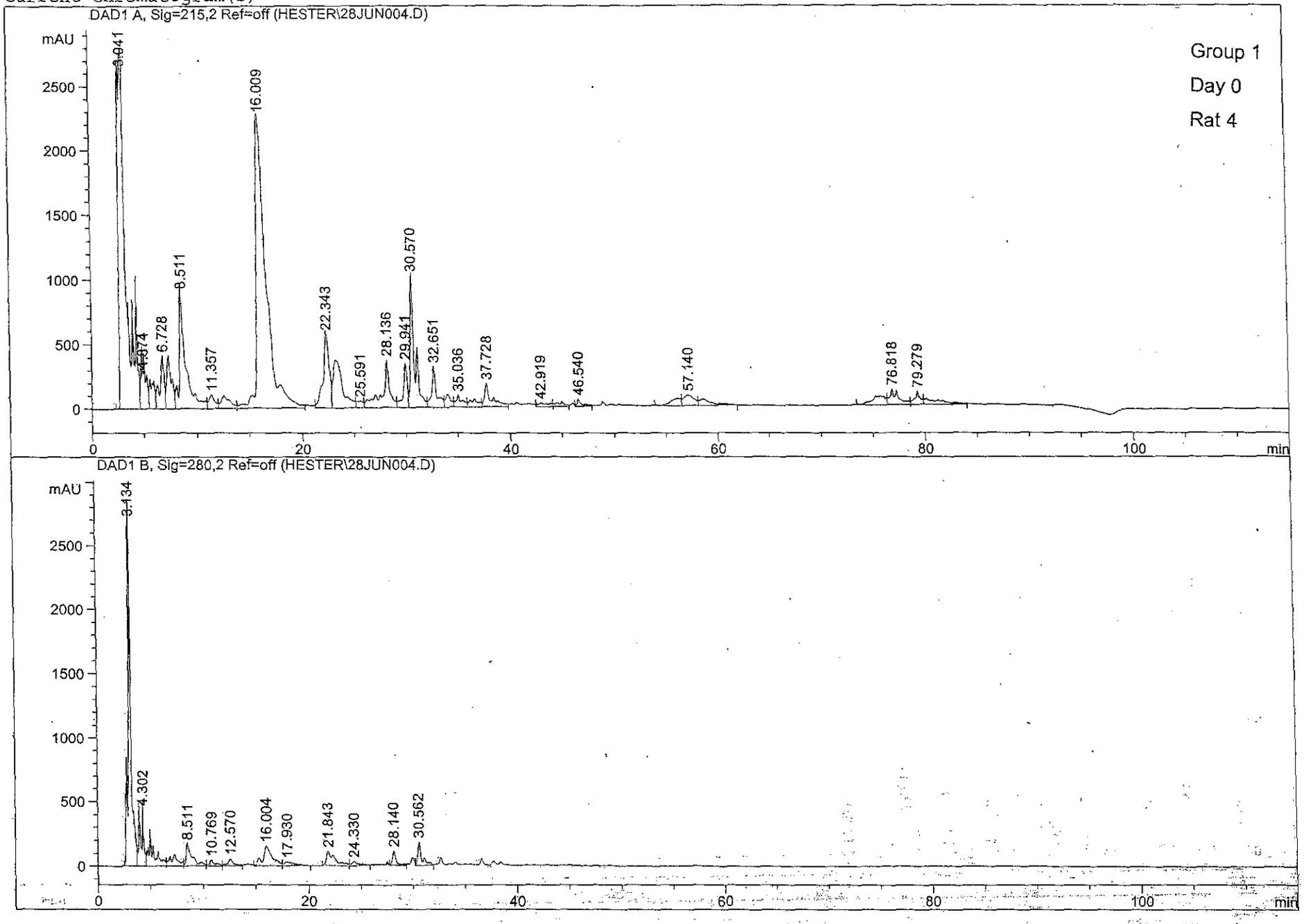

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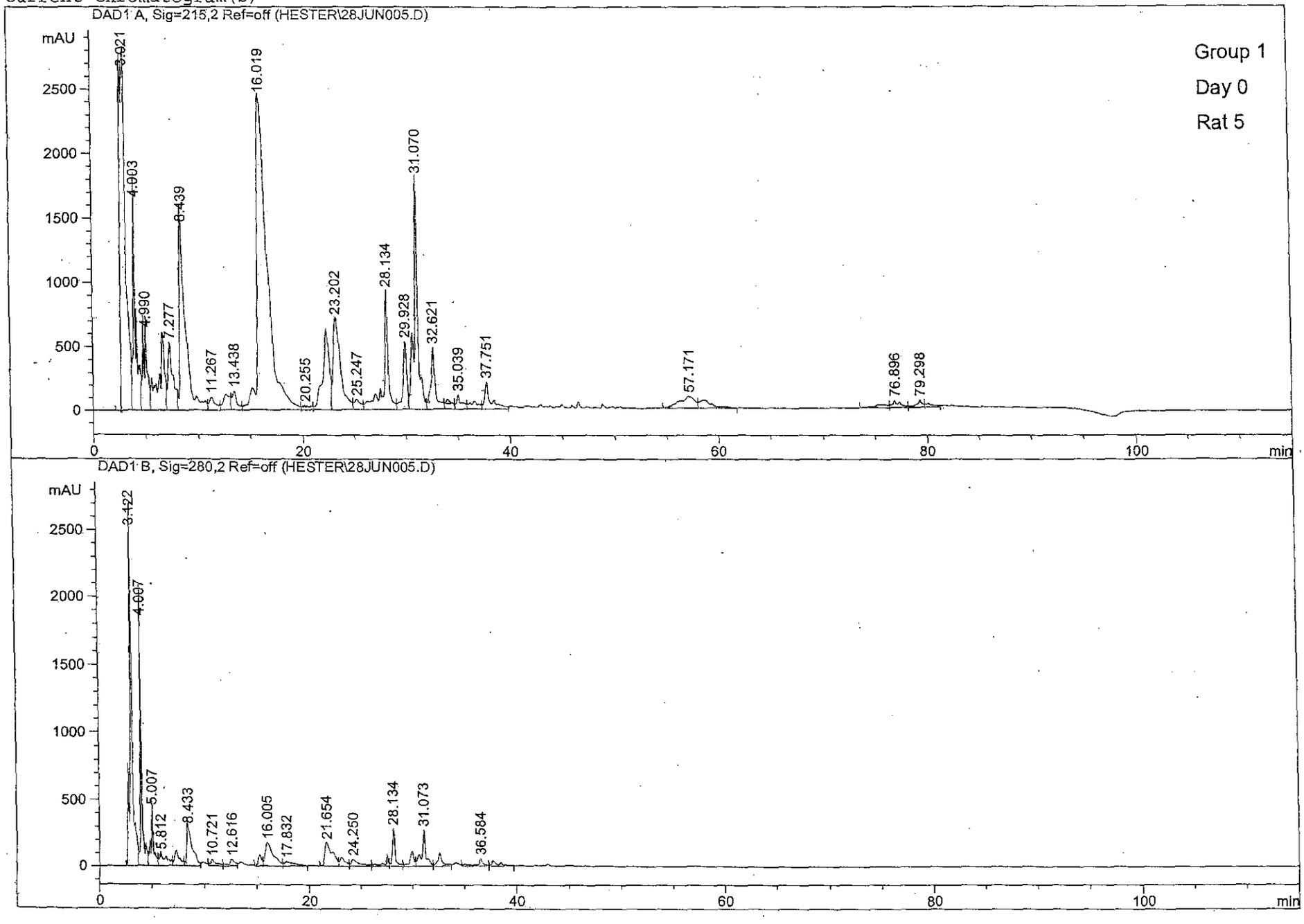

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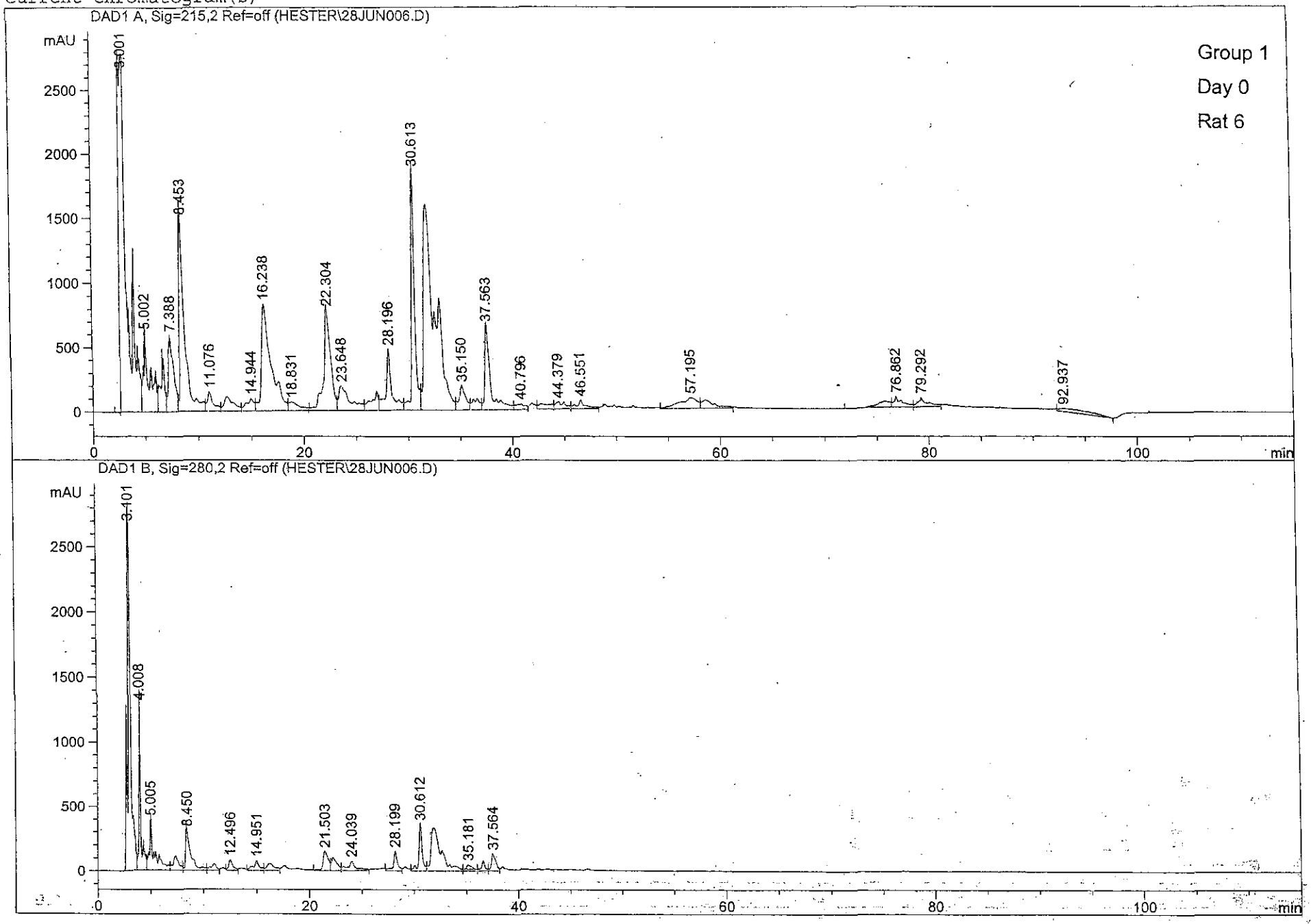

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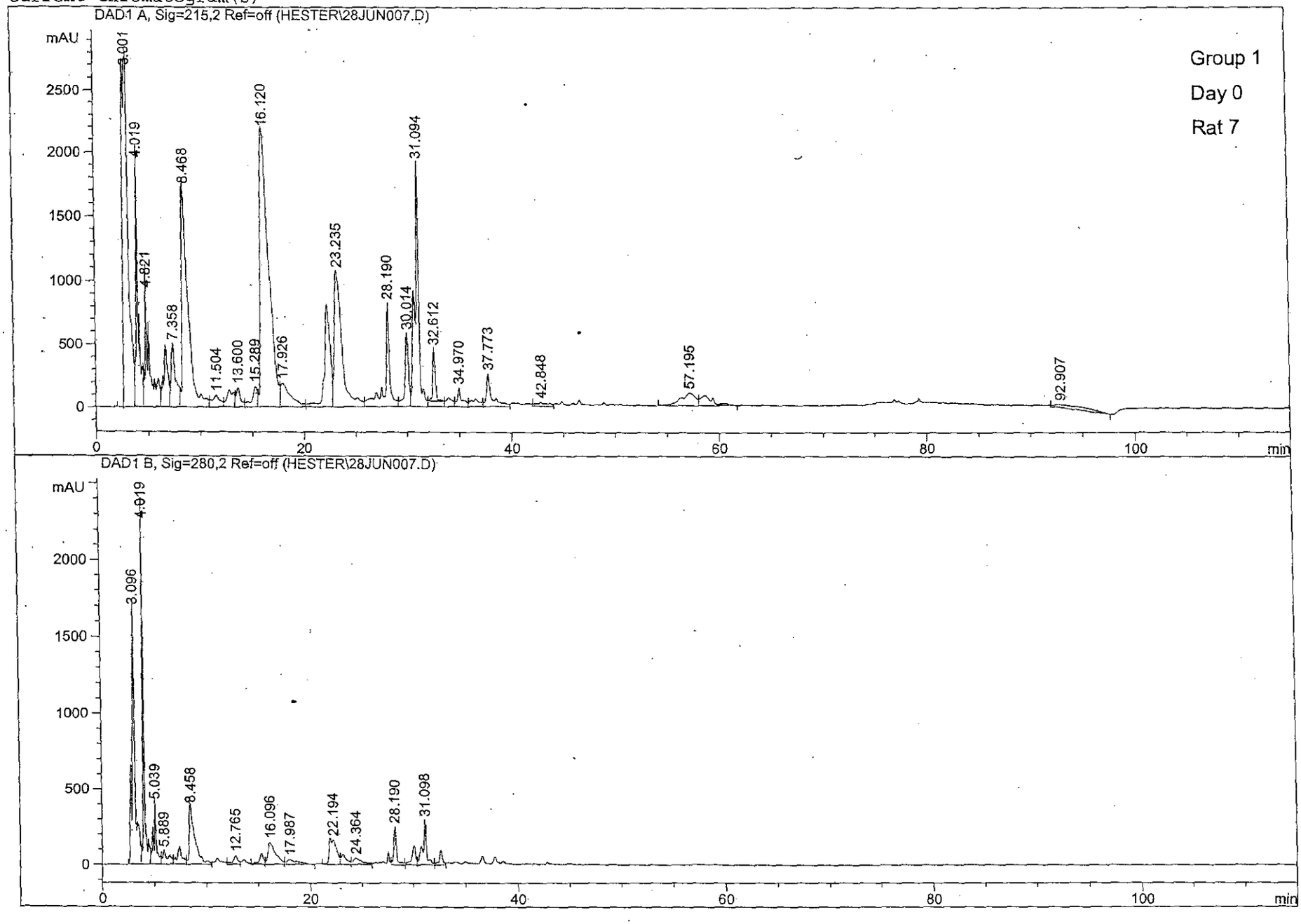

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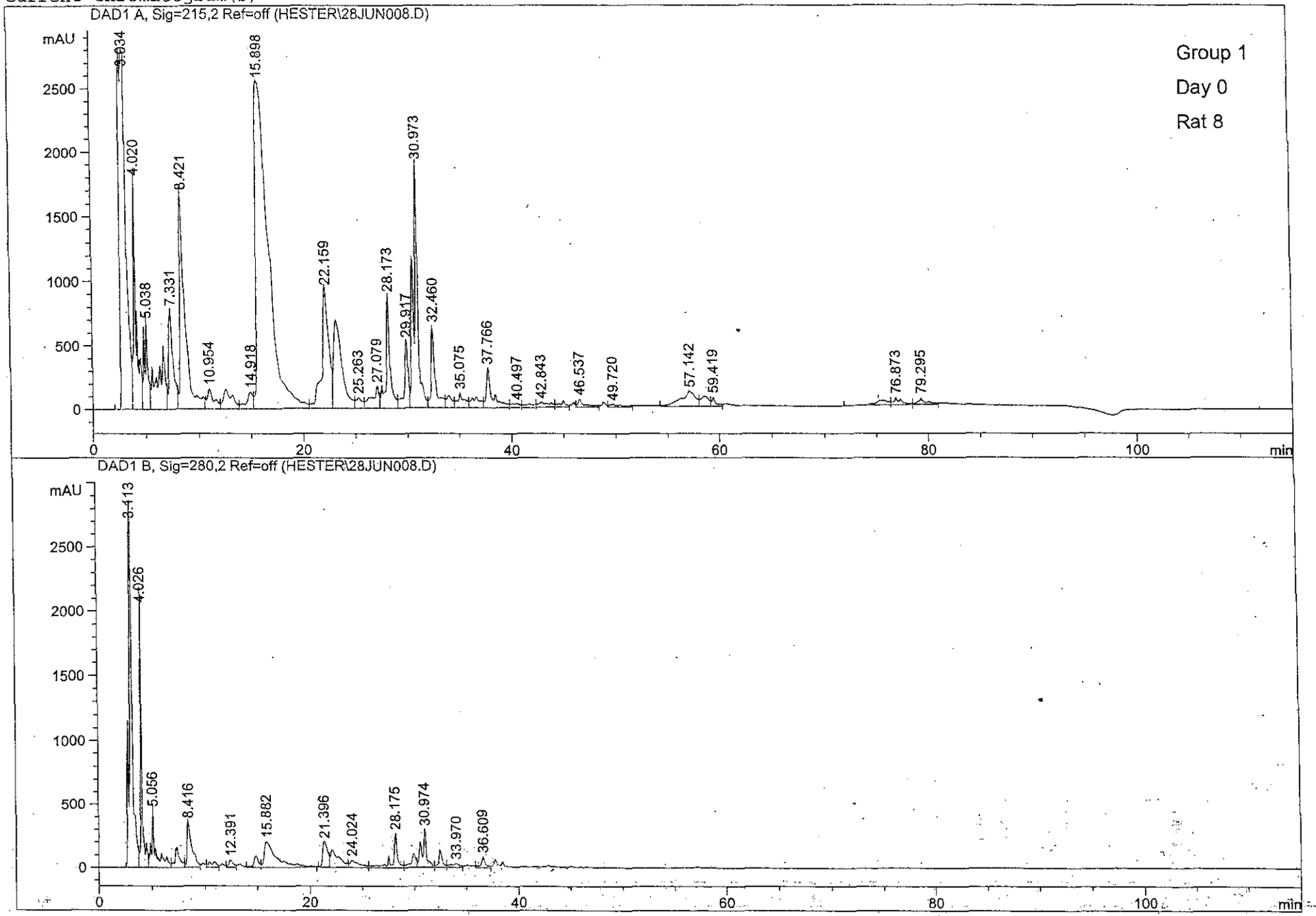

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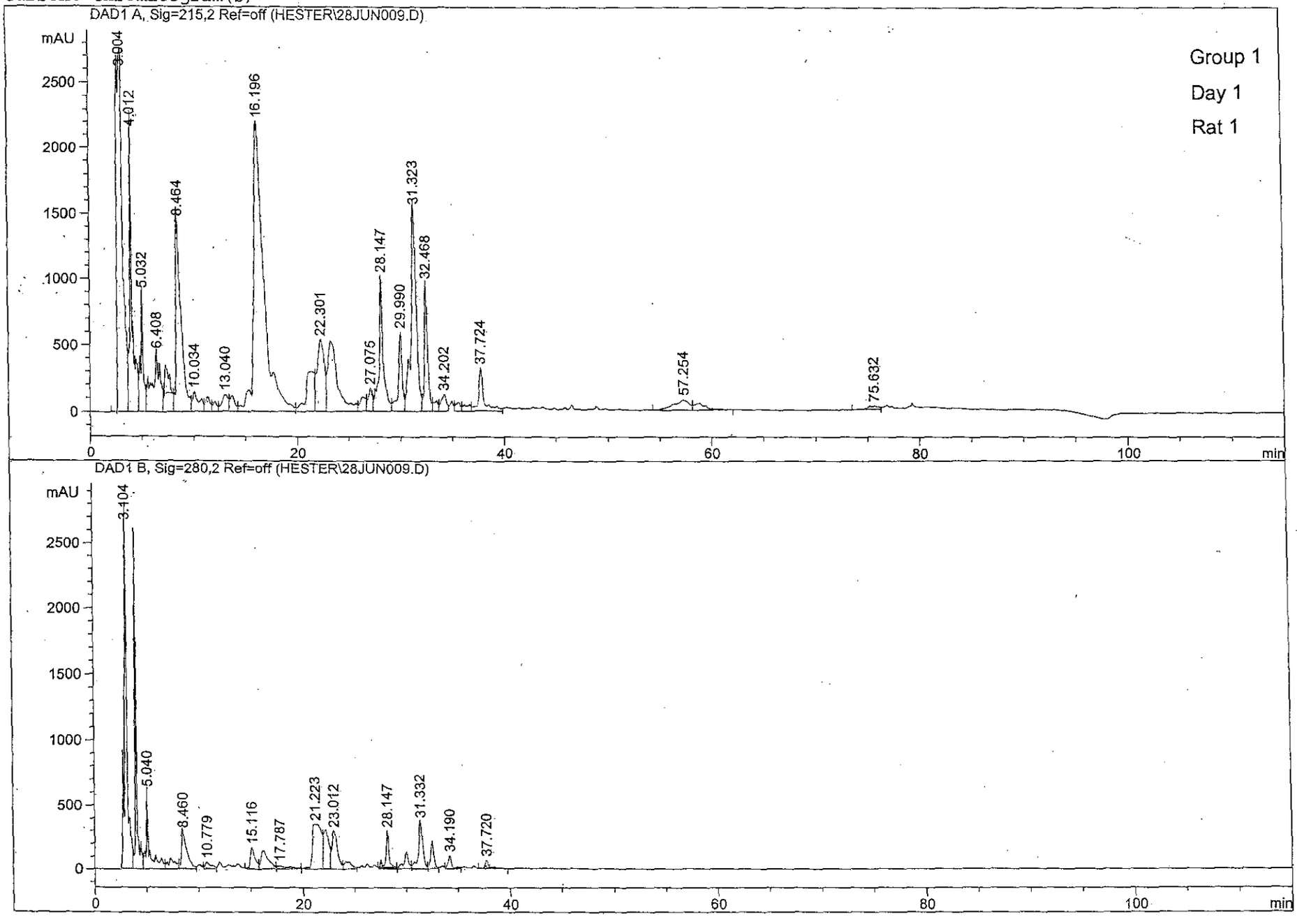

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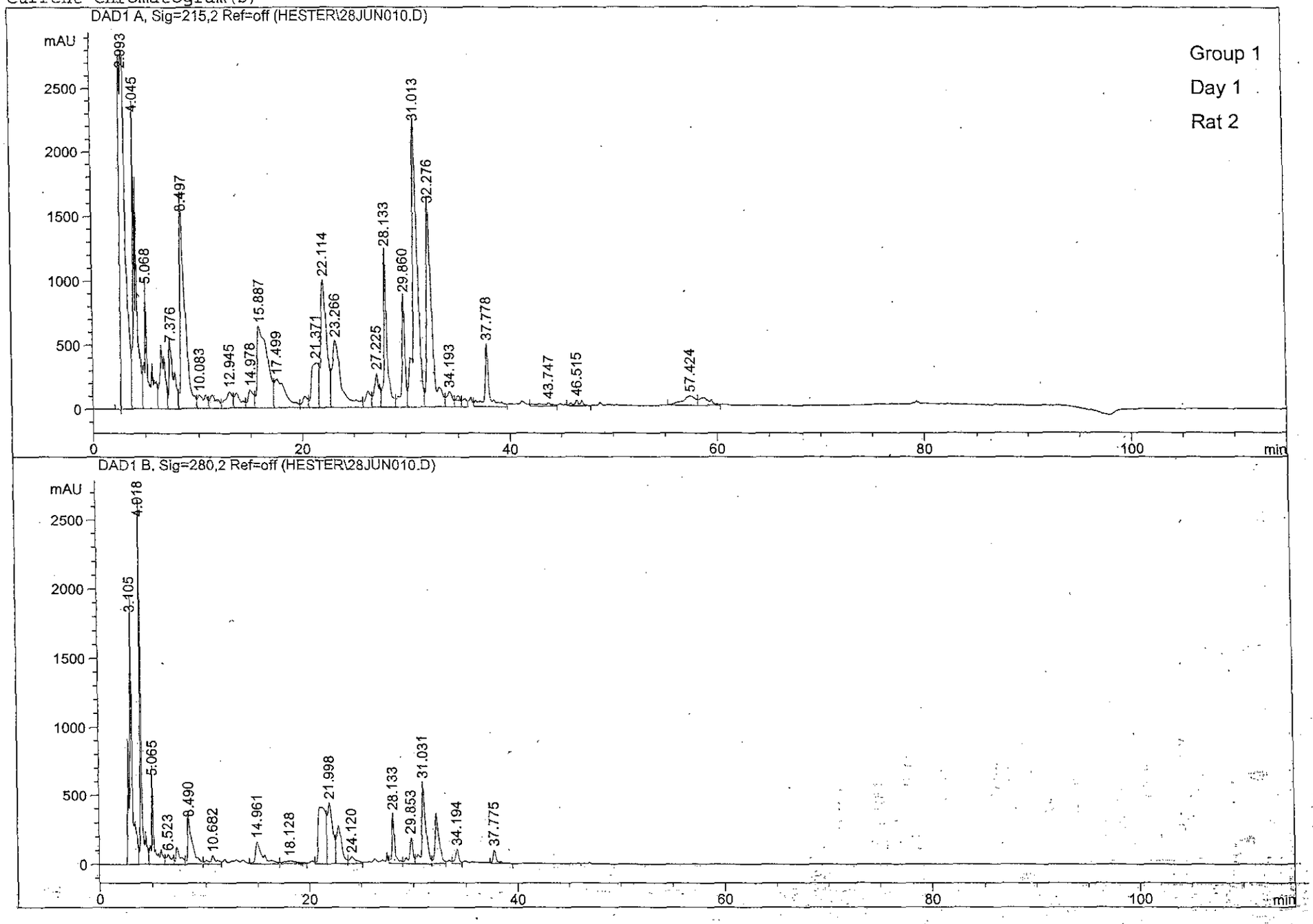

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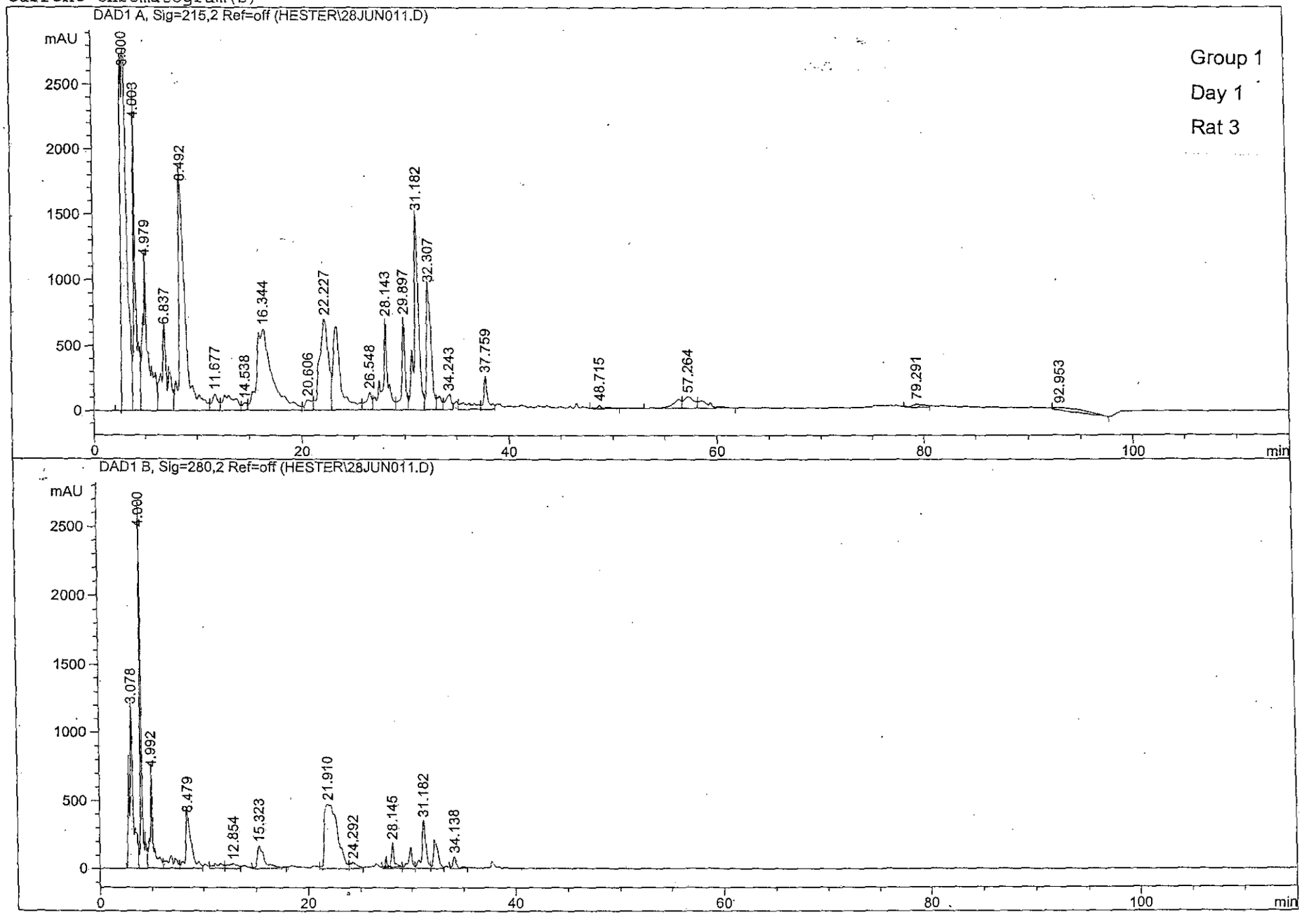

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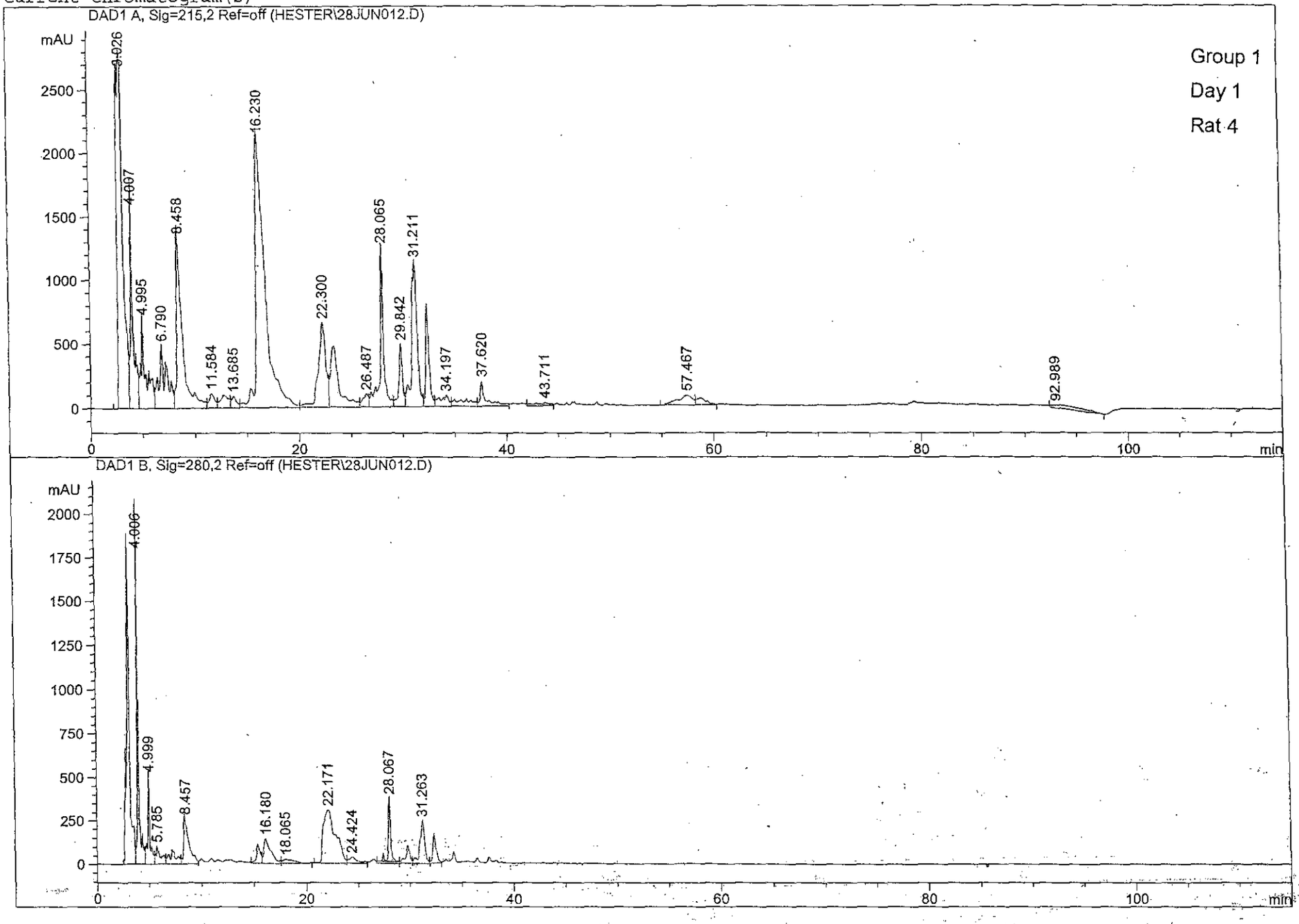

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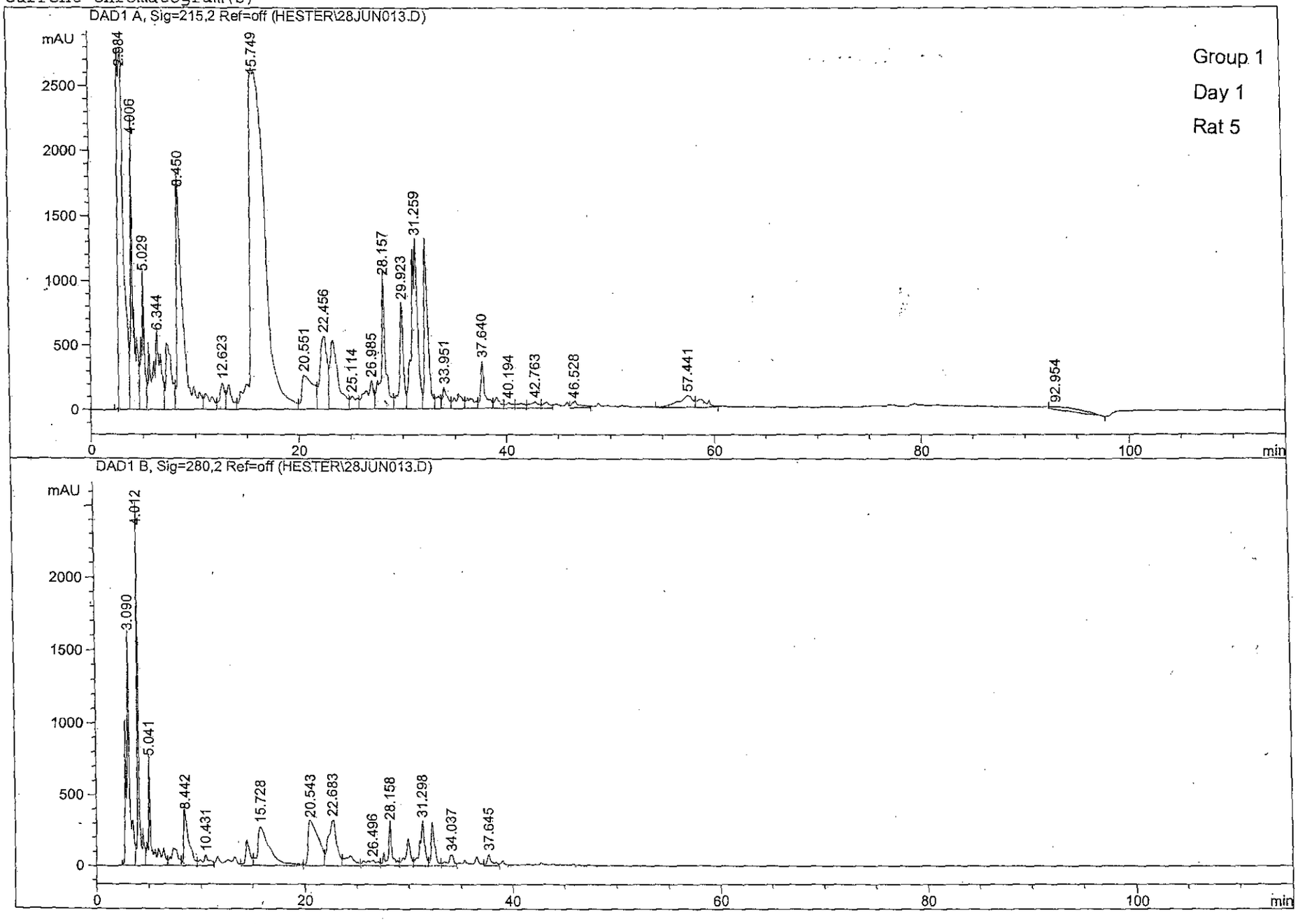

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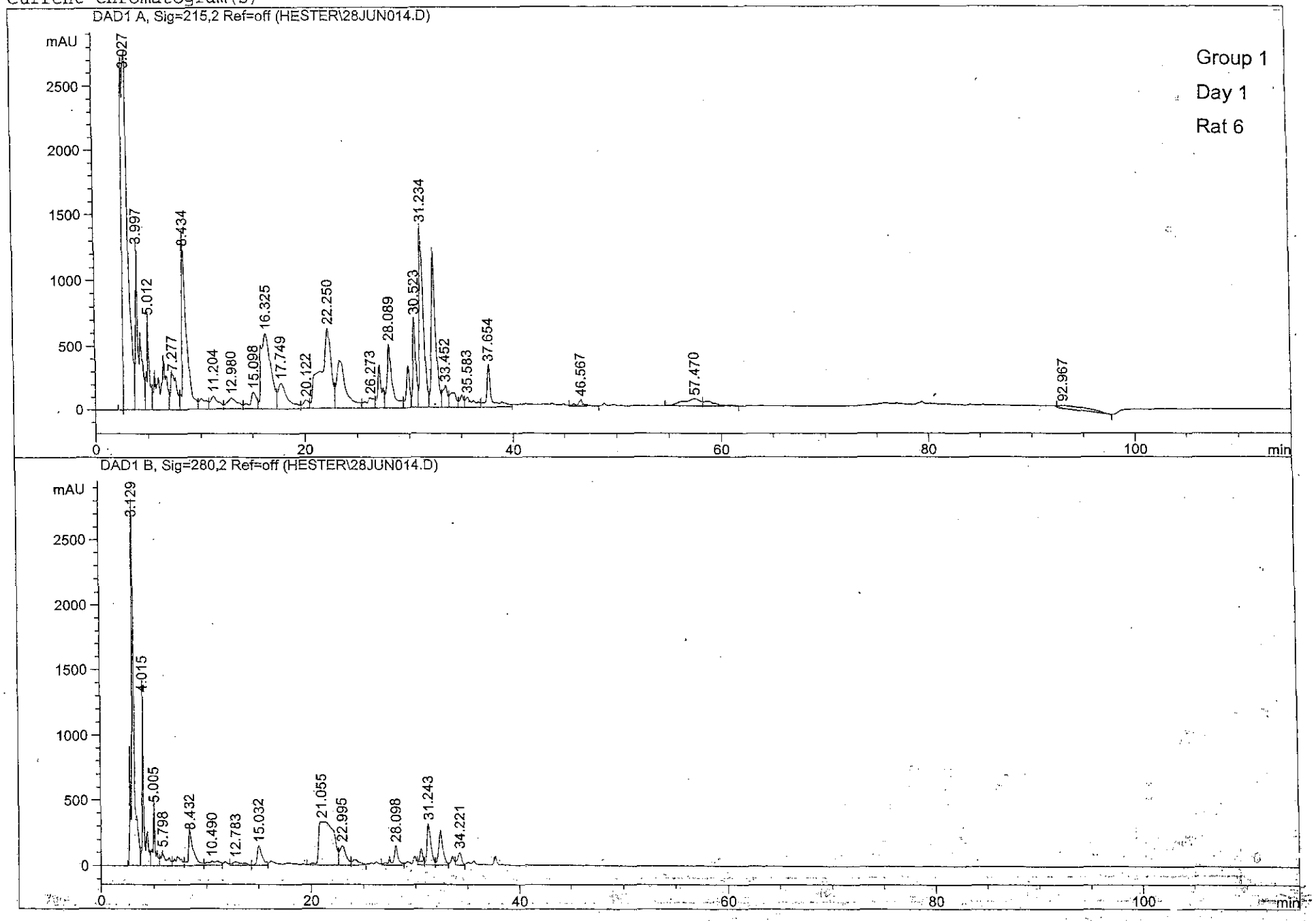

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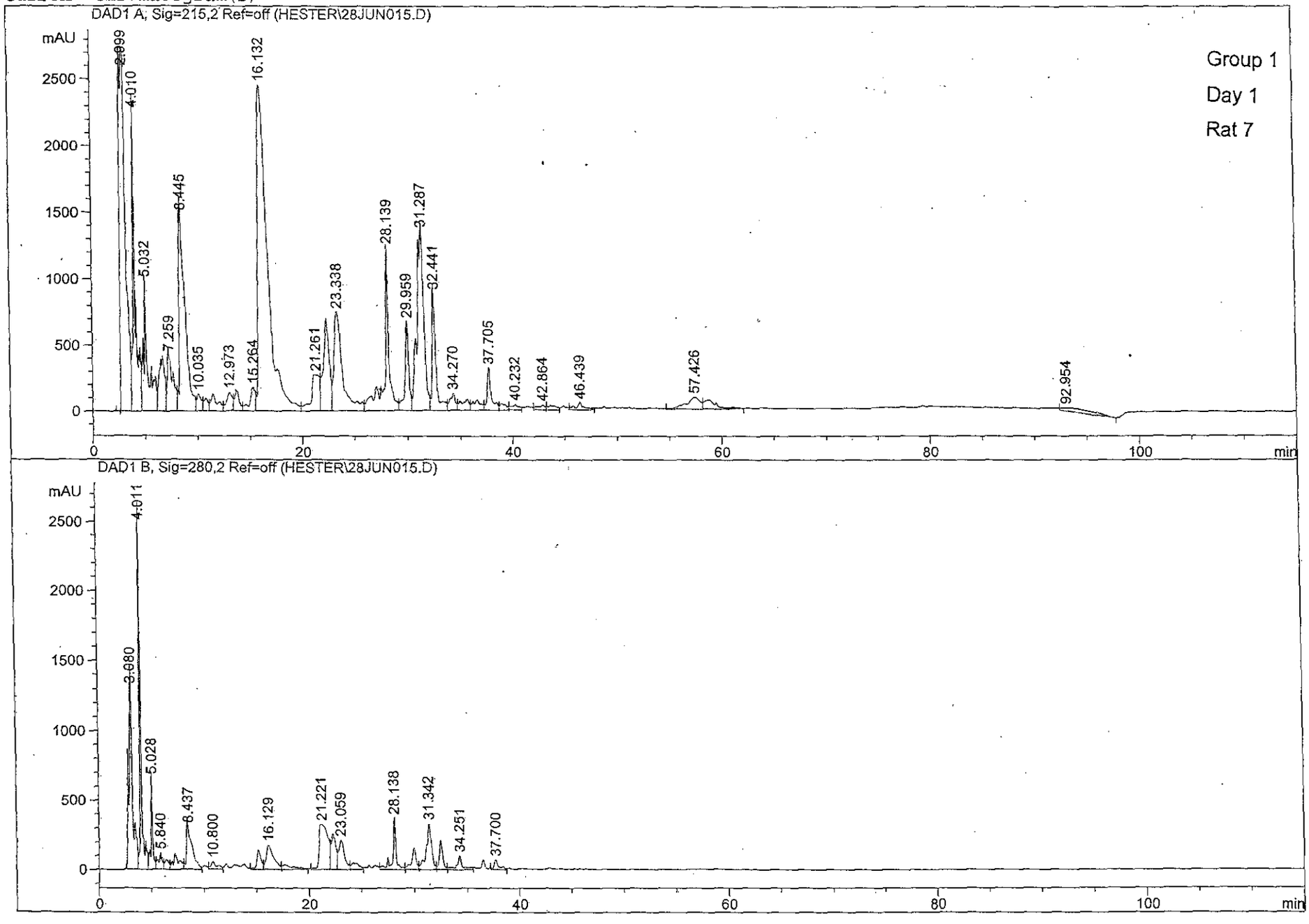

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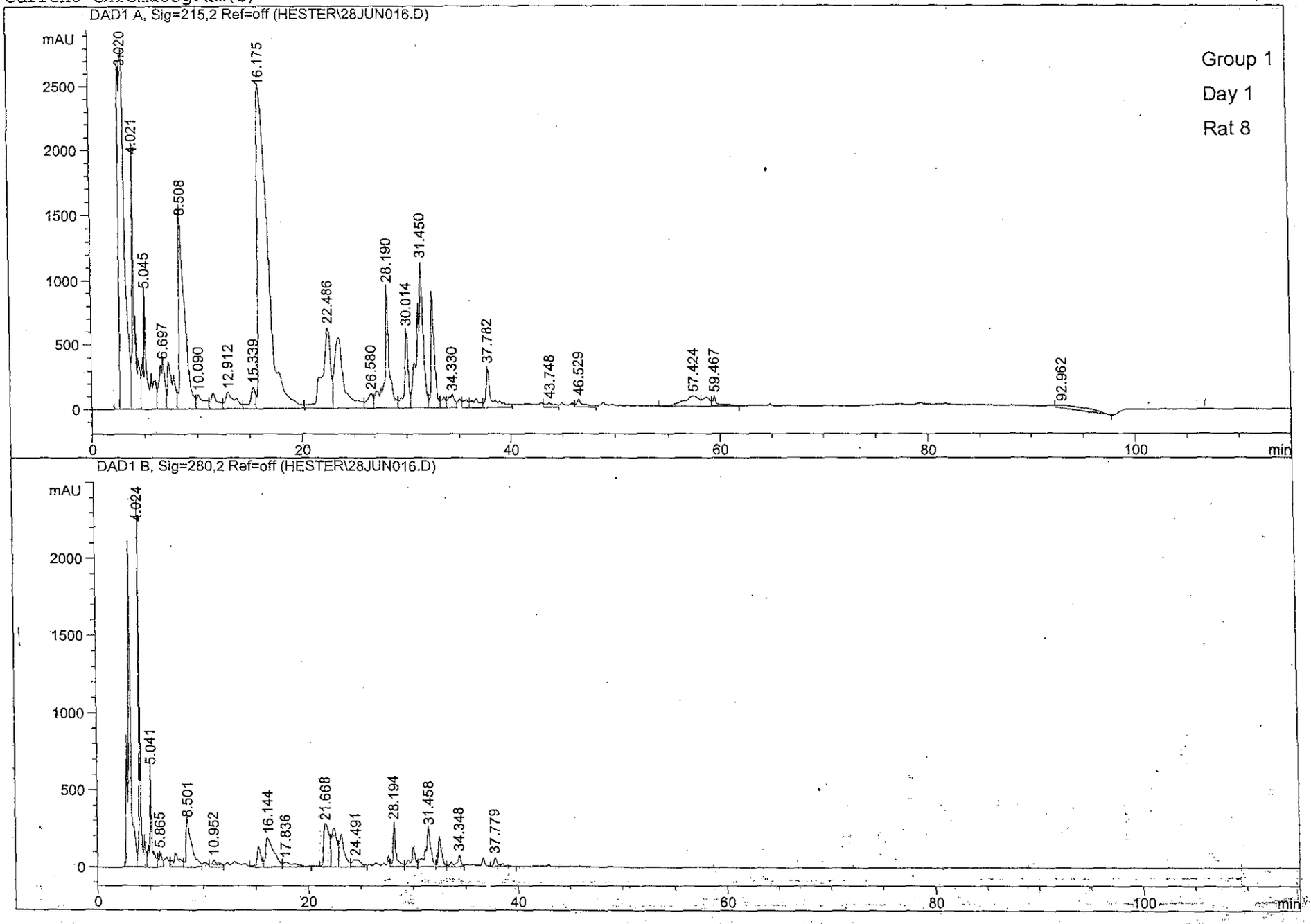

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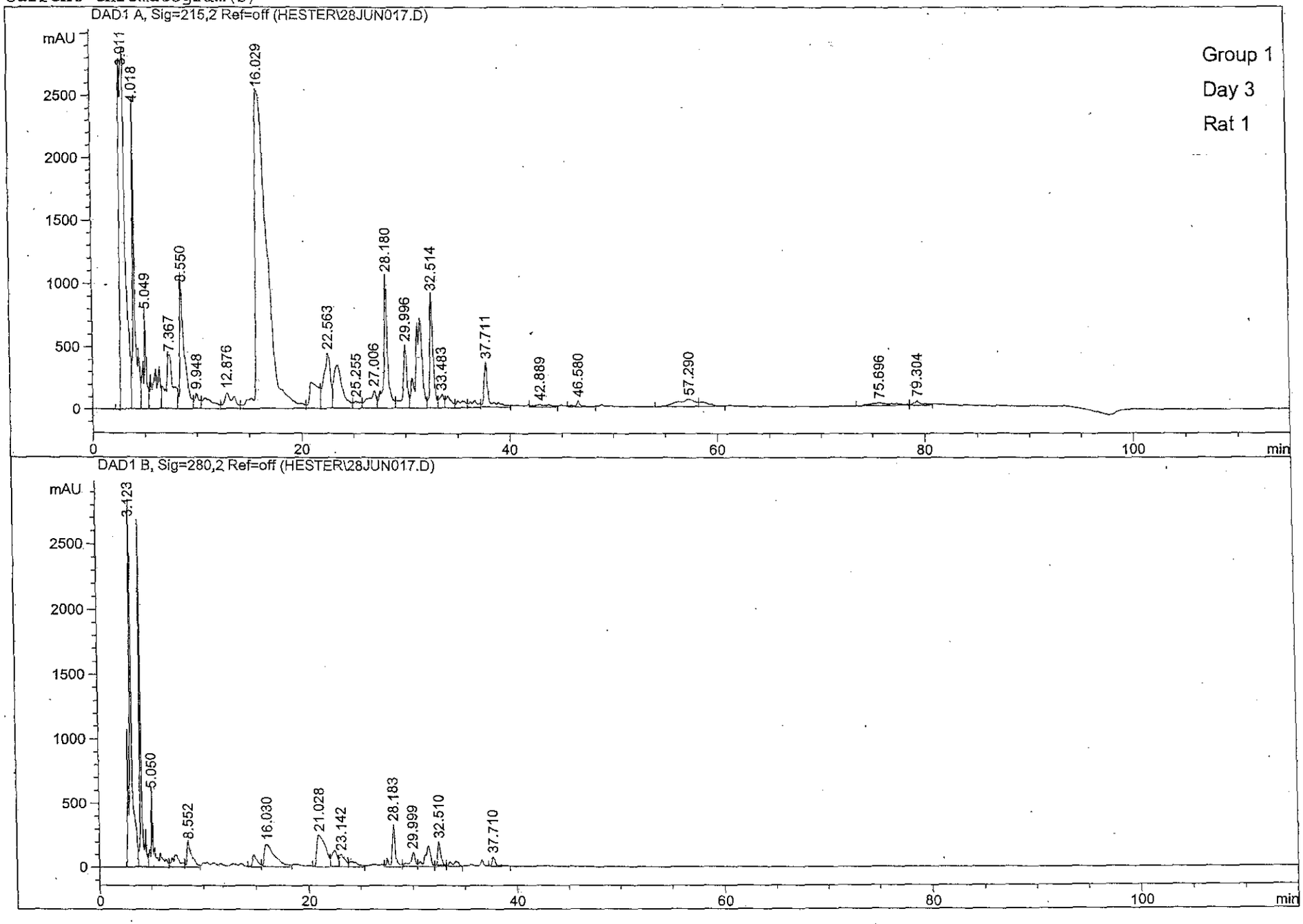

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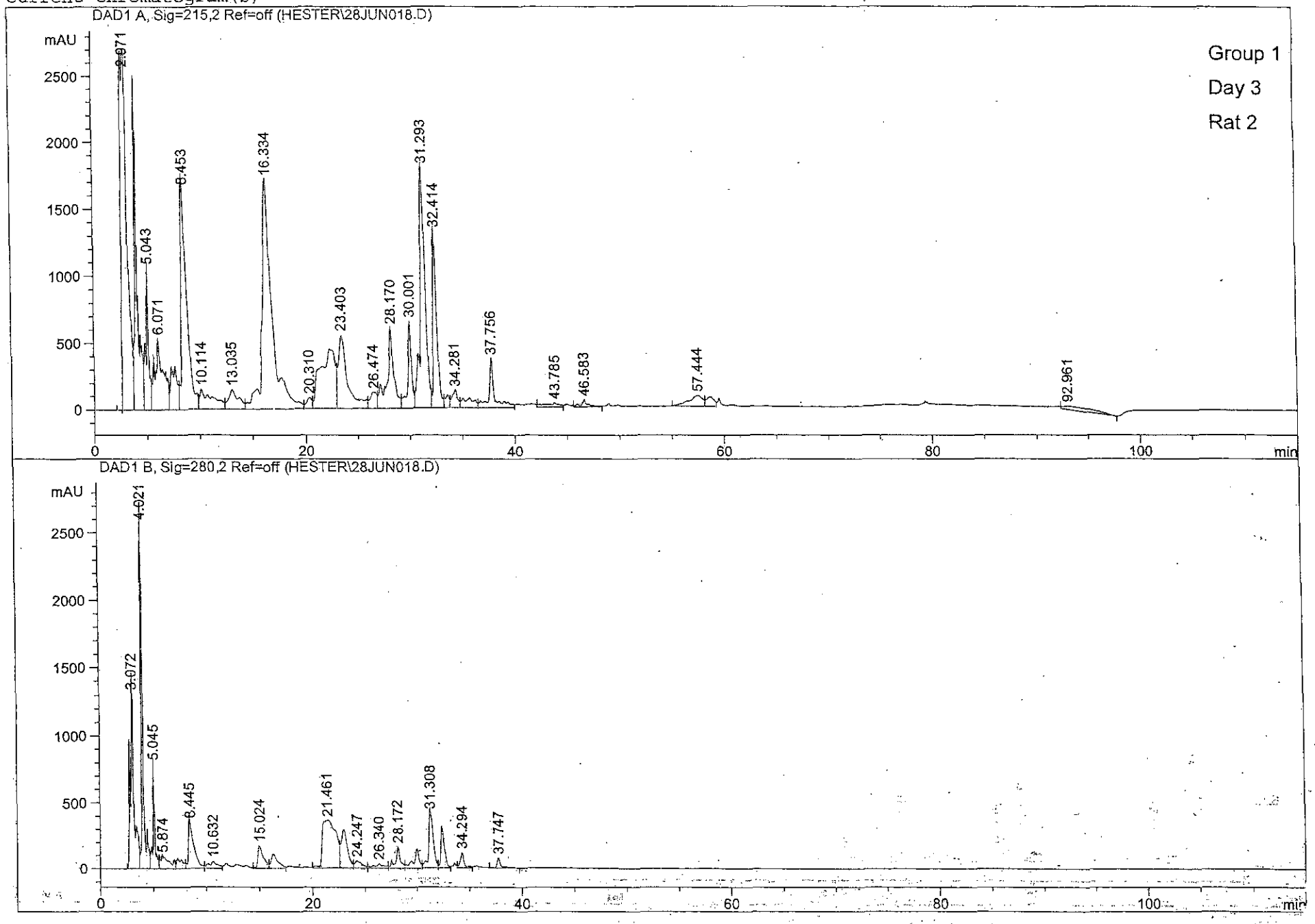

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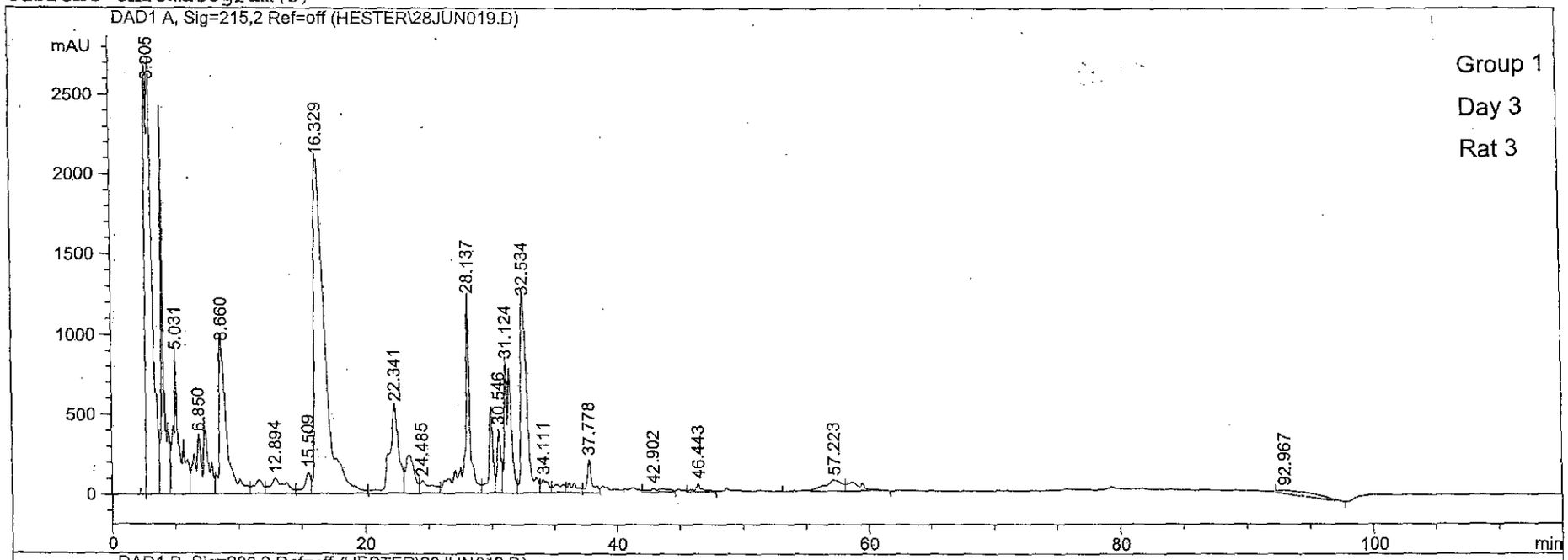


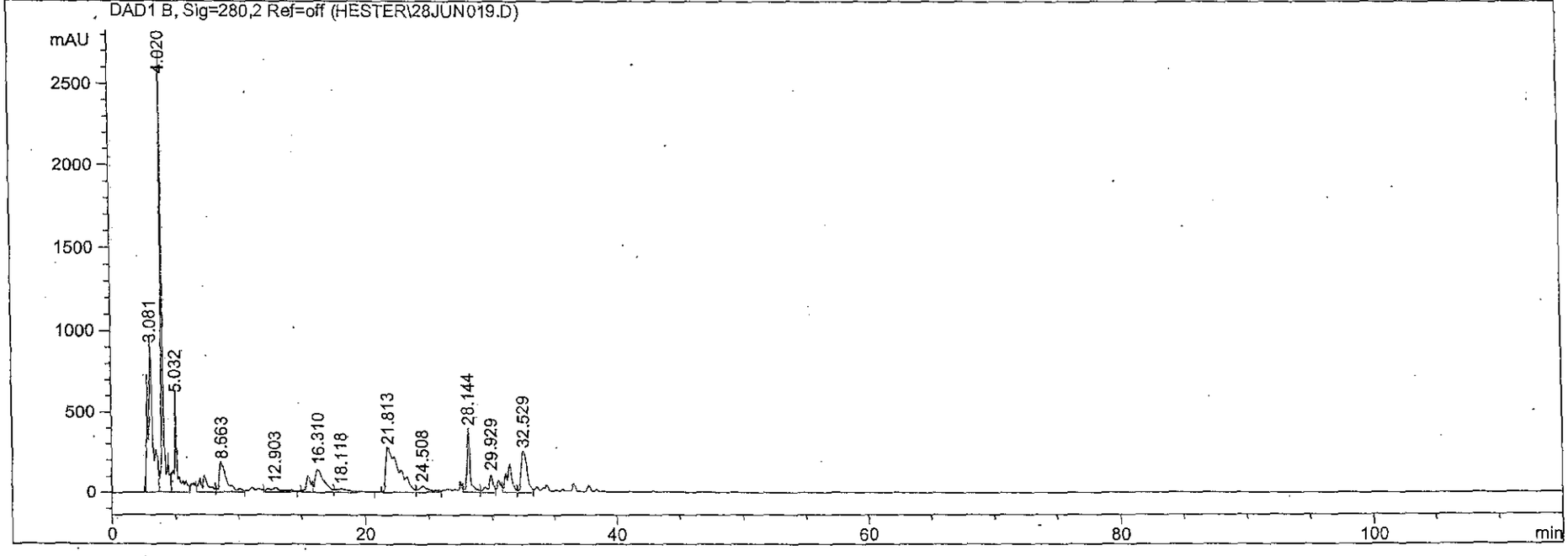

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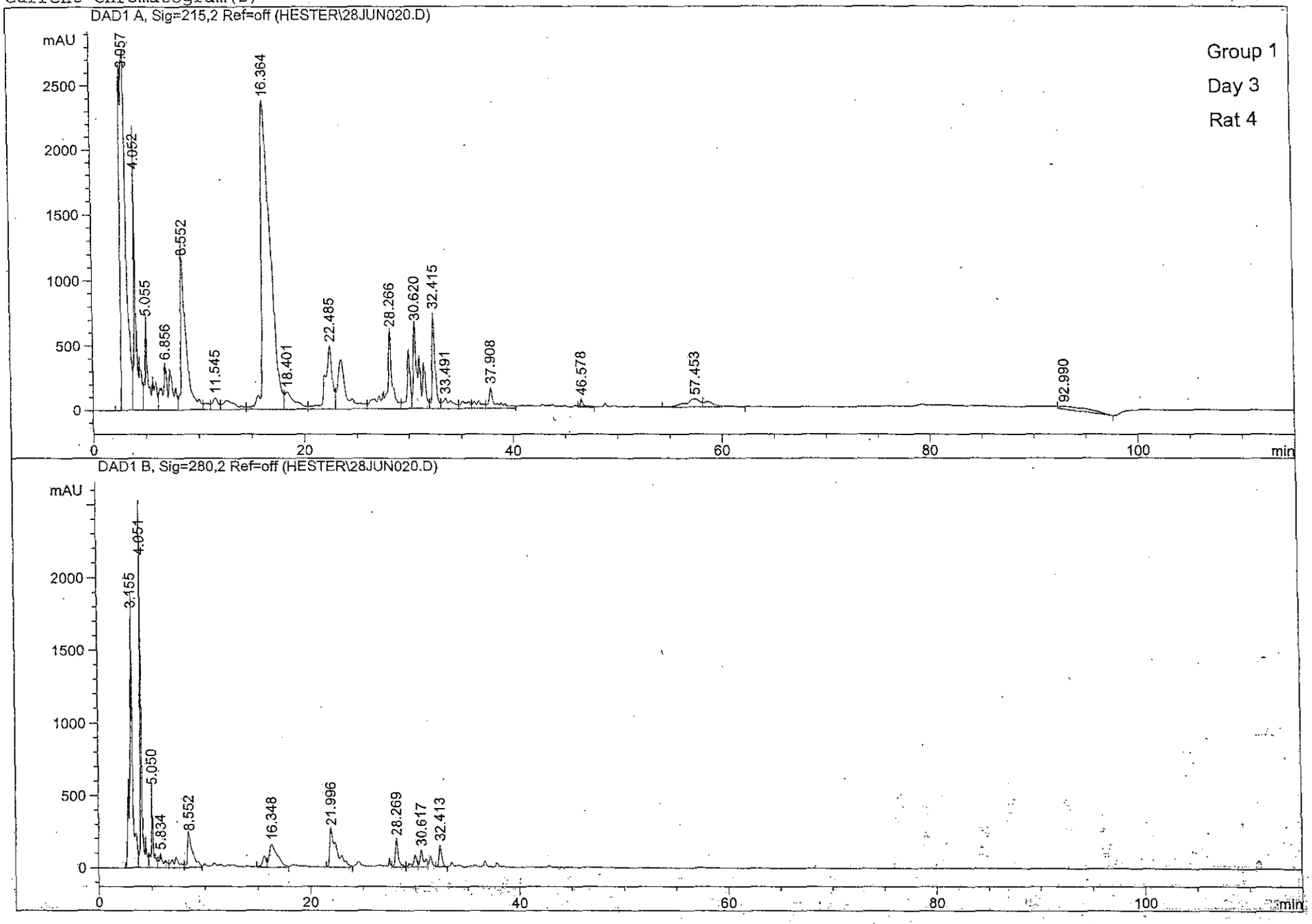

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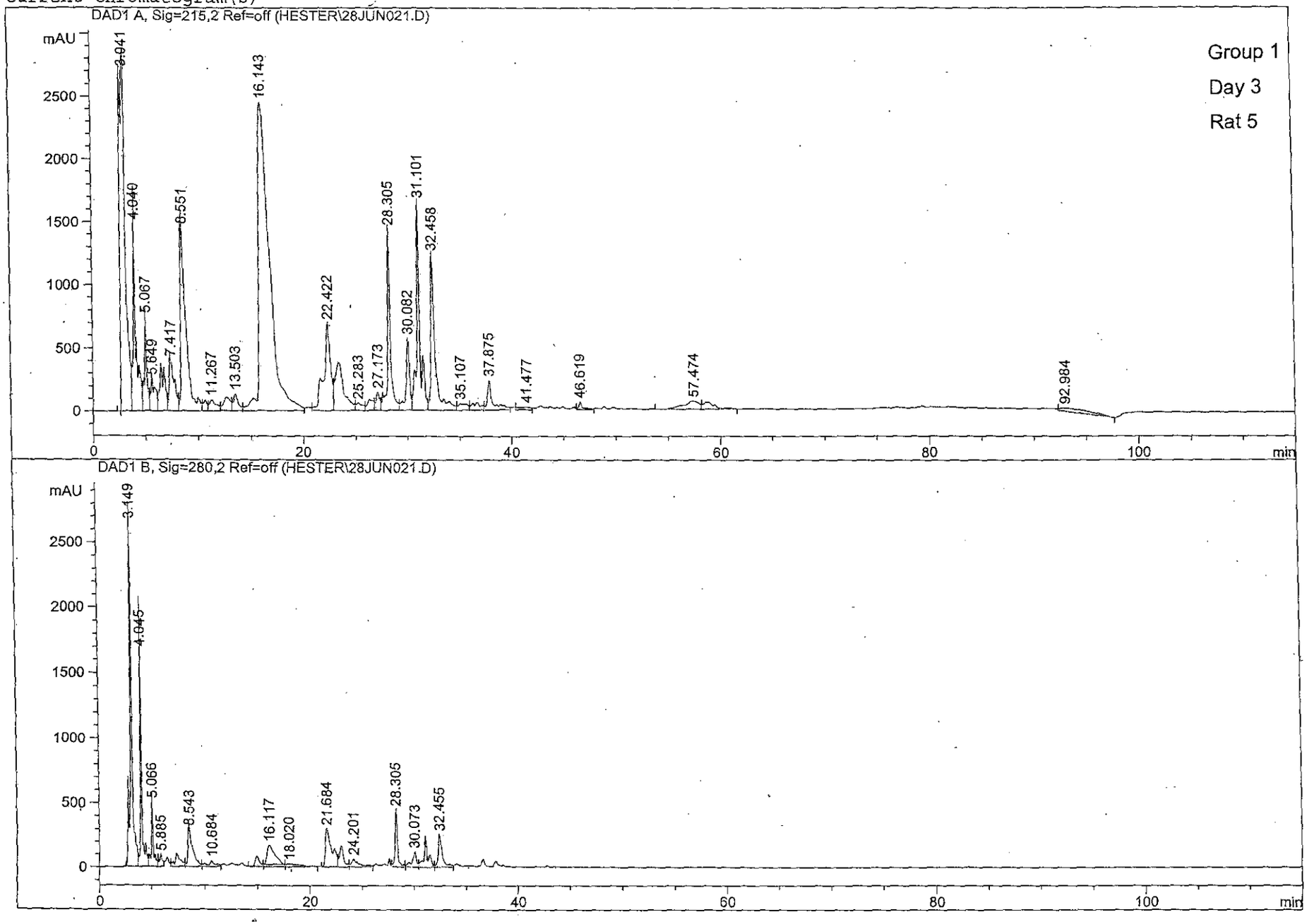

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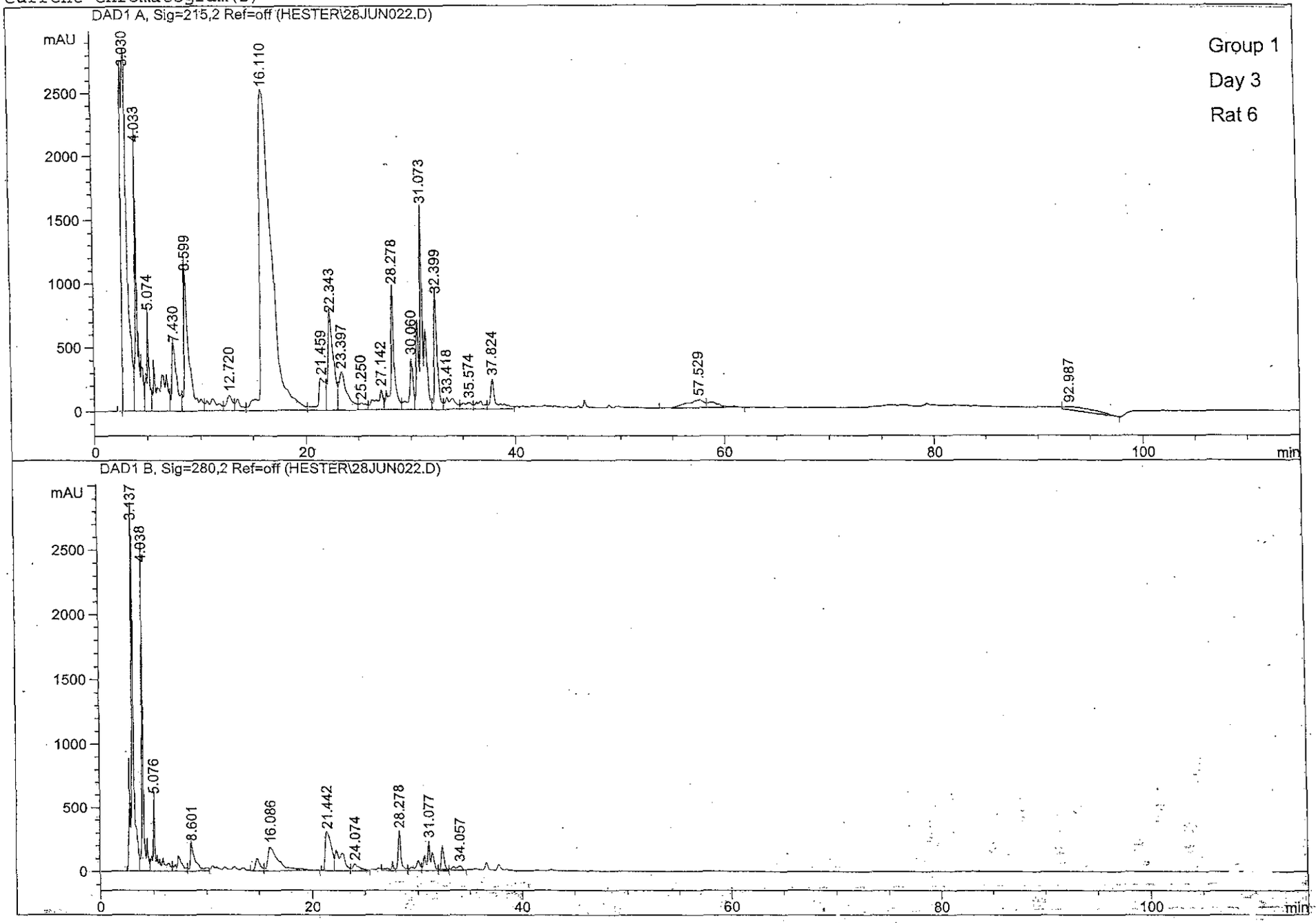

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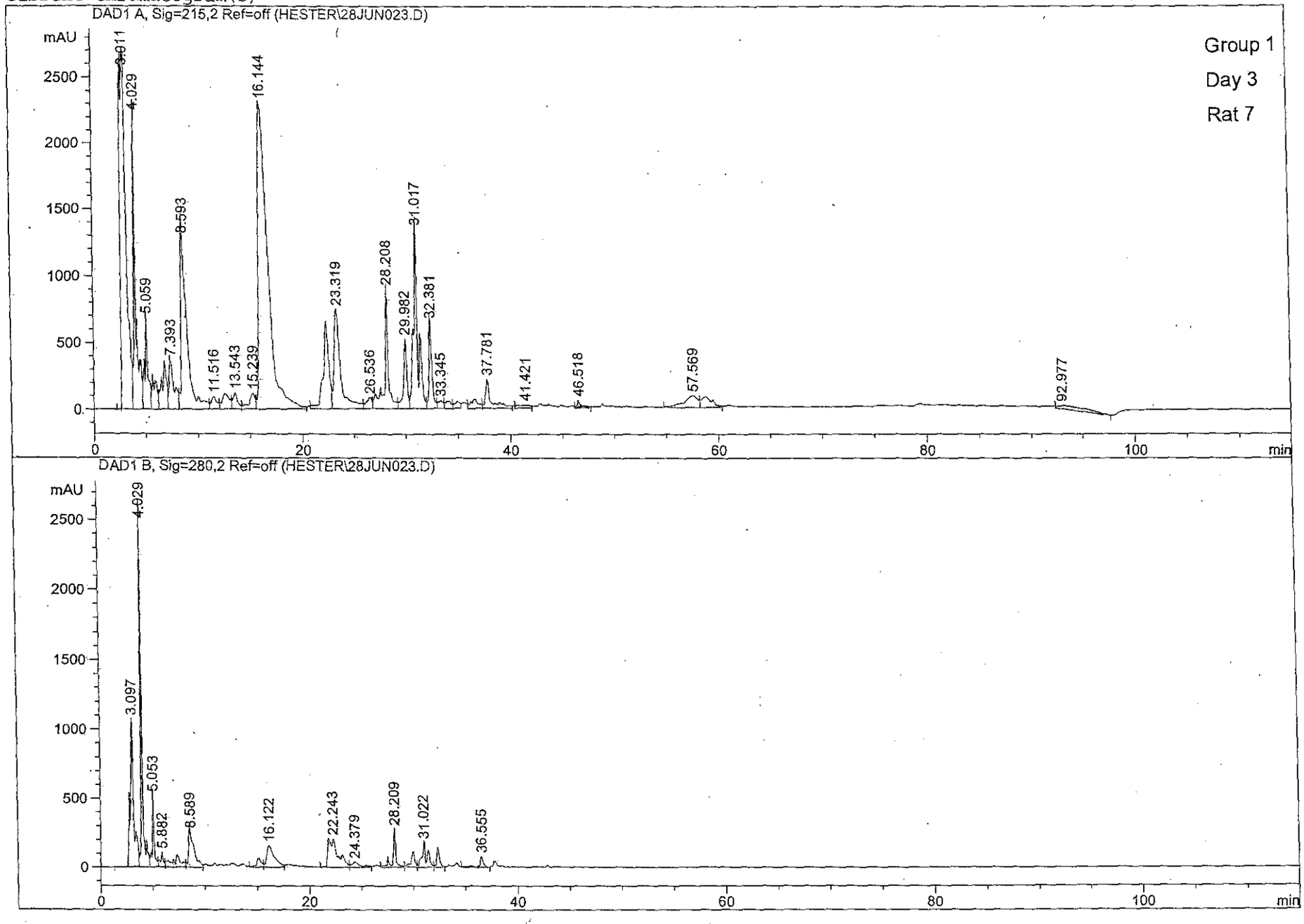

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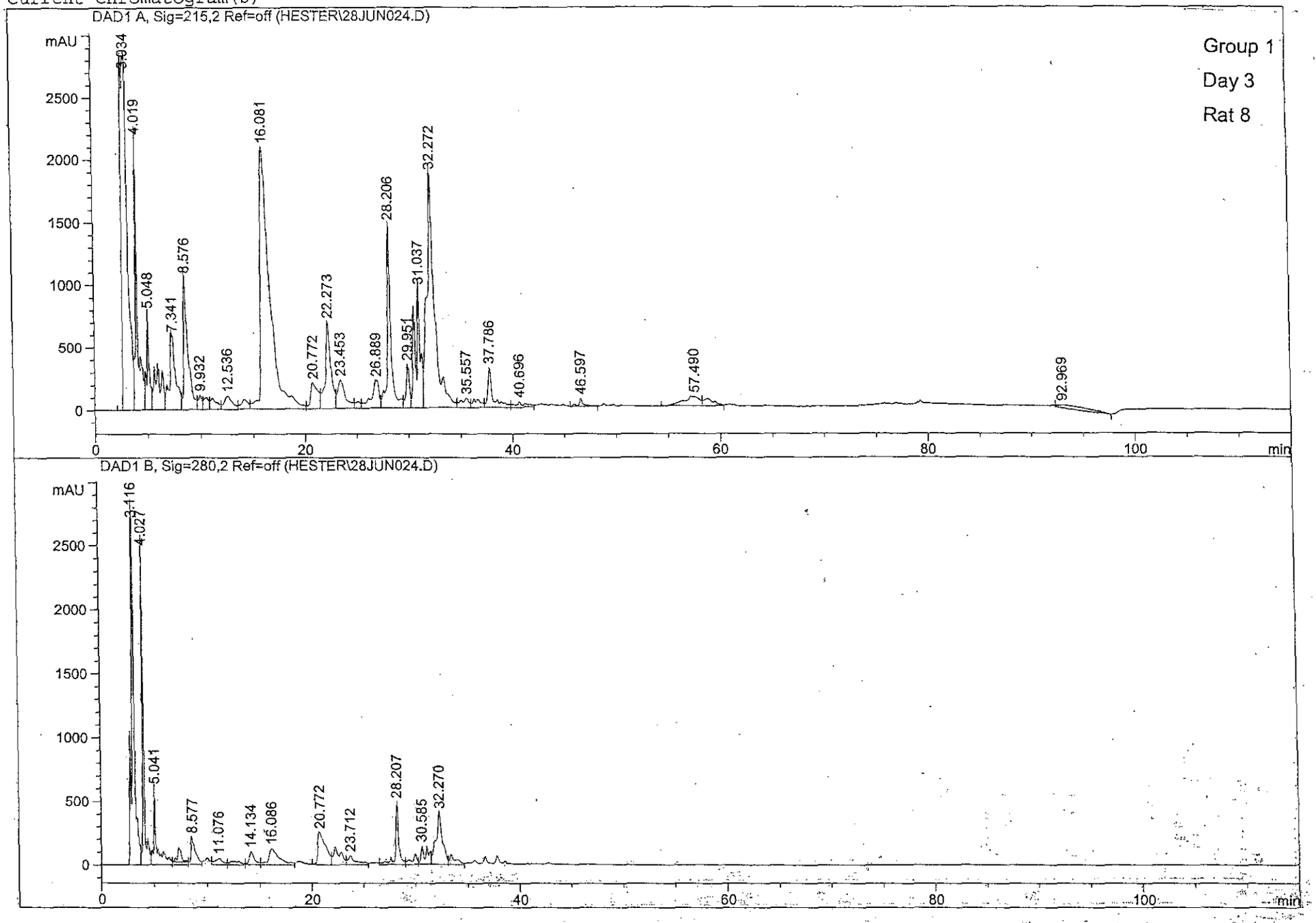

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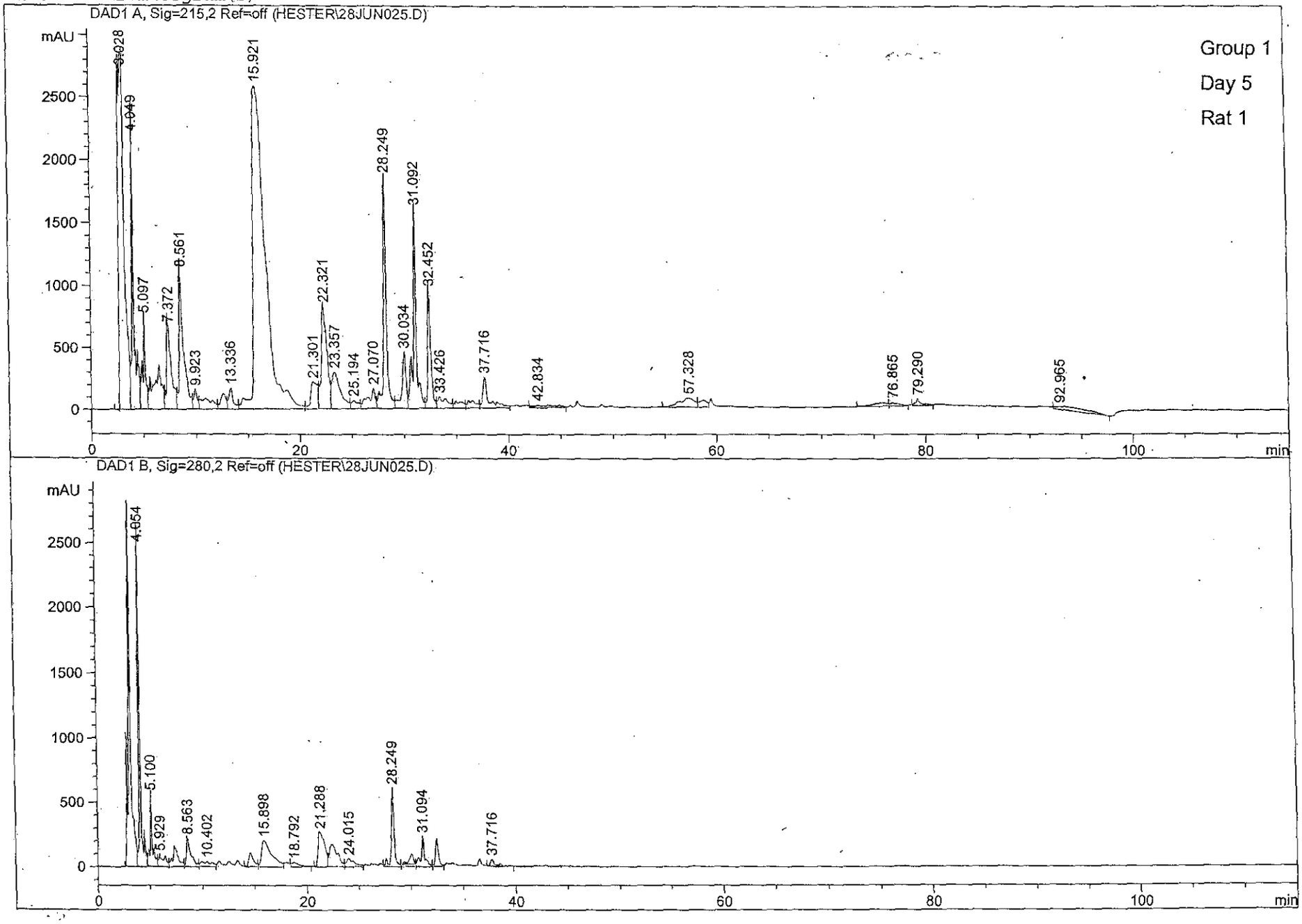

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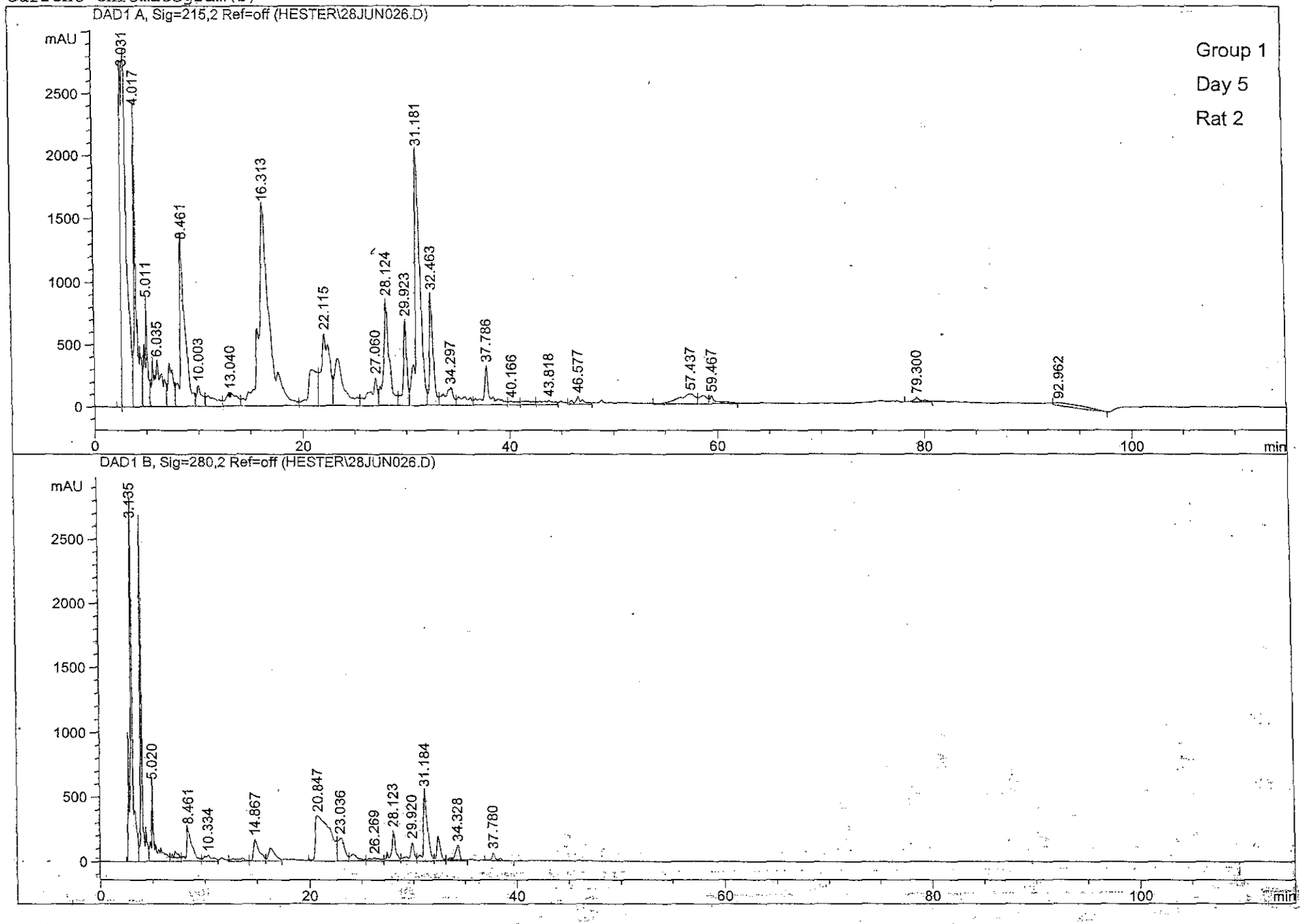

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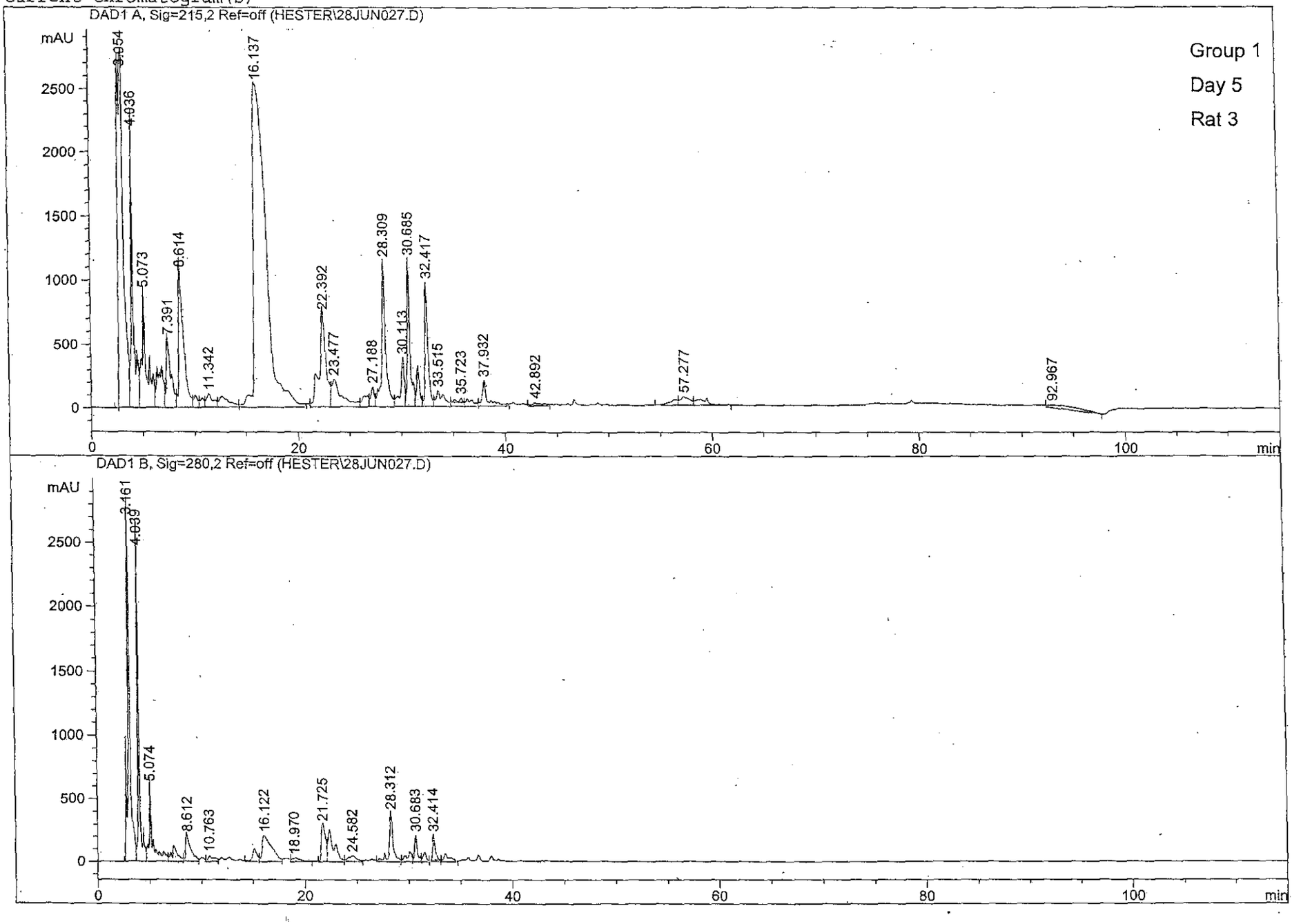

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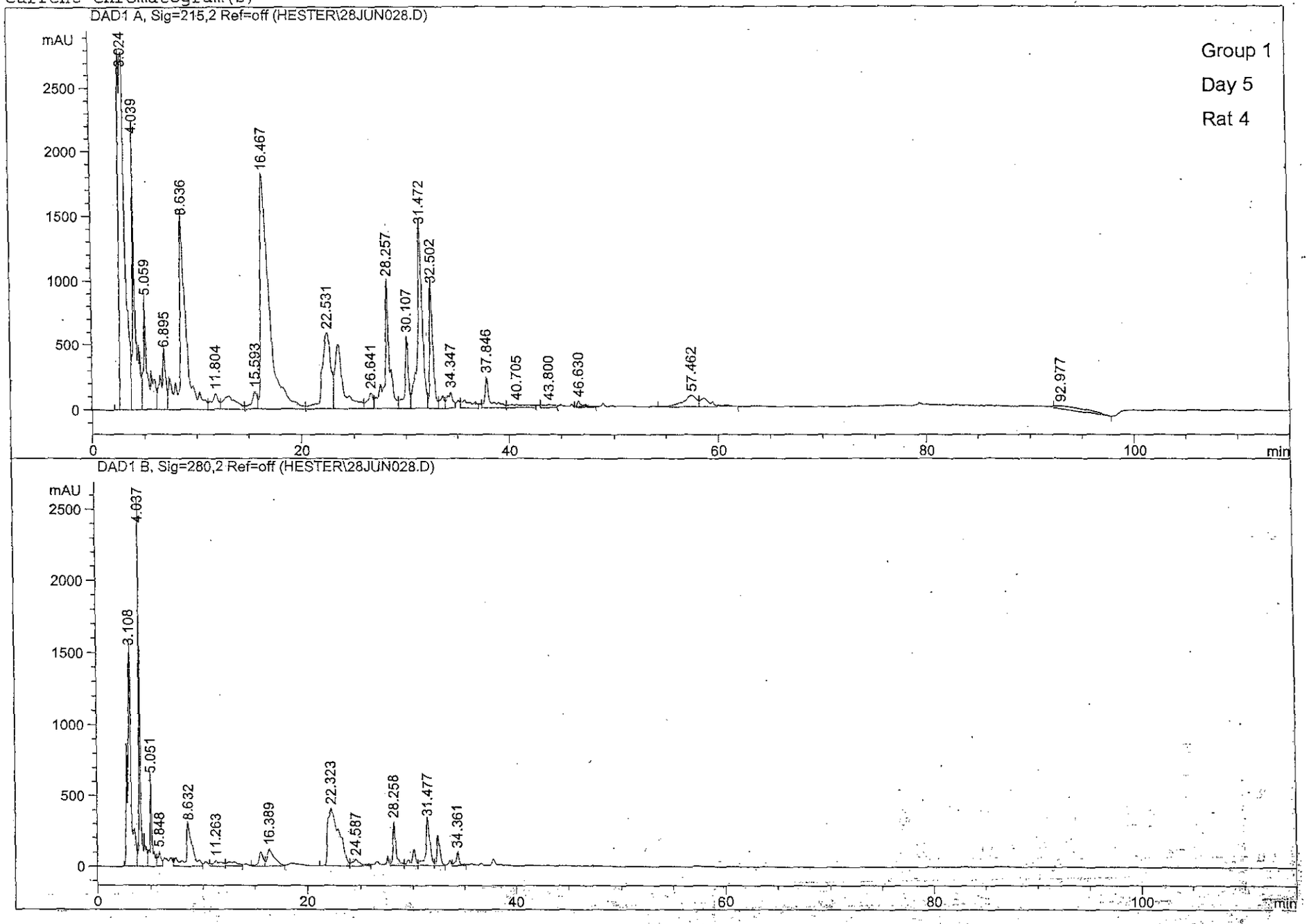
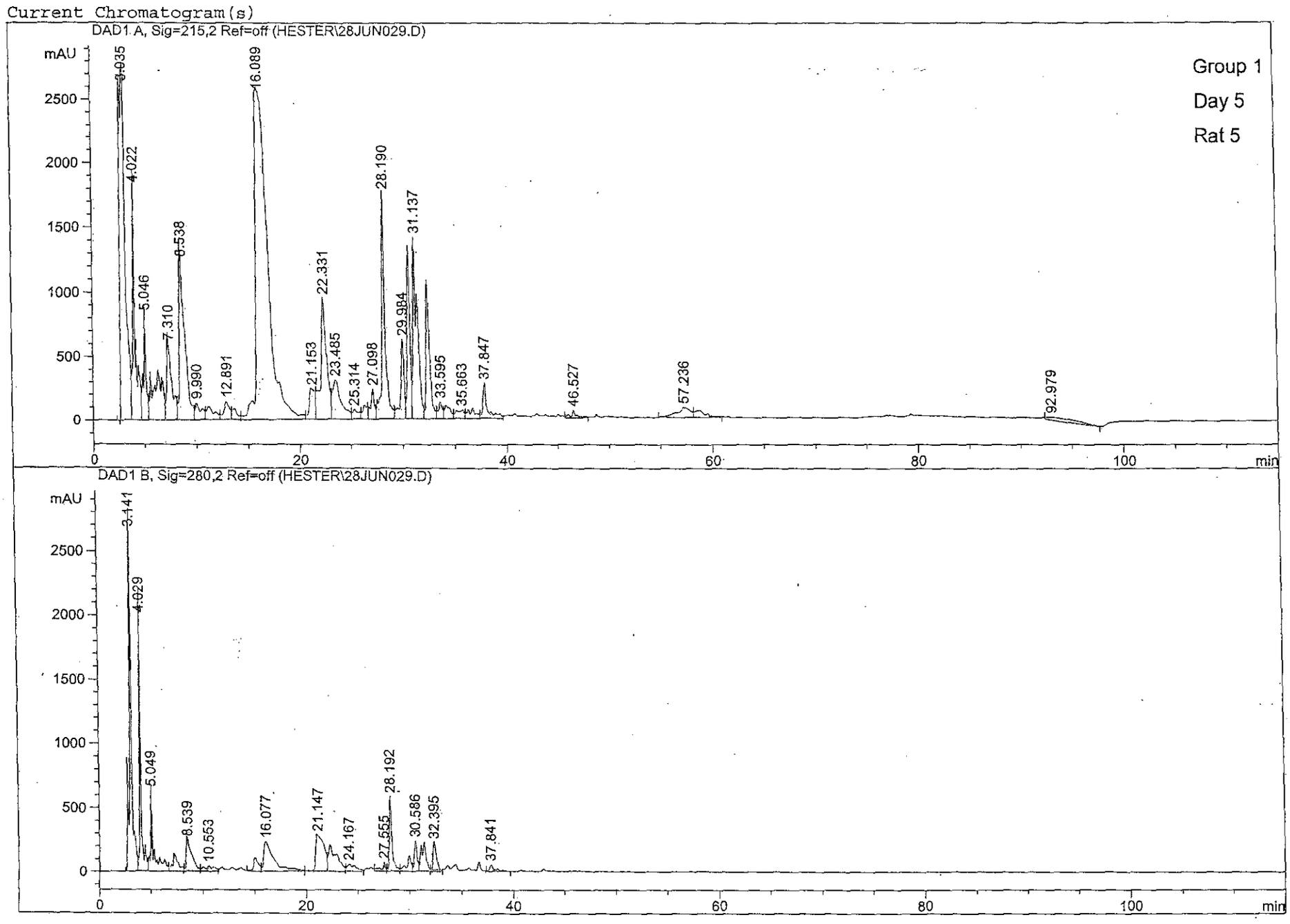
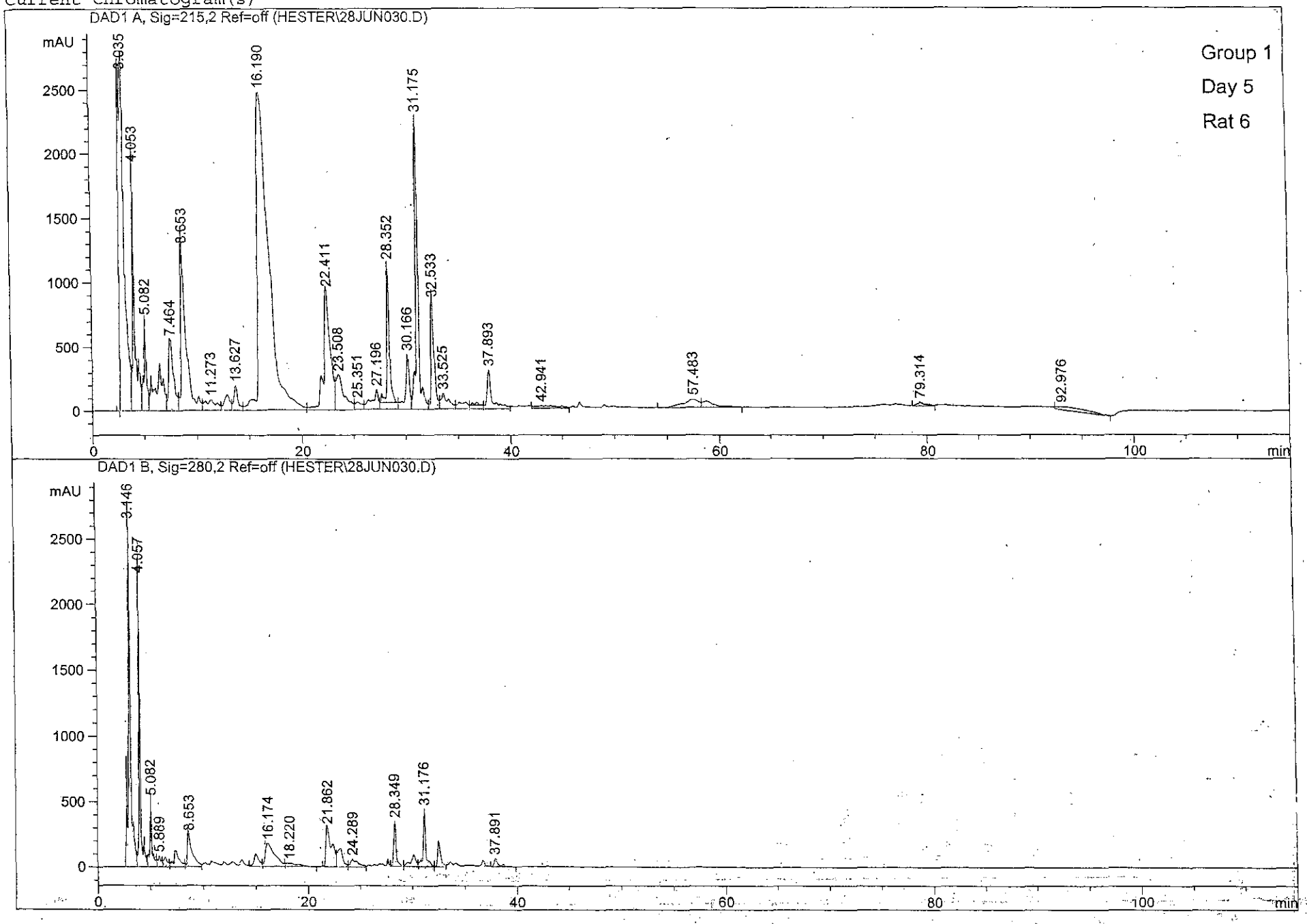



Current Chromatogram(s)
DAD1 A, Sig=215,2 Ref=off (HESTER\28JUN029.D)
mAU
Group 1
Day 5
Rat 5
3.935
4.022
5.046
7.310
8.538
9.990
12.891
16.089
21.153
22.331
23.485
25.314
27.098
28.190
29.984
31.137
33.595
35.663
37.847
46.527
57.236
92.979
min
DAD1 B, Sig=280,2 Ref=off (HESTER\28JUN029.D)
mAU
3.141
4.029
5.049
8.539
10.553
16.077
21.147
24.167
27.555
28.192
30.586
32.395
37.841
min

Current Chromatogram(s)



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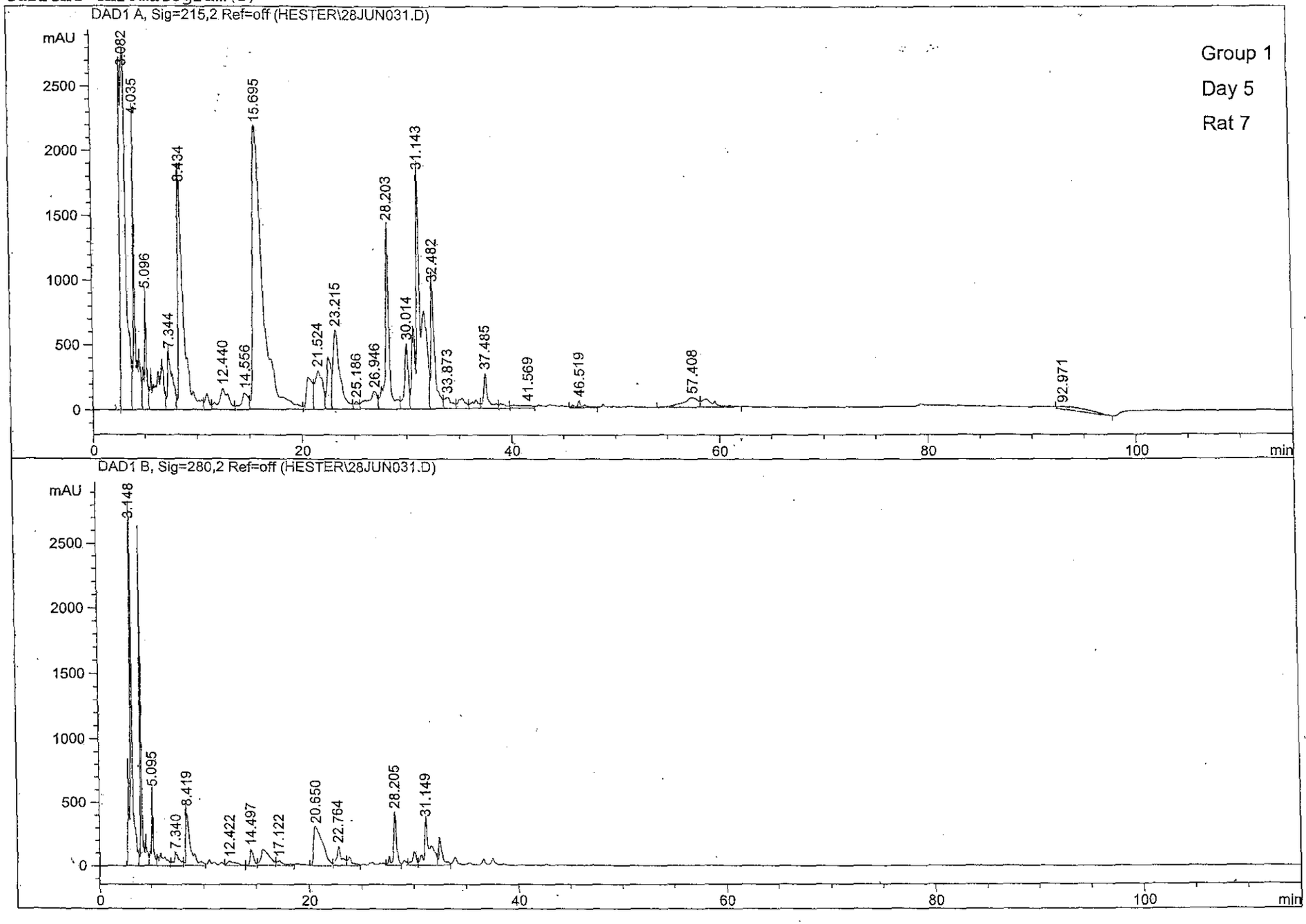
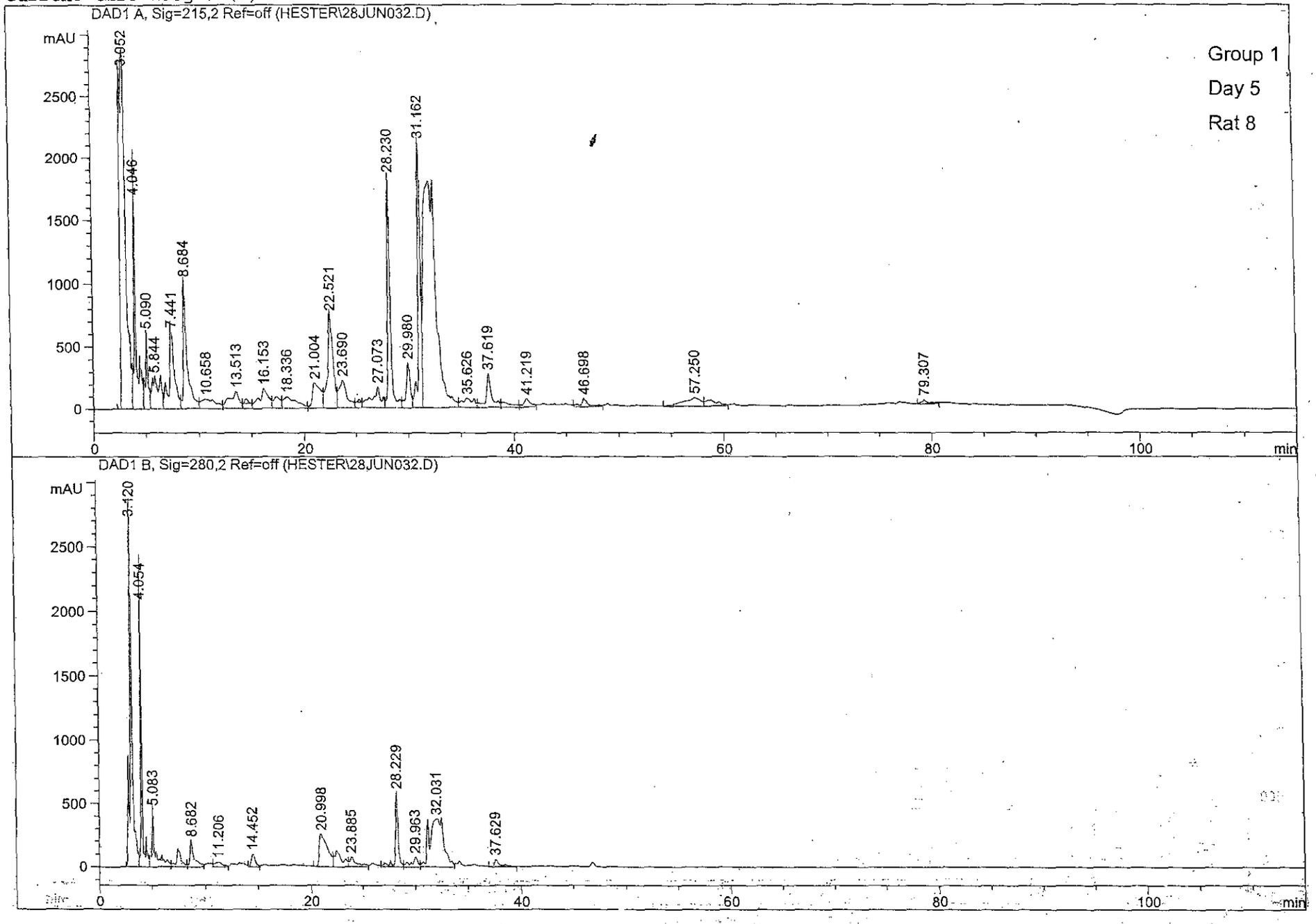
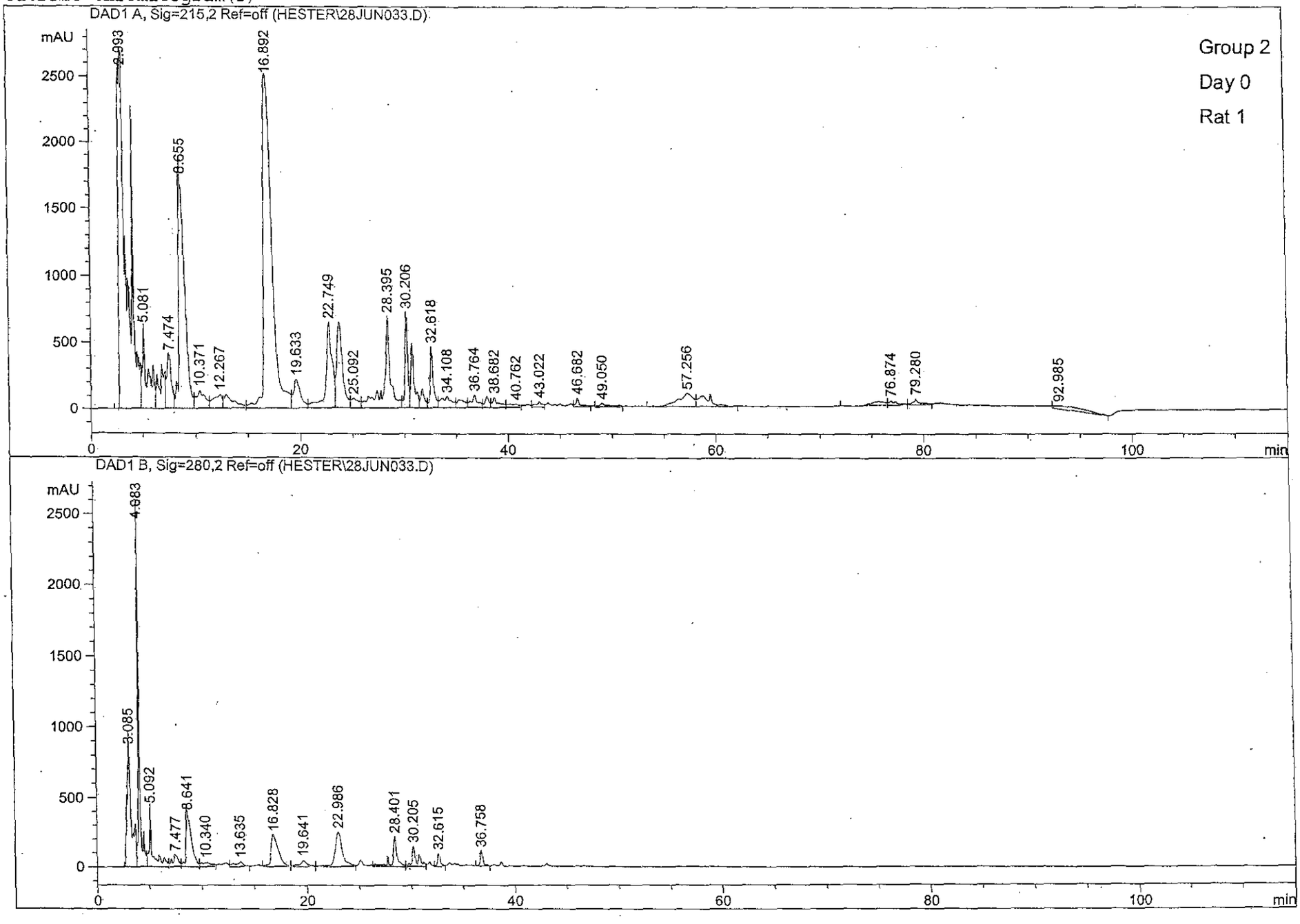


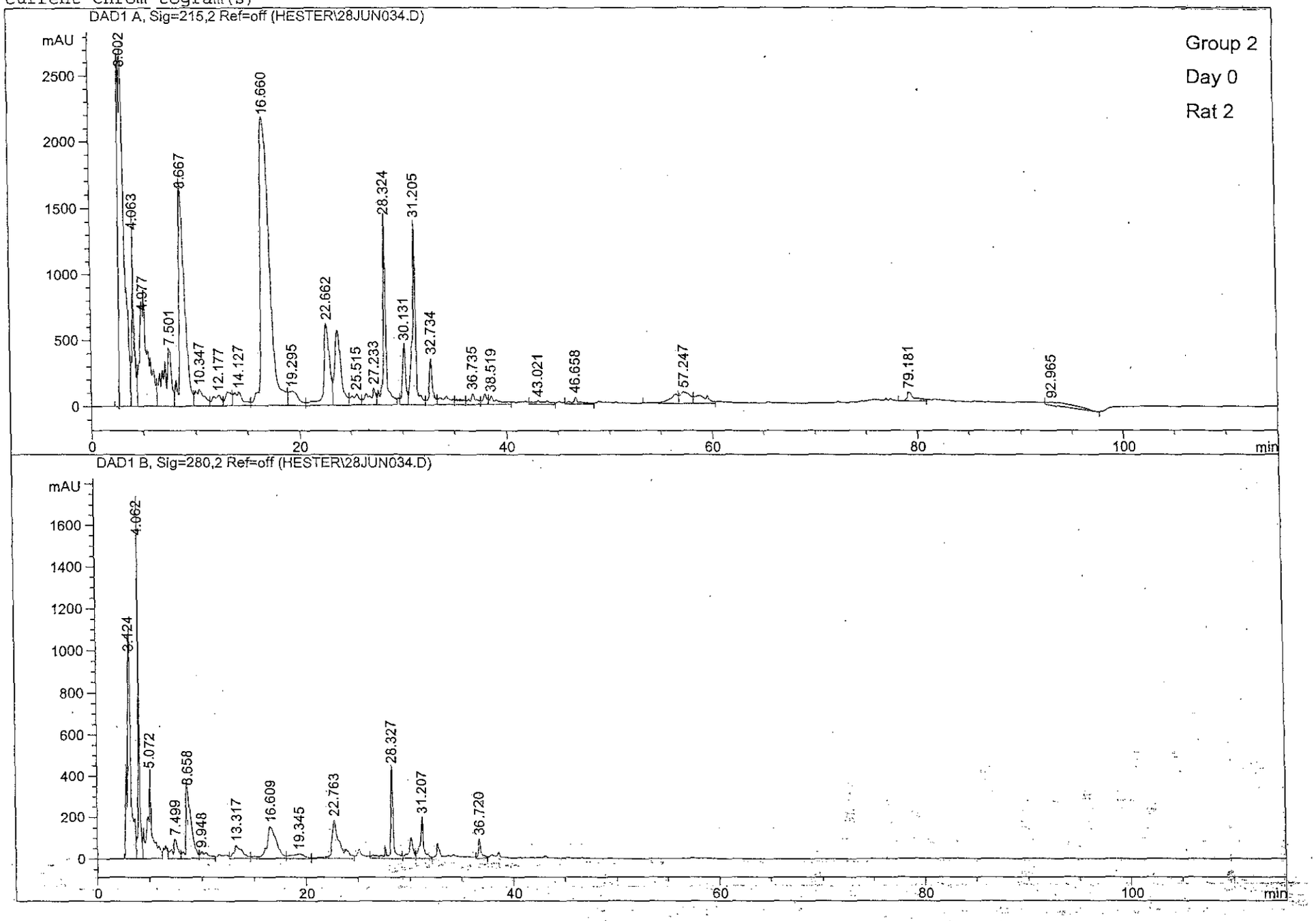
Current Chromatogram(s)



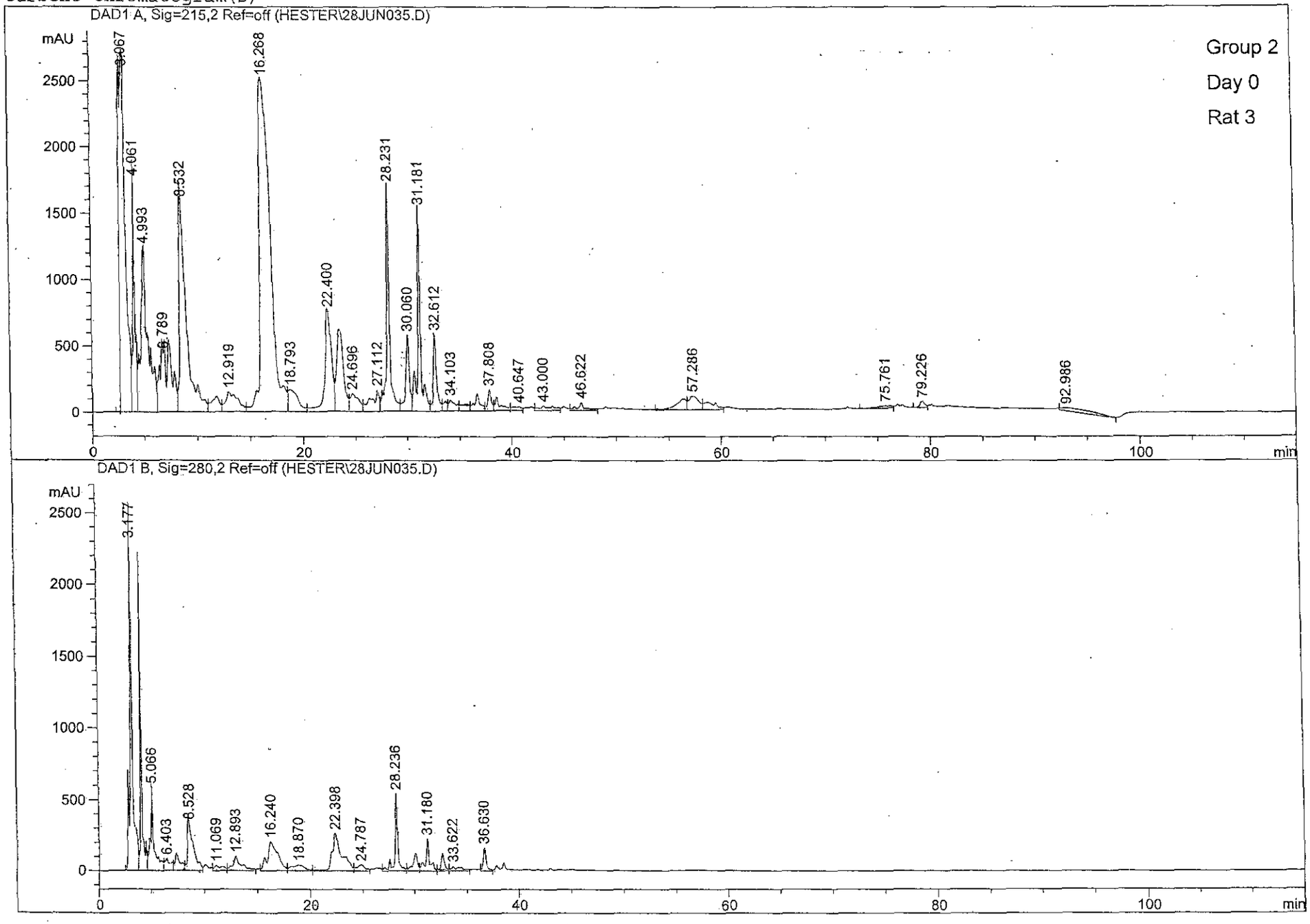
# Casein and cyclosporine

Current Chromatogram(s)




Current Chrom togram(s)
DAD1 A, Sig=215,2 Ref=off (HESTER\28JUN034.D)
mAU
Group 2
Day 0
Rat 2
3.902
4.063
4.977
7.501
8.667
10.347
12.177
14.127
16.660
19.295
22.662
25.515
27.233
28.324
30.131
31.205
32.734
36.735
38.519
43.021
46.658
57.247
79.181
92.965
min
DAD1 B, Sig=280,2 Ref=off (HESTER\28JUN034.D)
mAU
3.424
4.062
5.072
6.658
7.499
9.948
13.317
16.609
19.345
22.763
28.327
31.207
36.720
min

Current Chromatogram(s)



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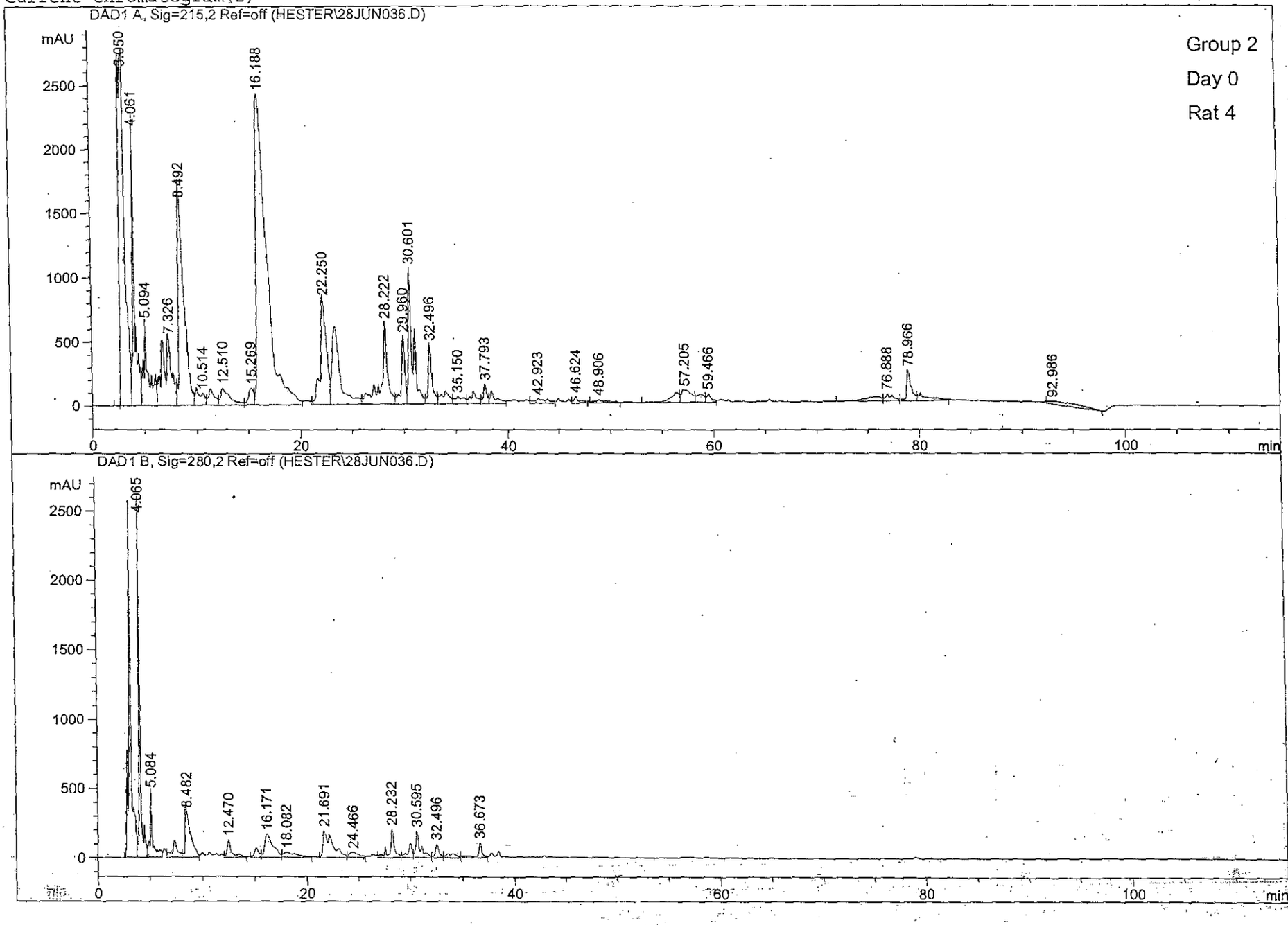

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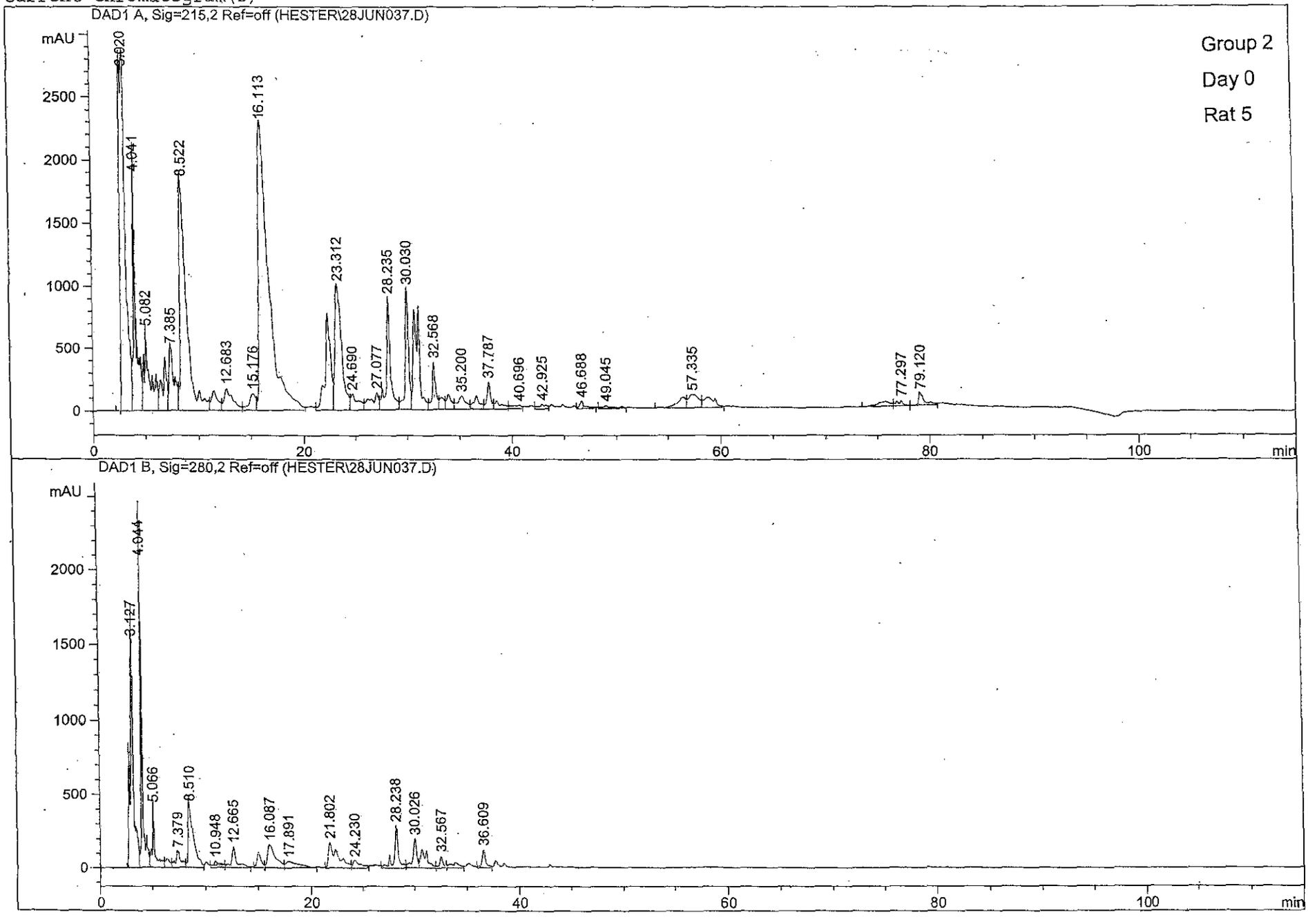

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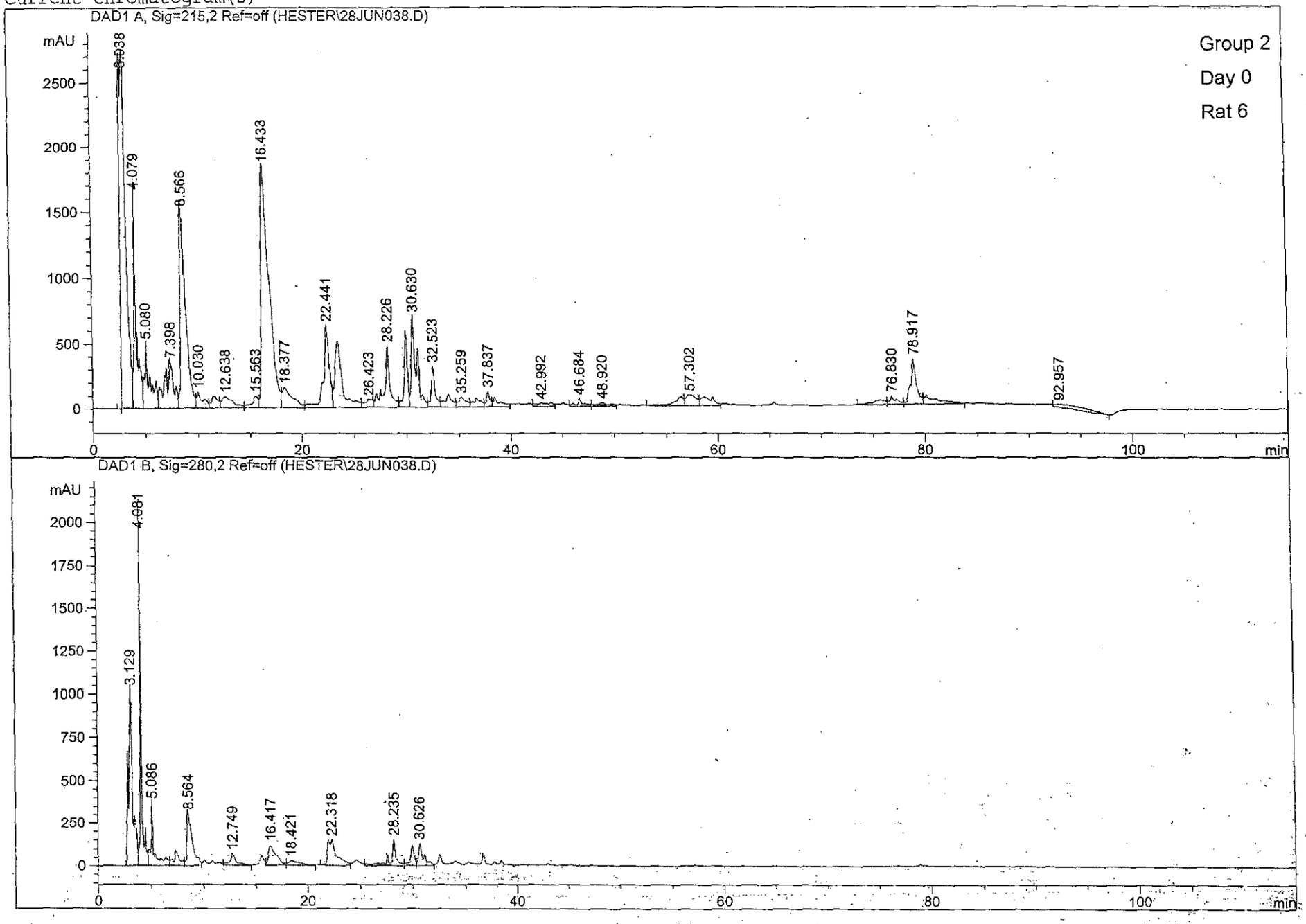
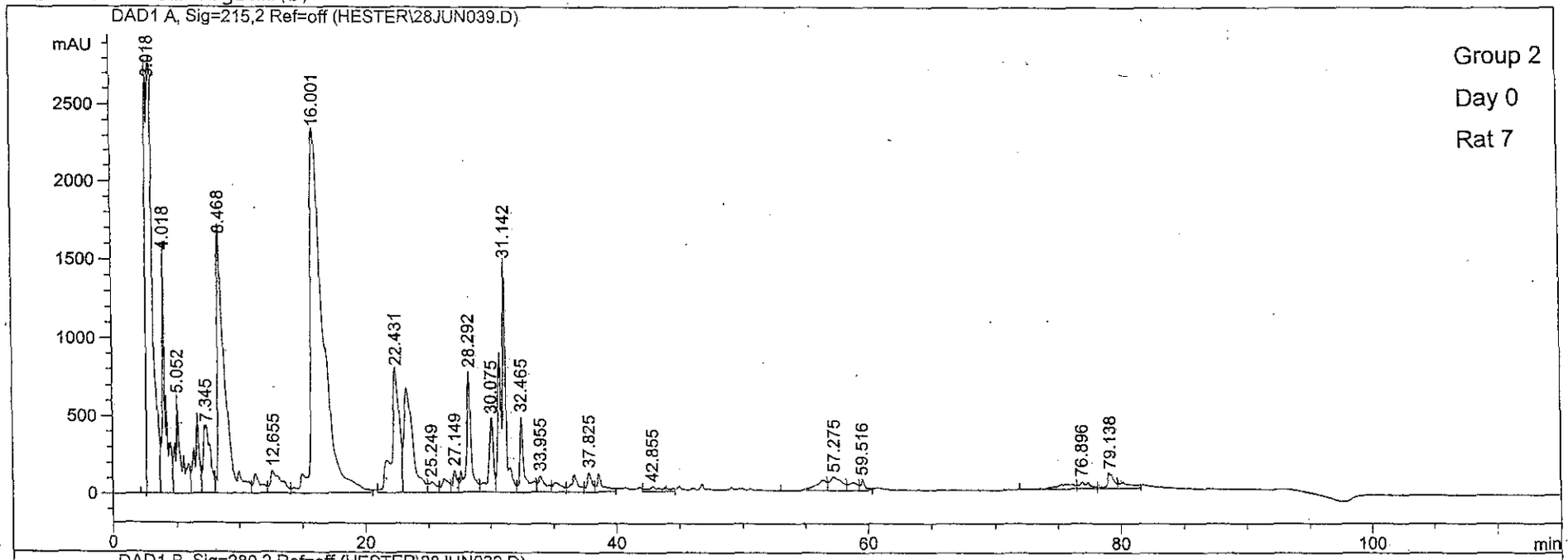
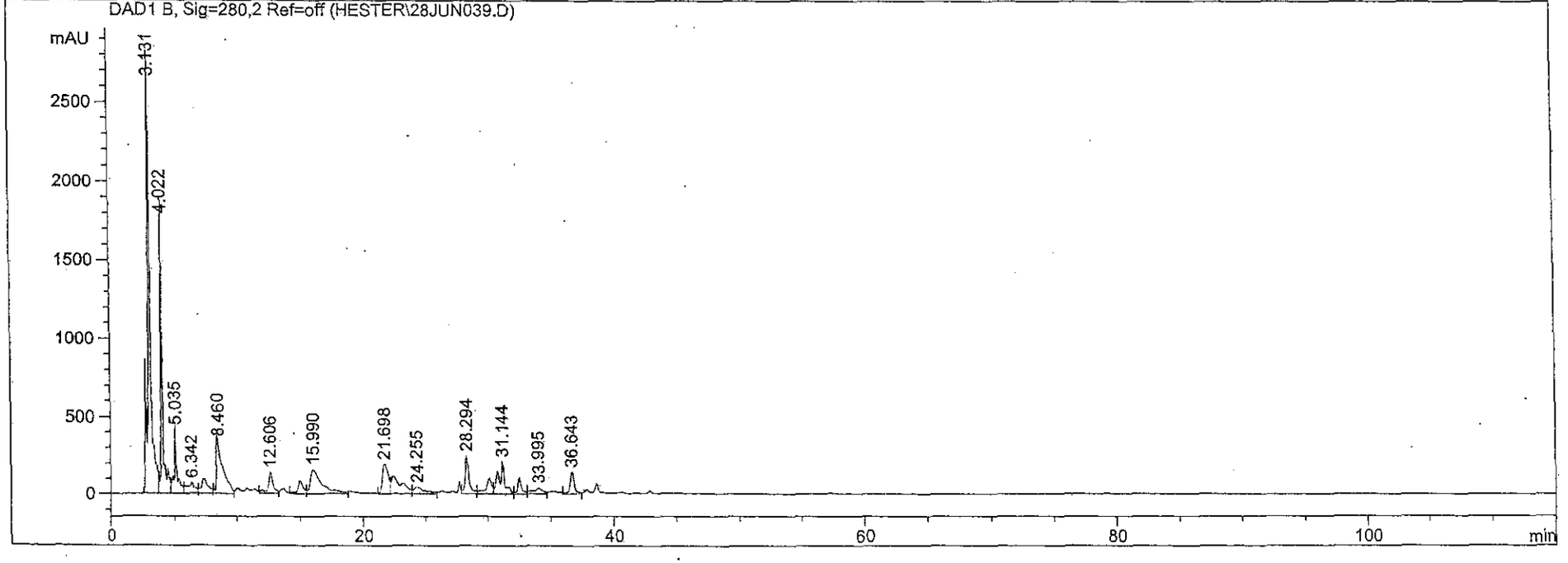


Current Chromatogram(s)



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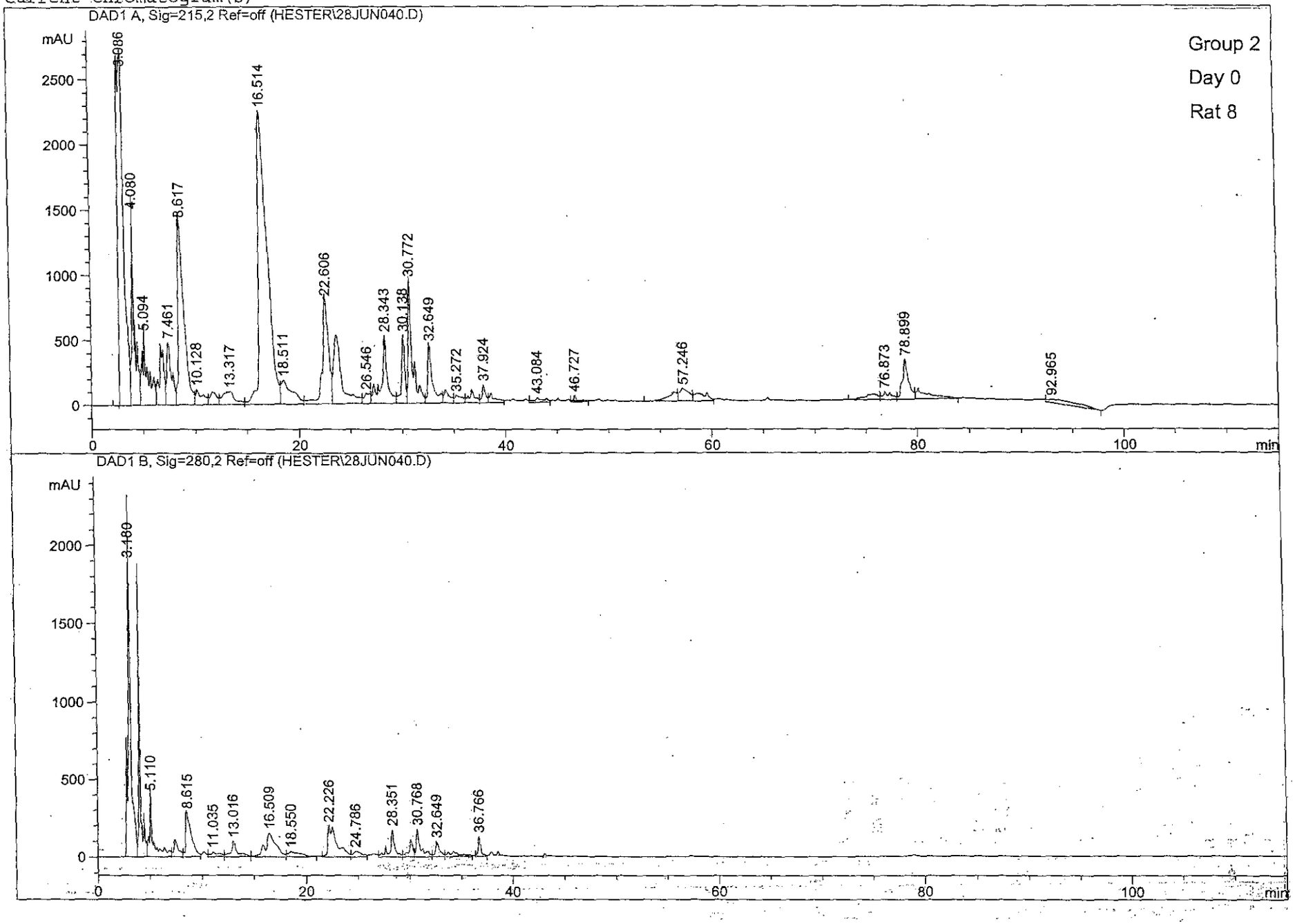

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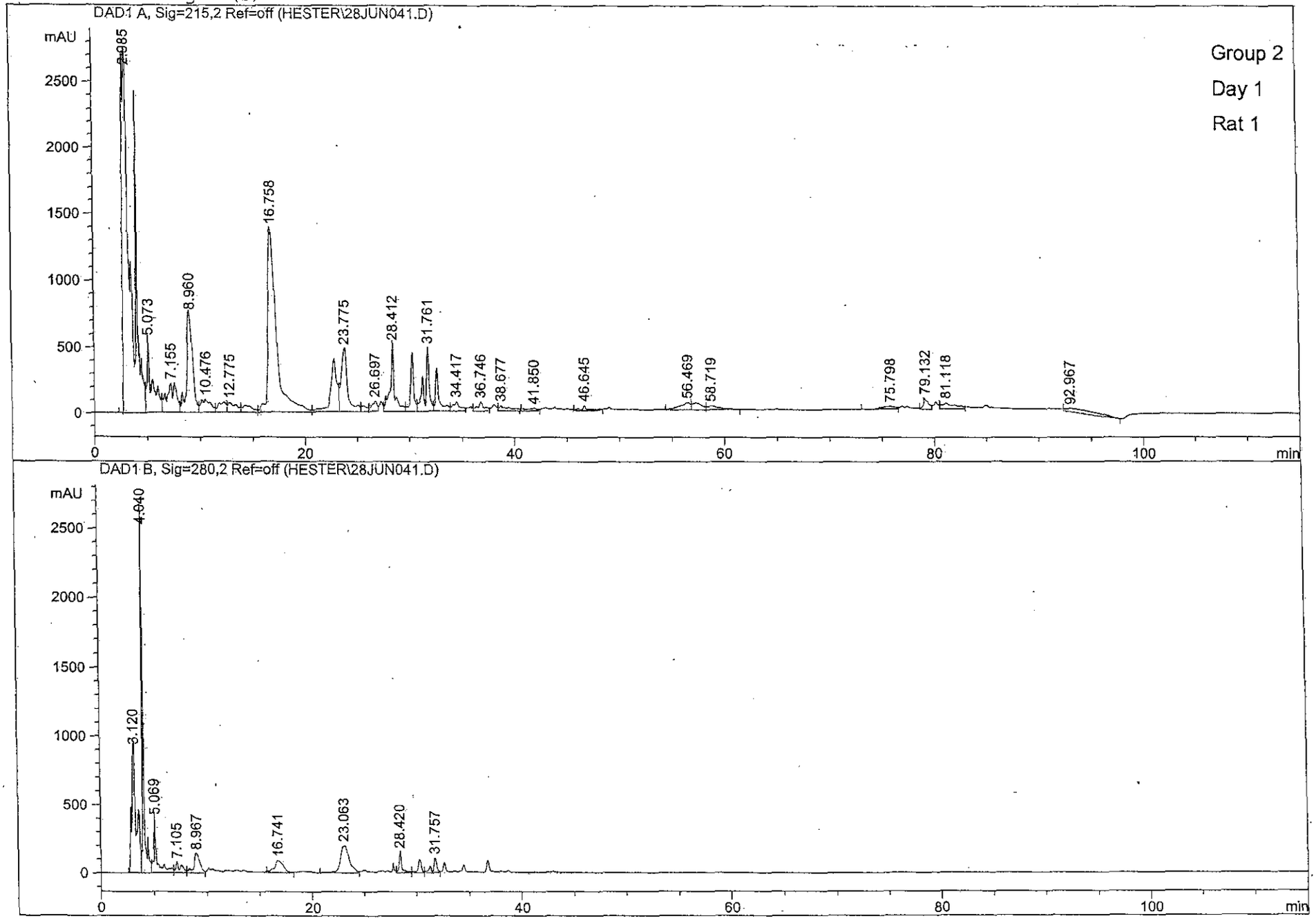

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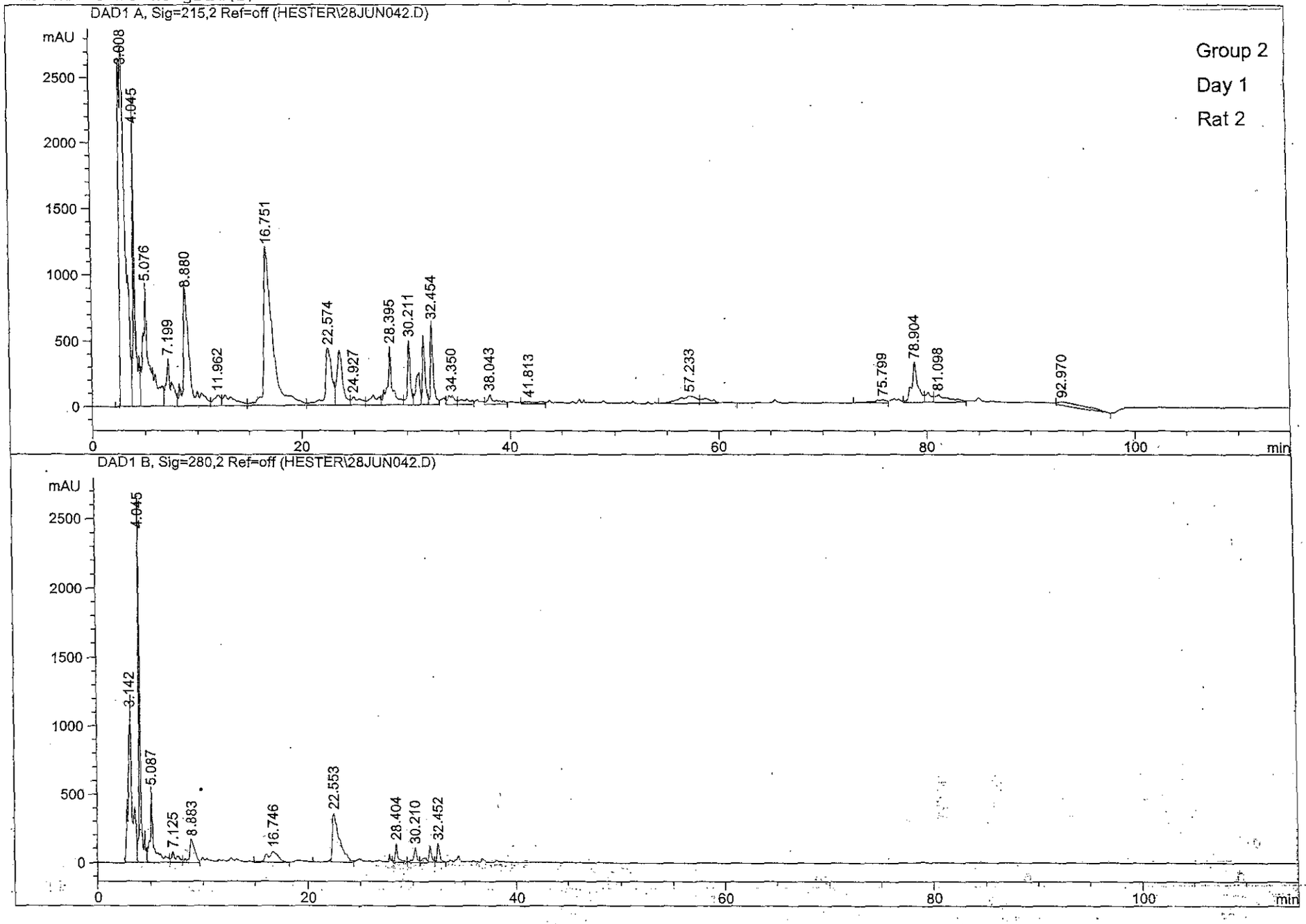
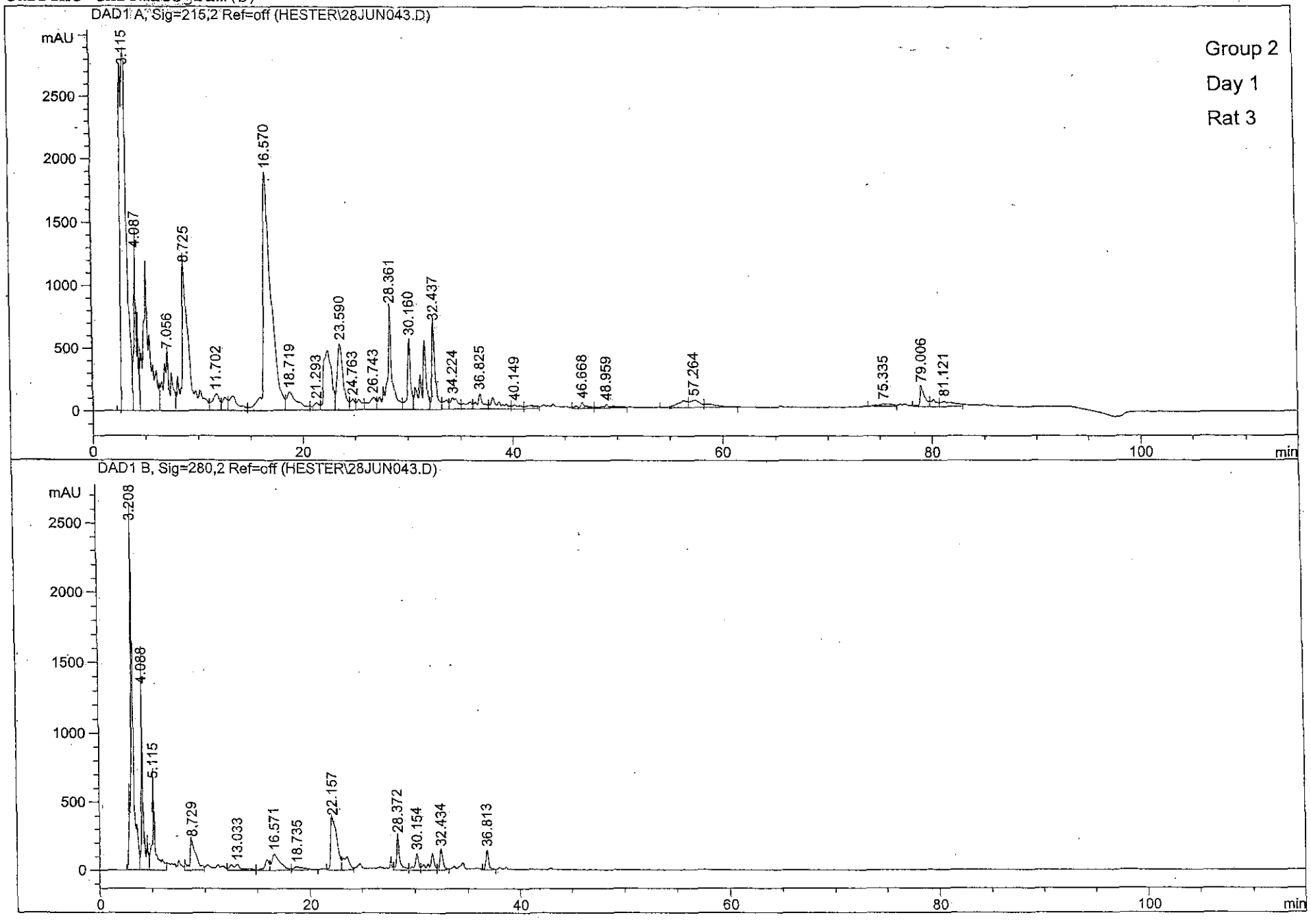
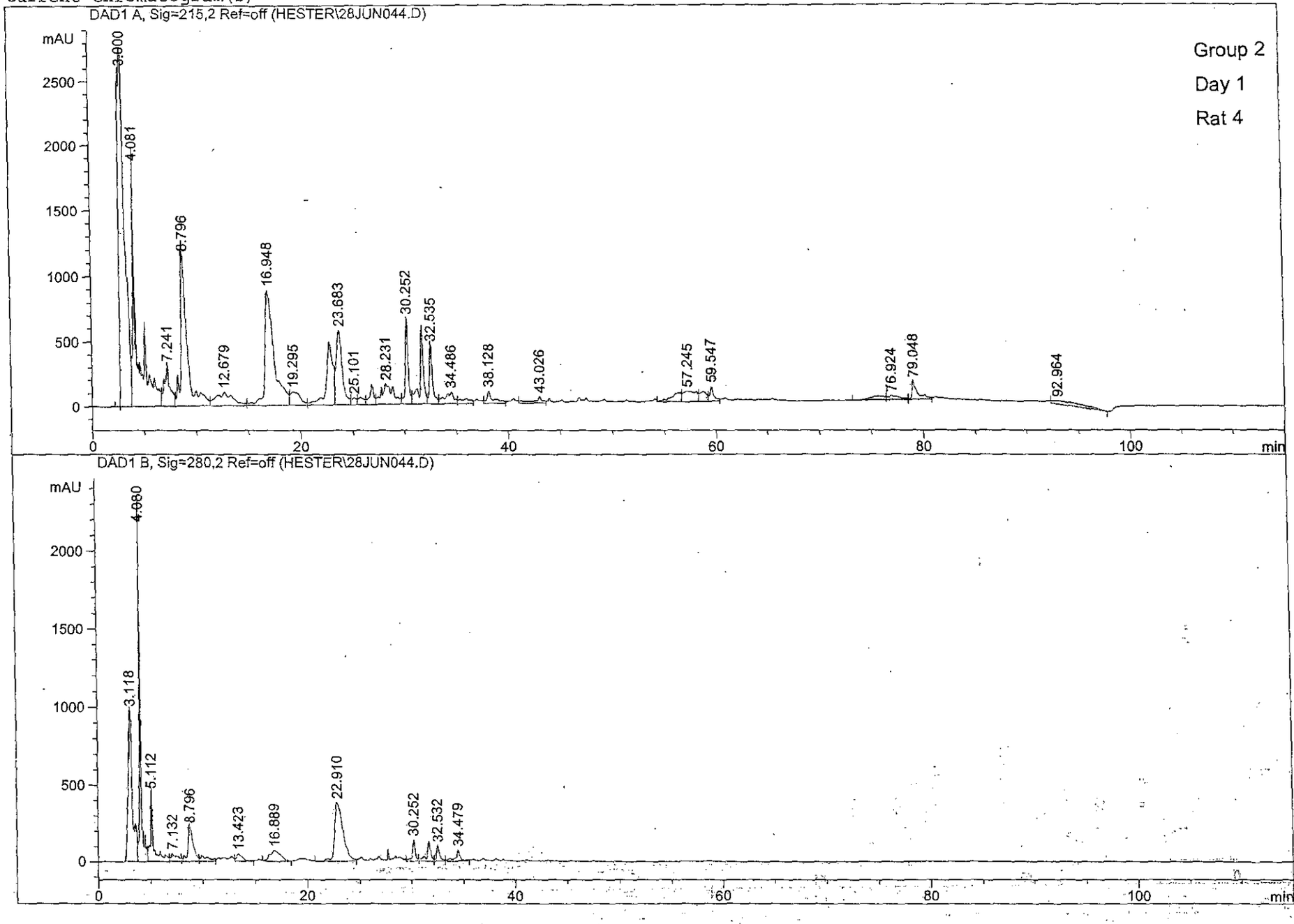


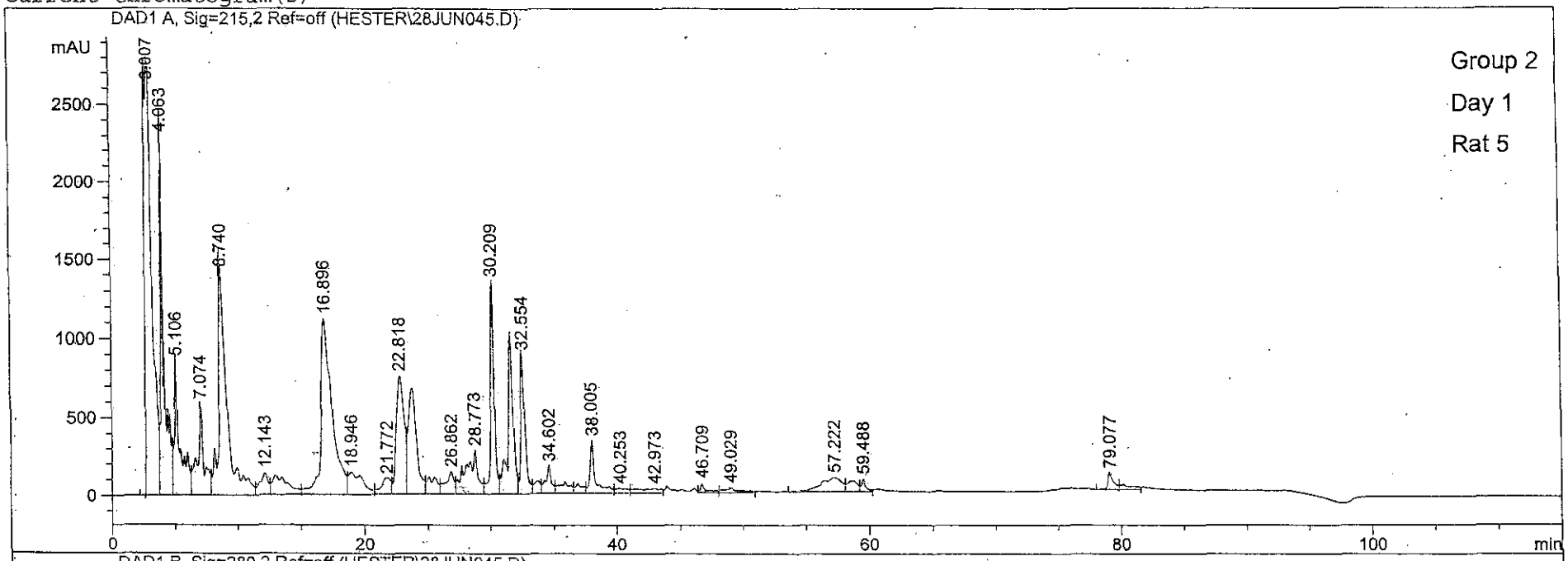
Current Chromatogram(s)

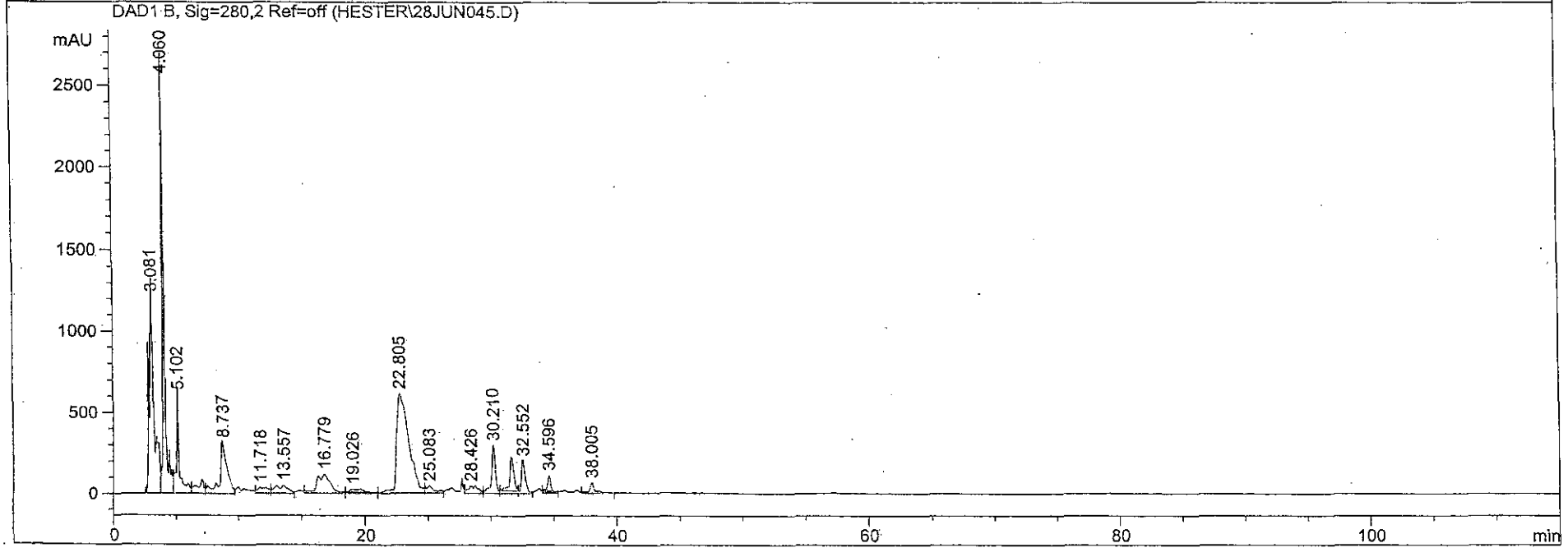


Current Chromatogram(s)

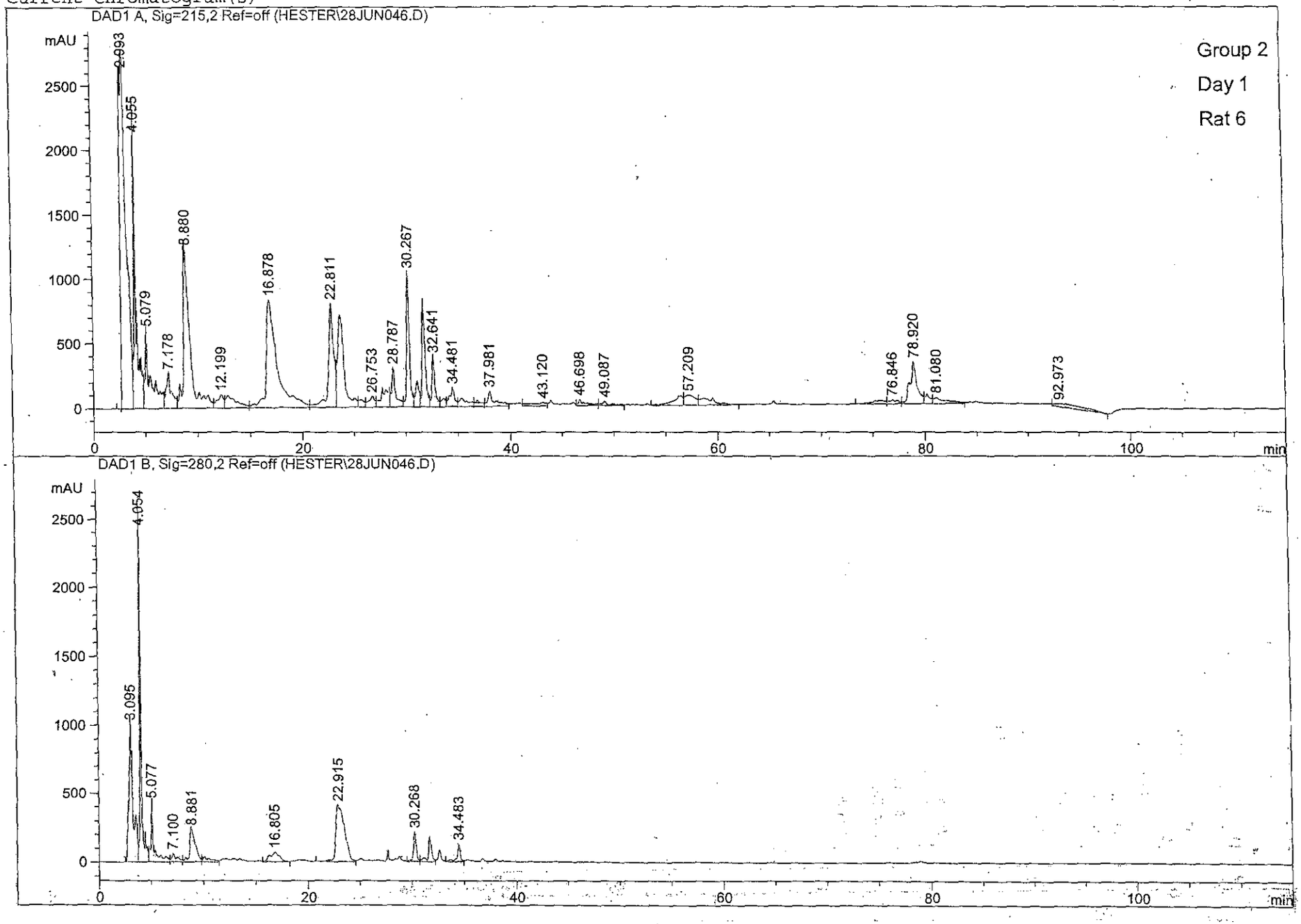


Current Chromatogram(s)



Current Chromatogram(s)



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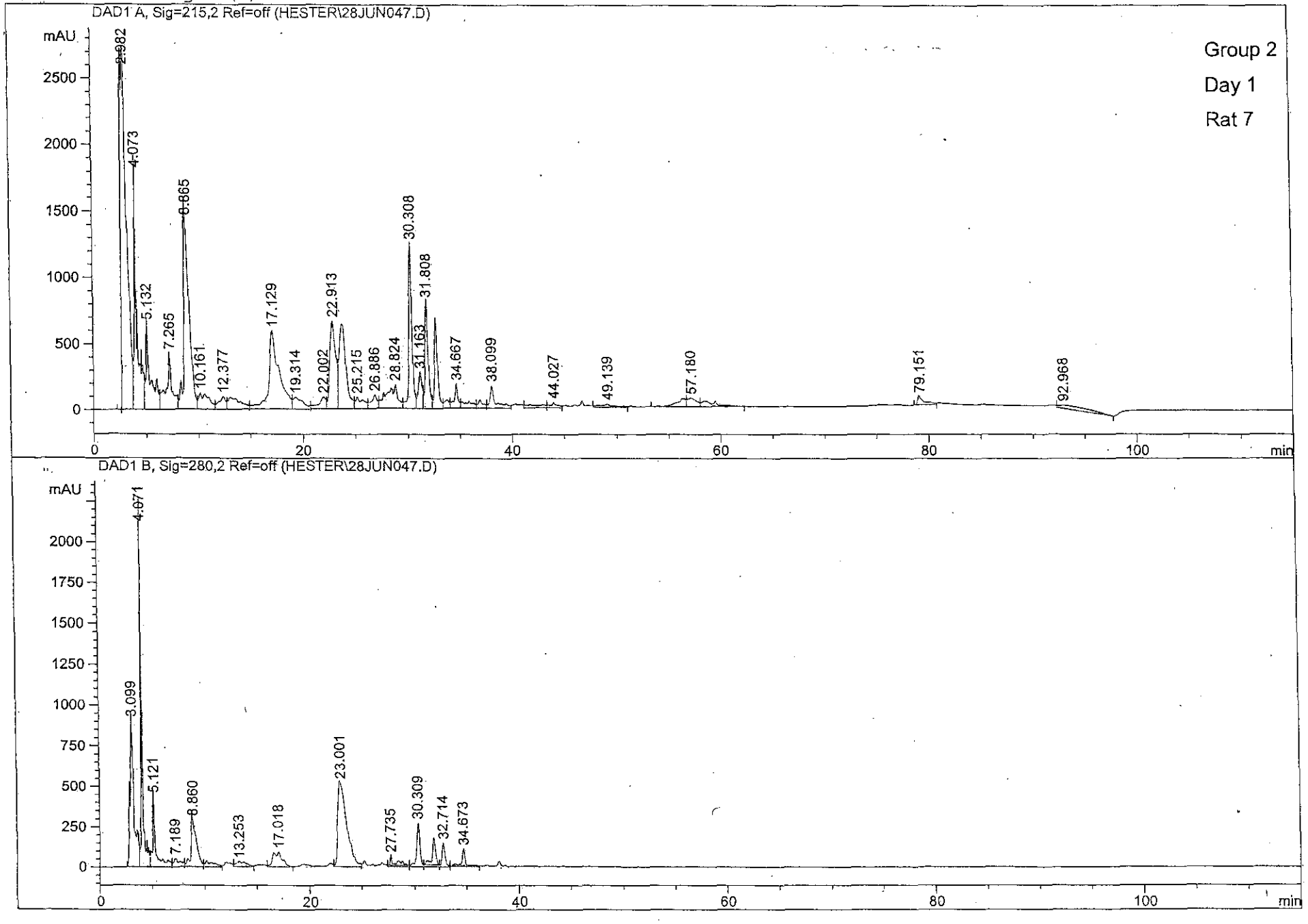

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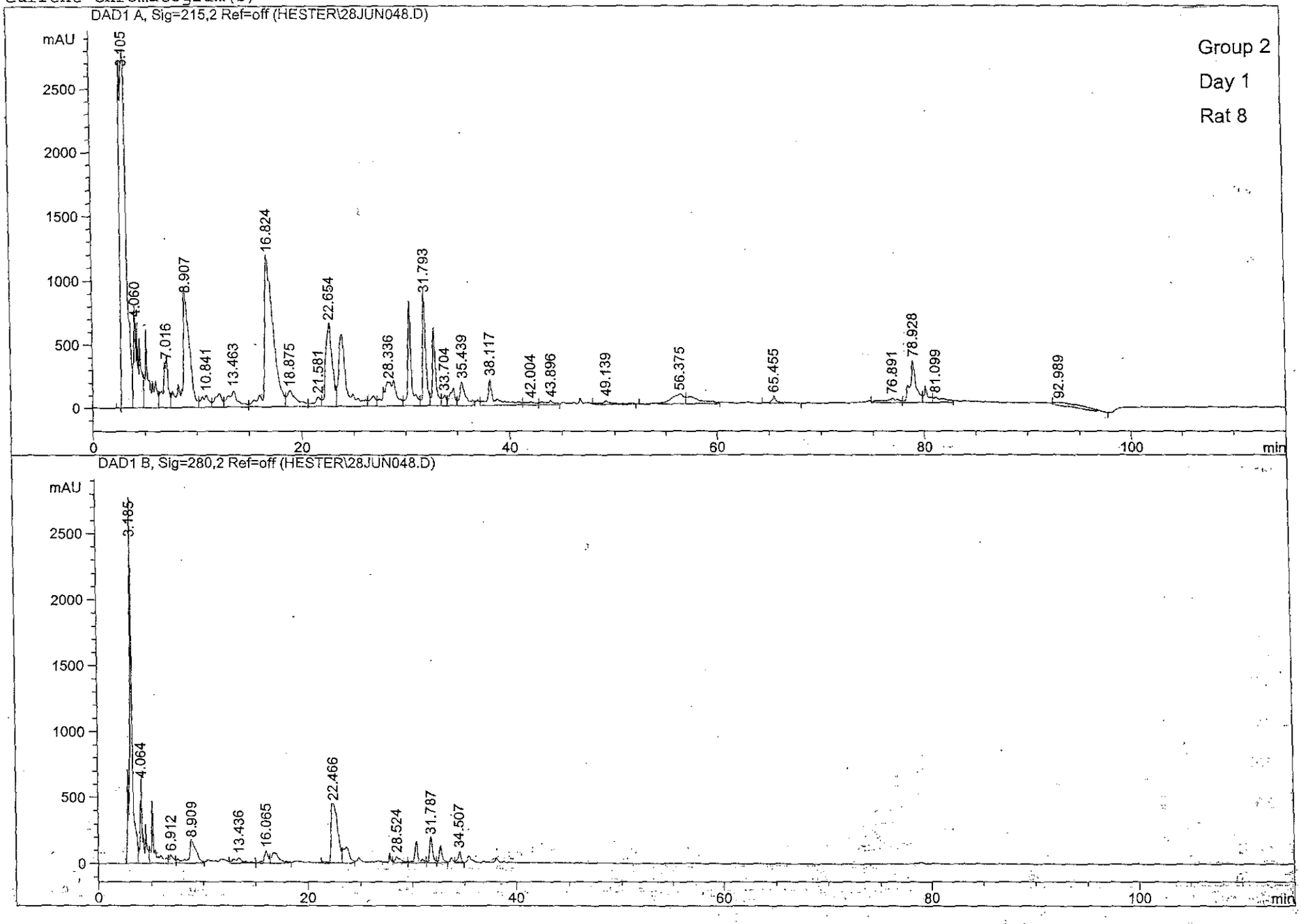

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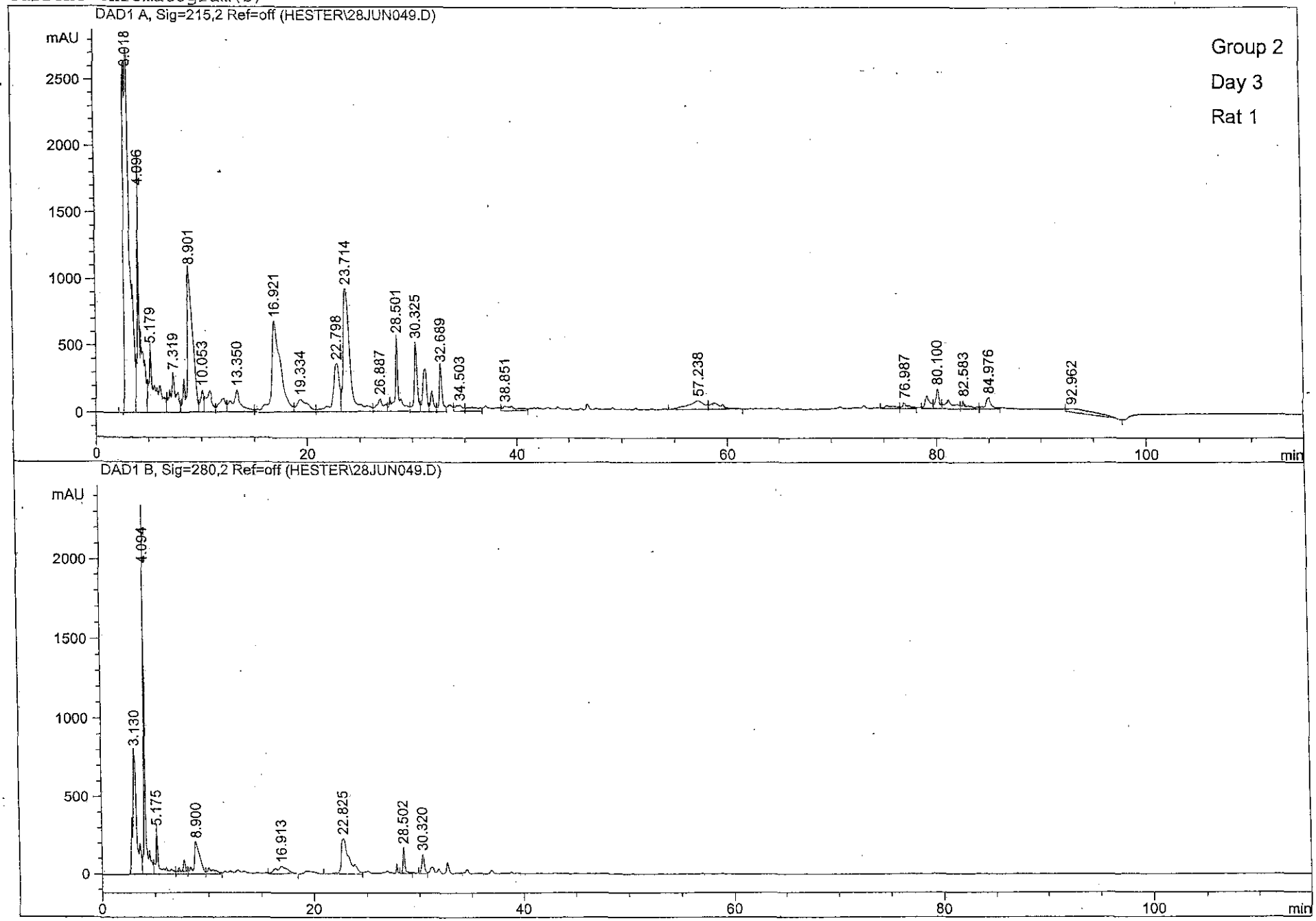

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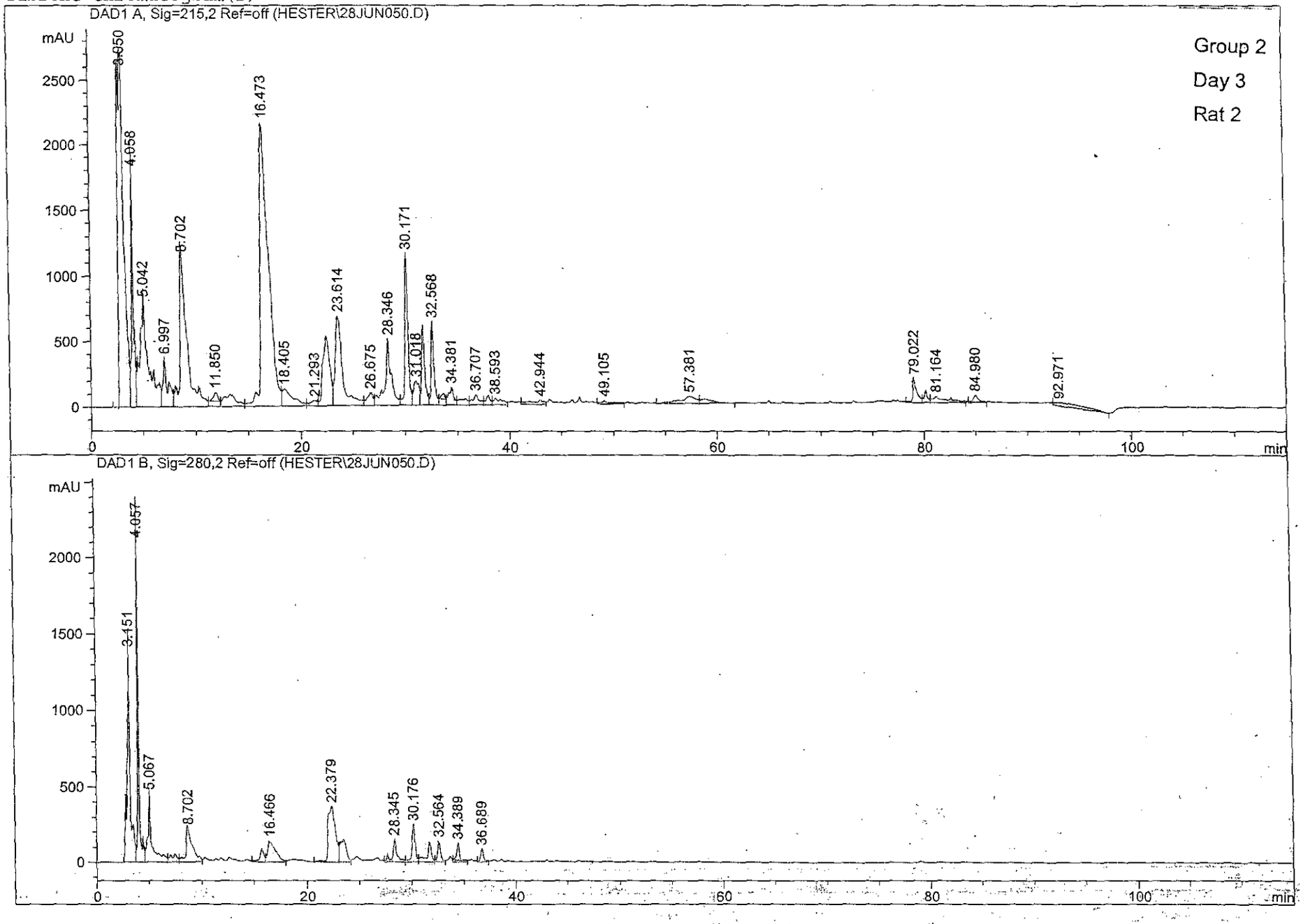

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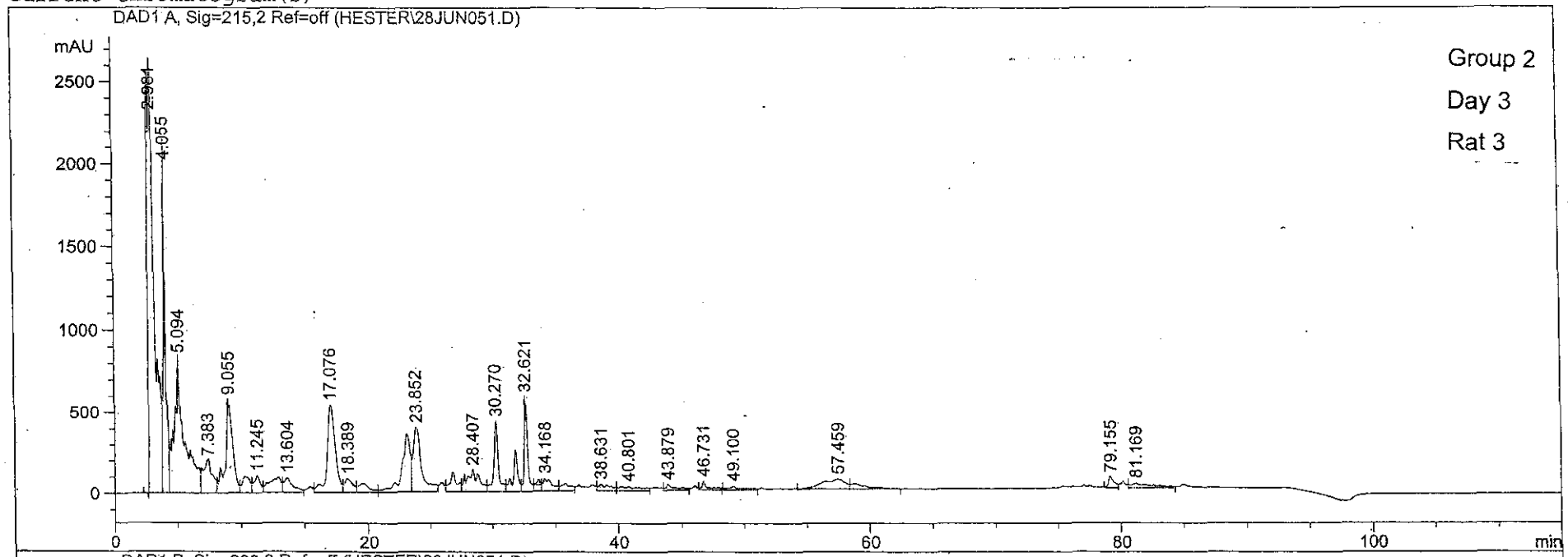


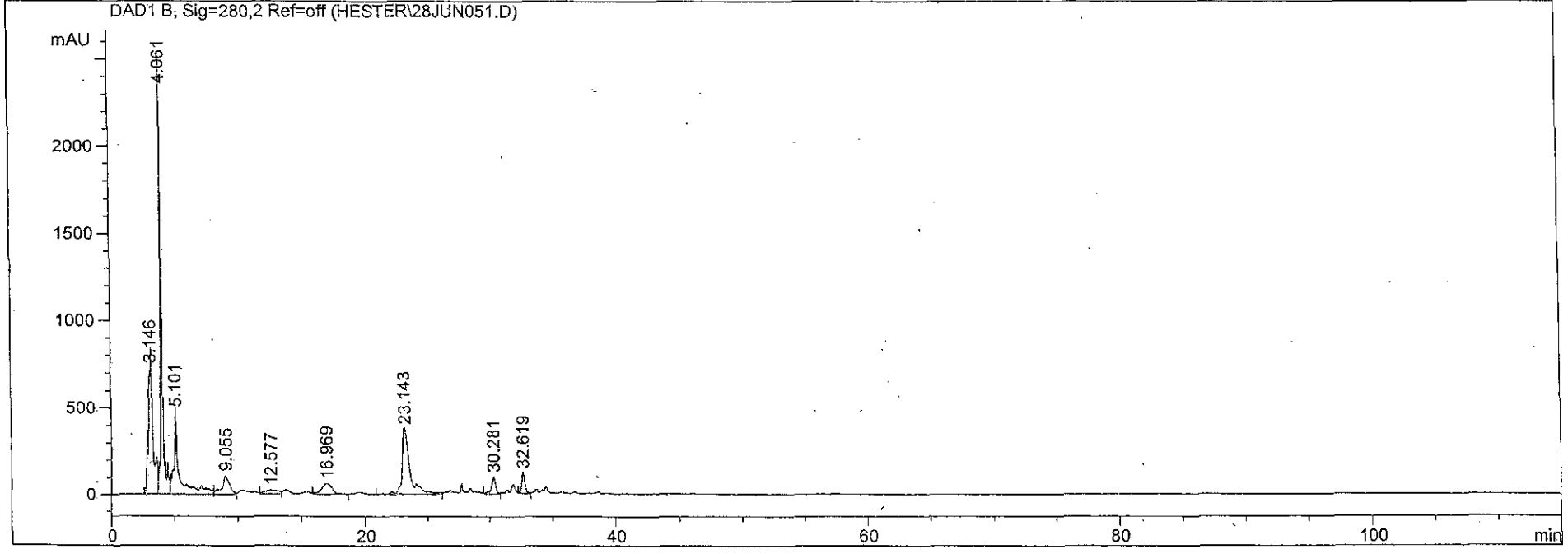

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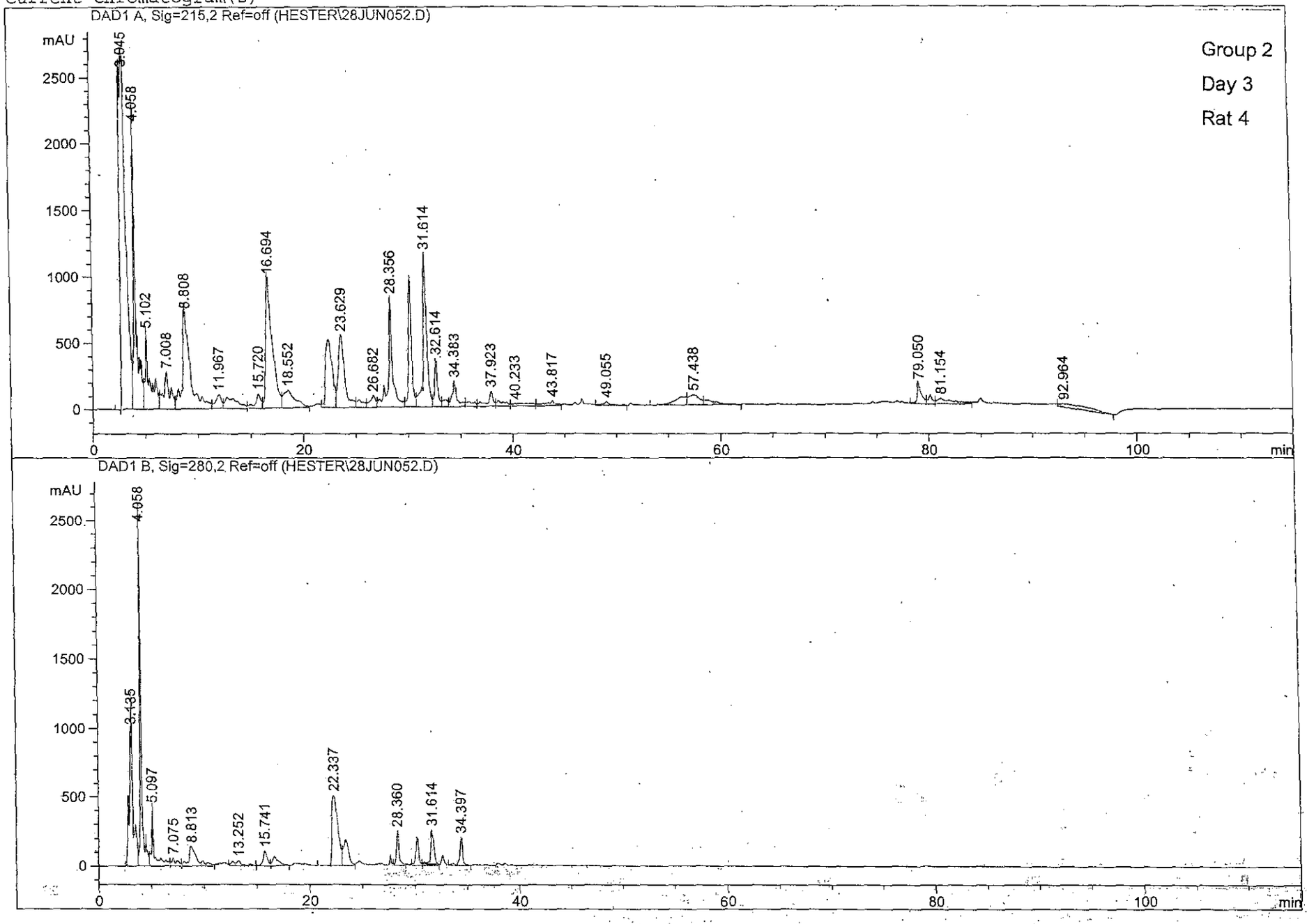
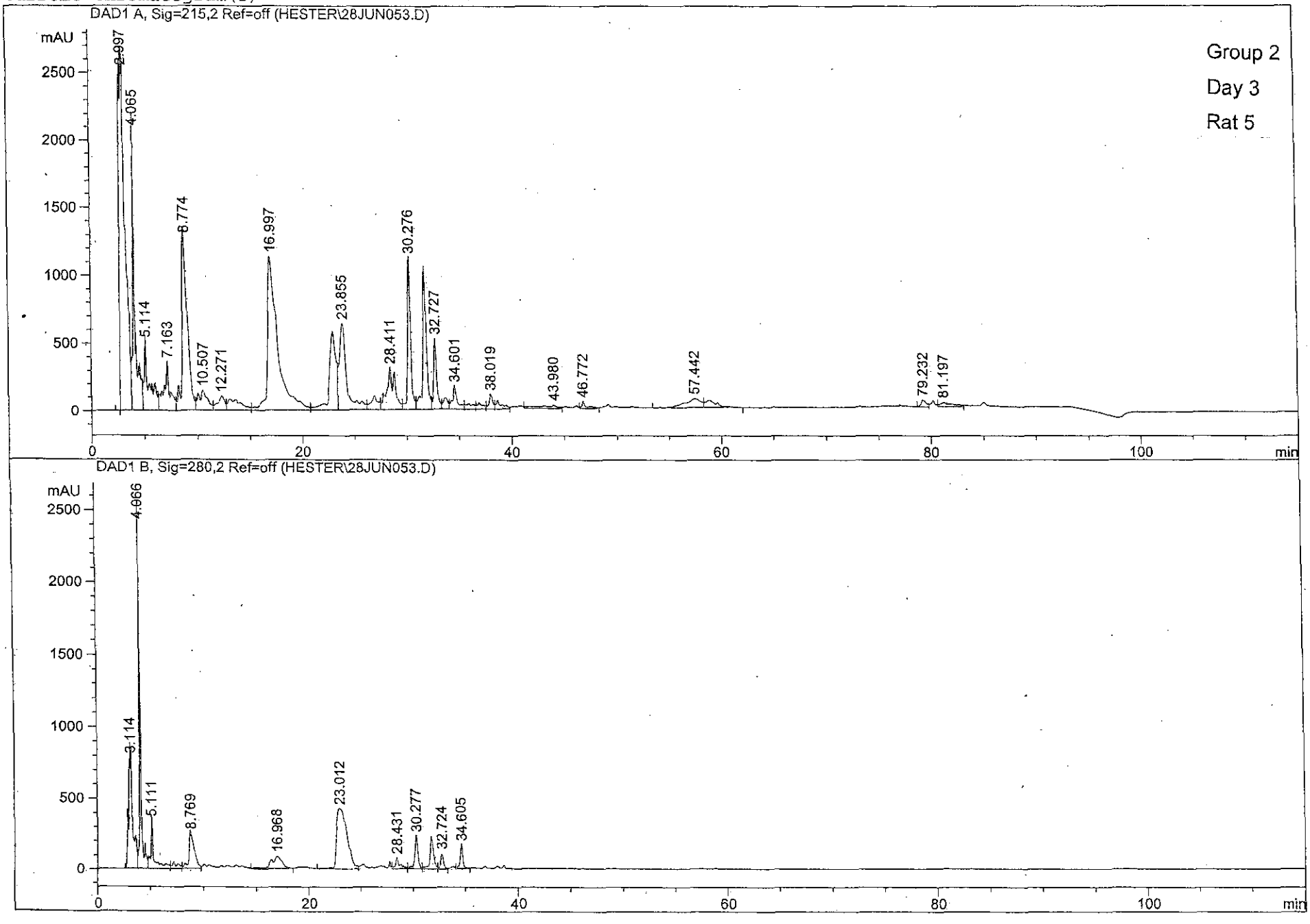
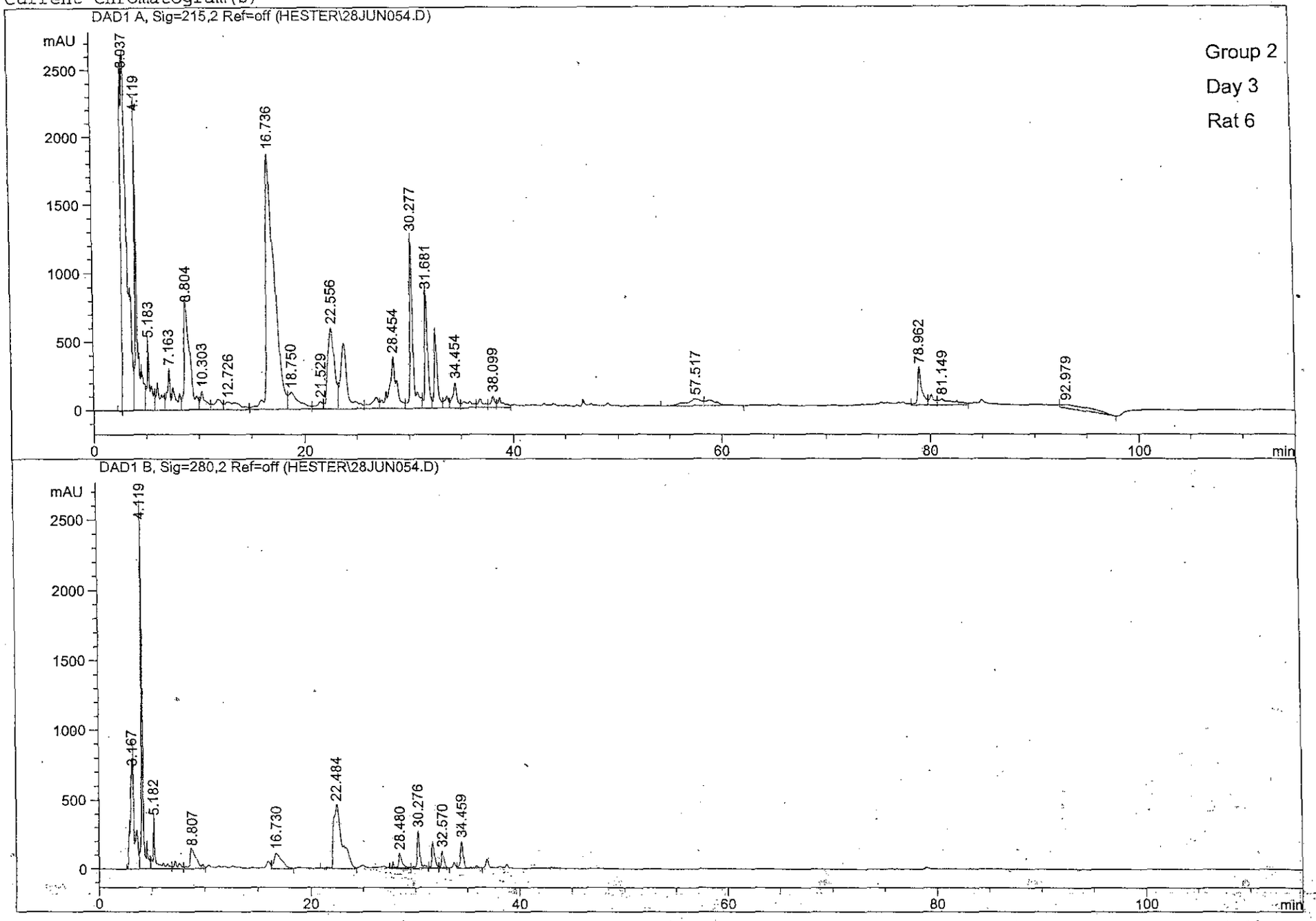


Current Chromatogram(s)



Current Chromatogram(s)



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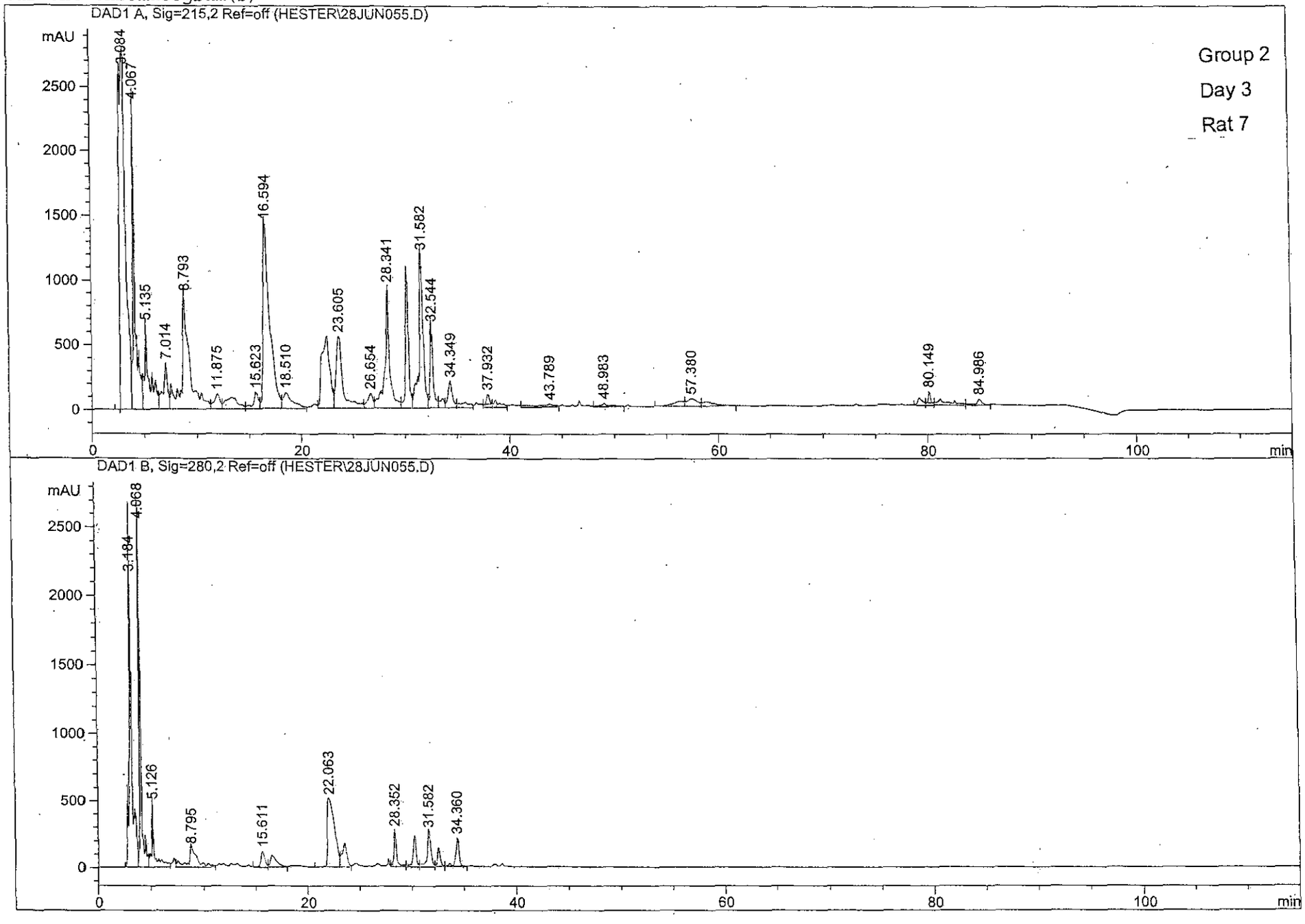

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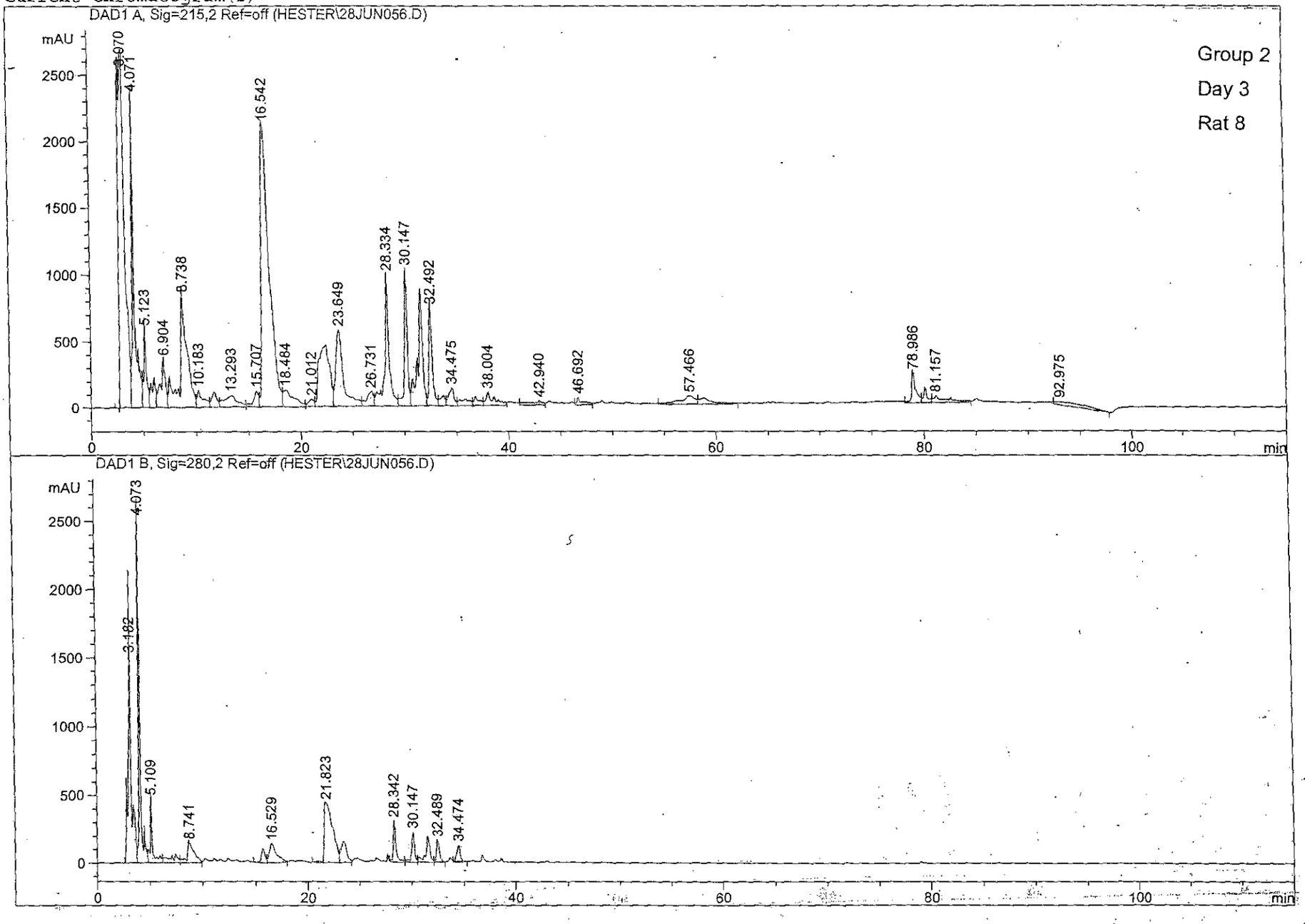

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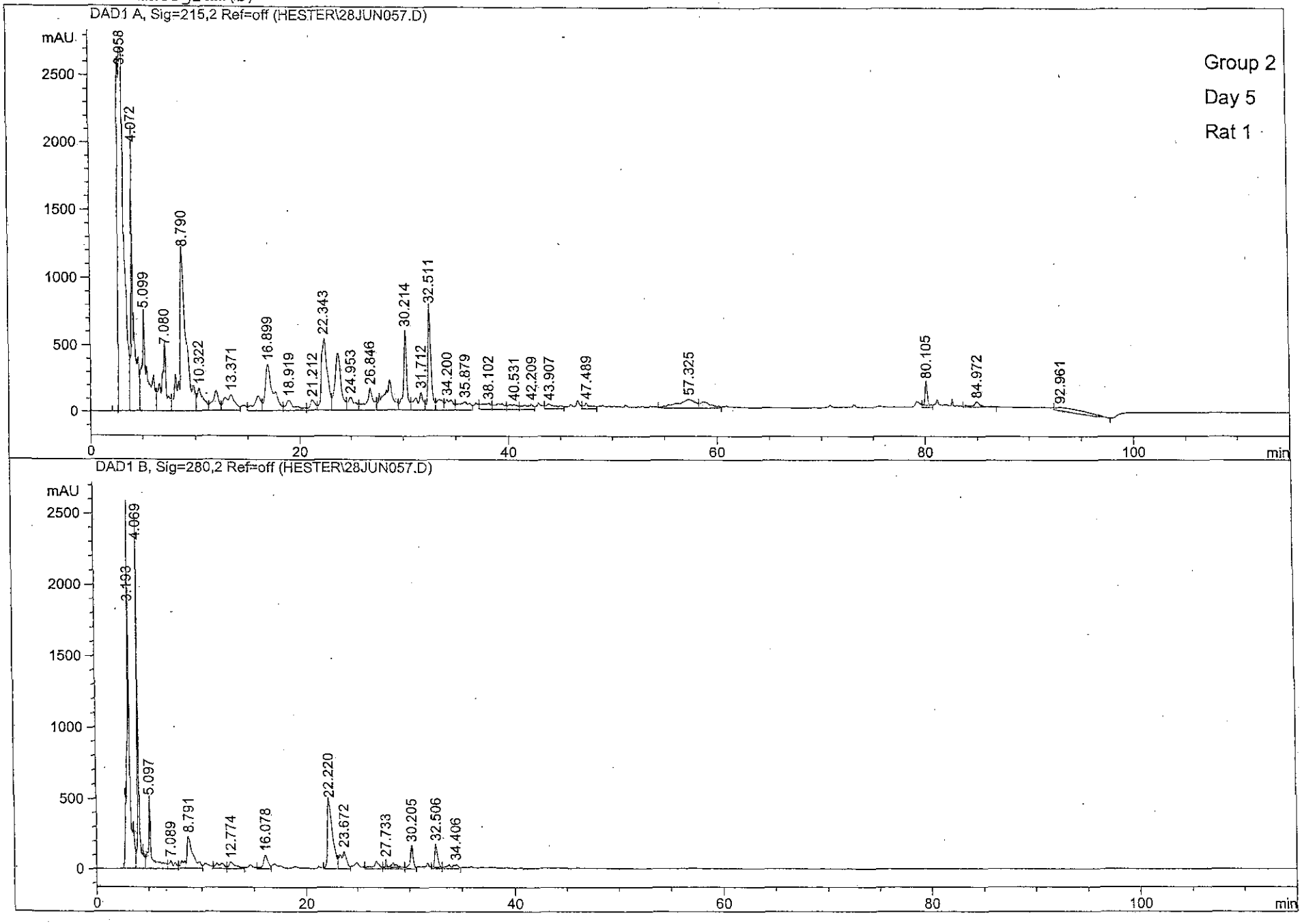

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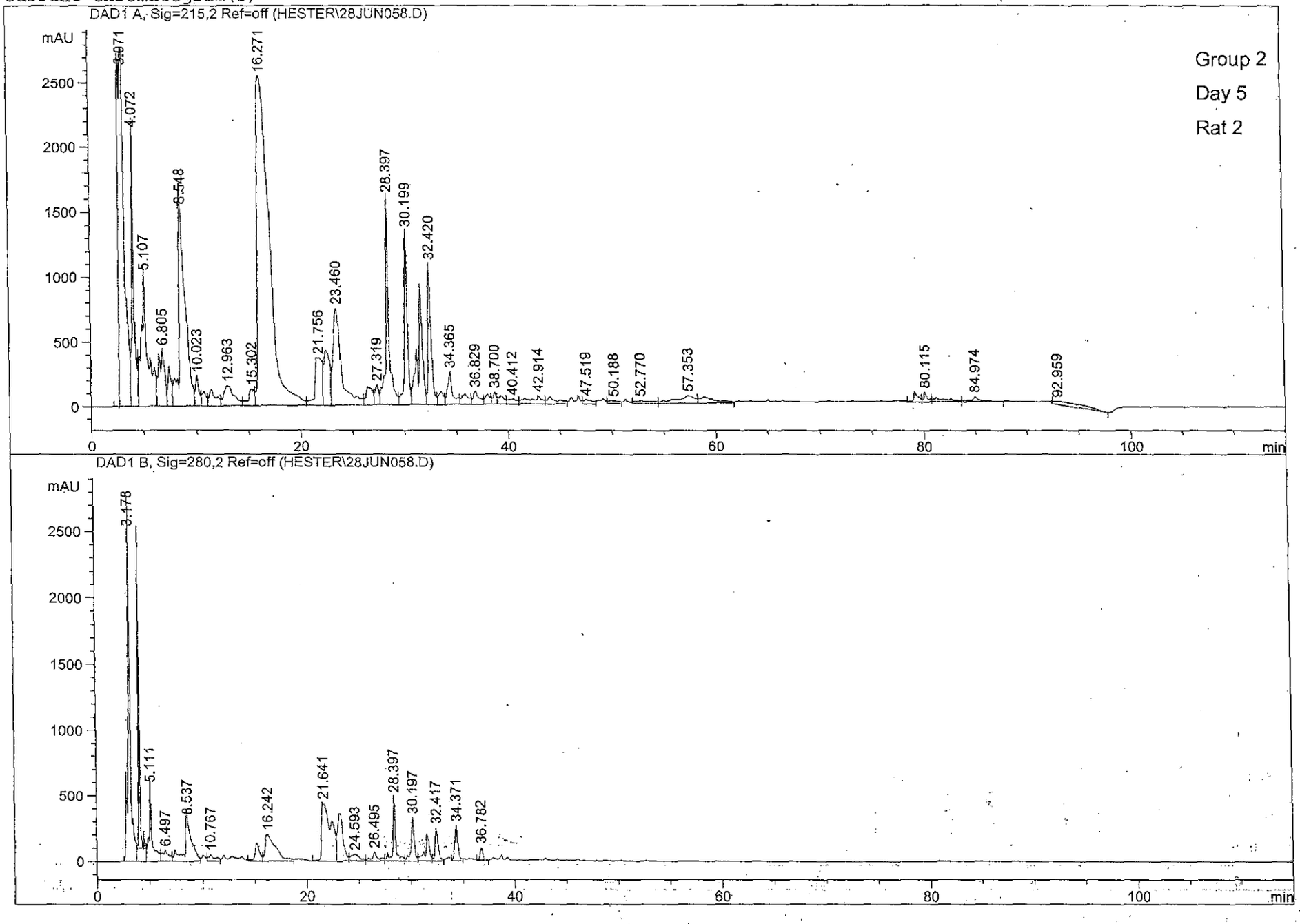

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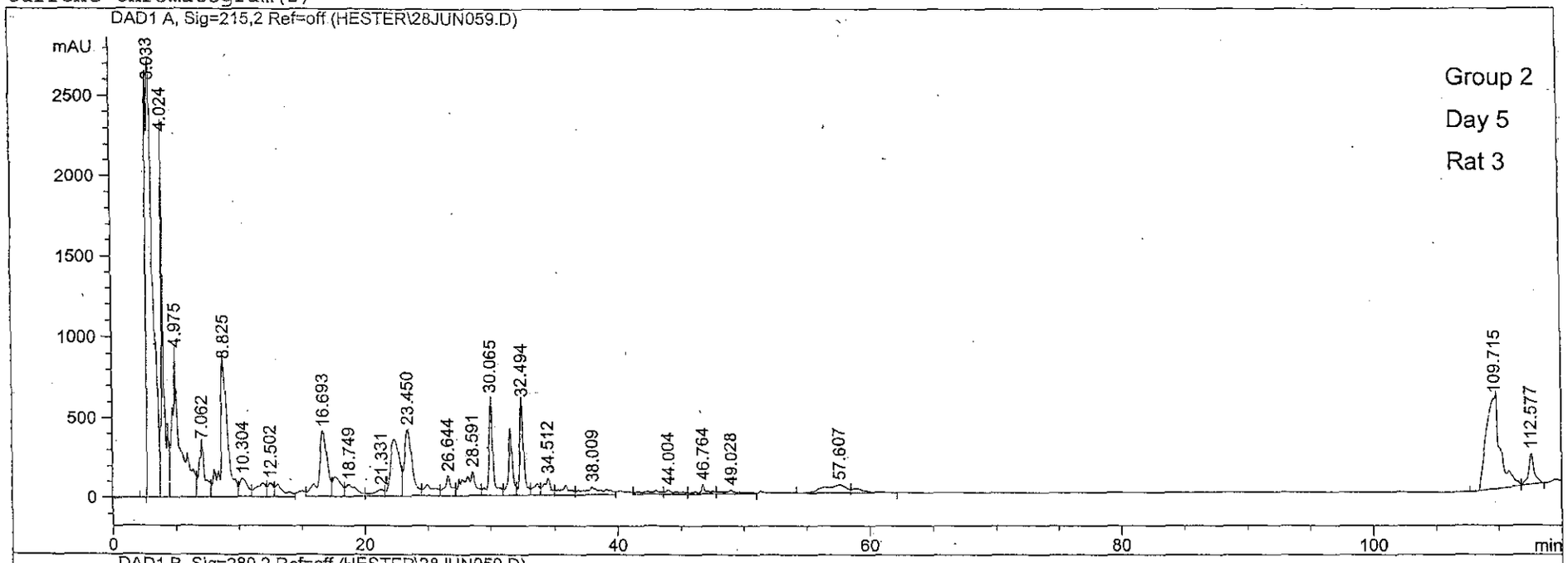


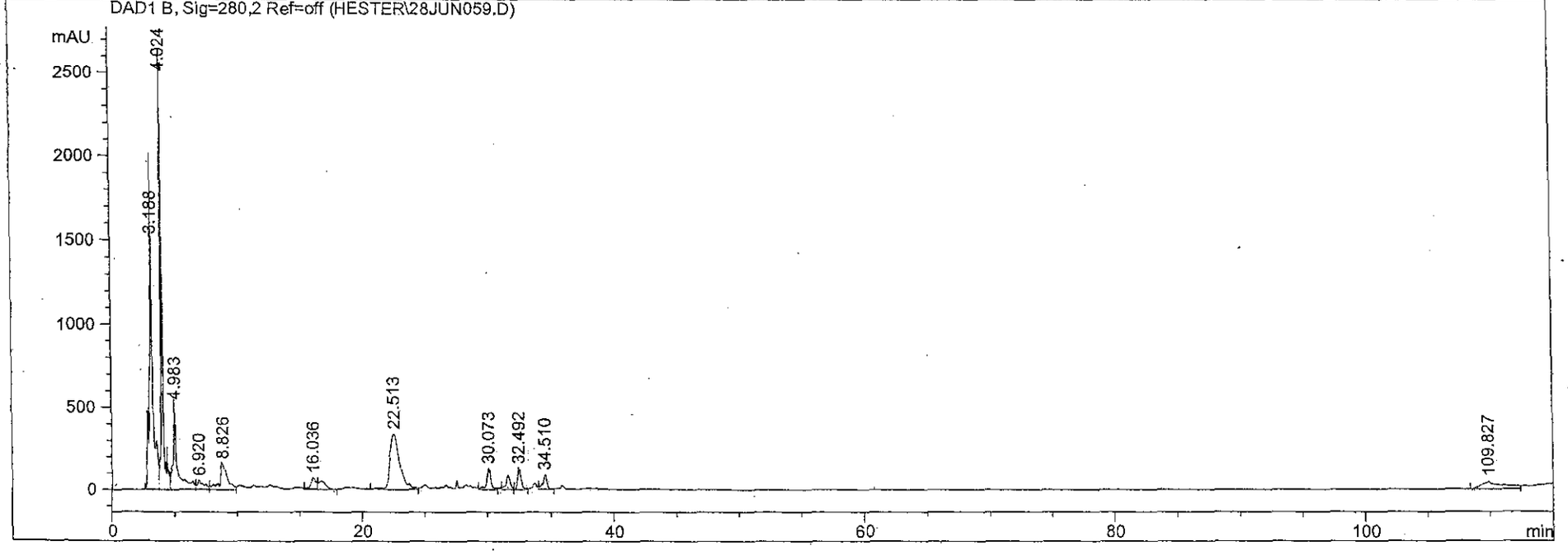

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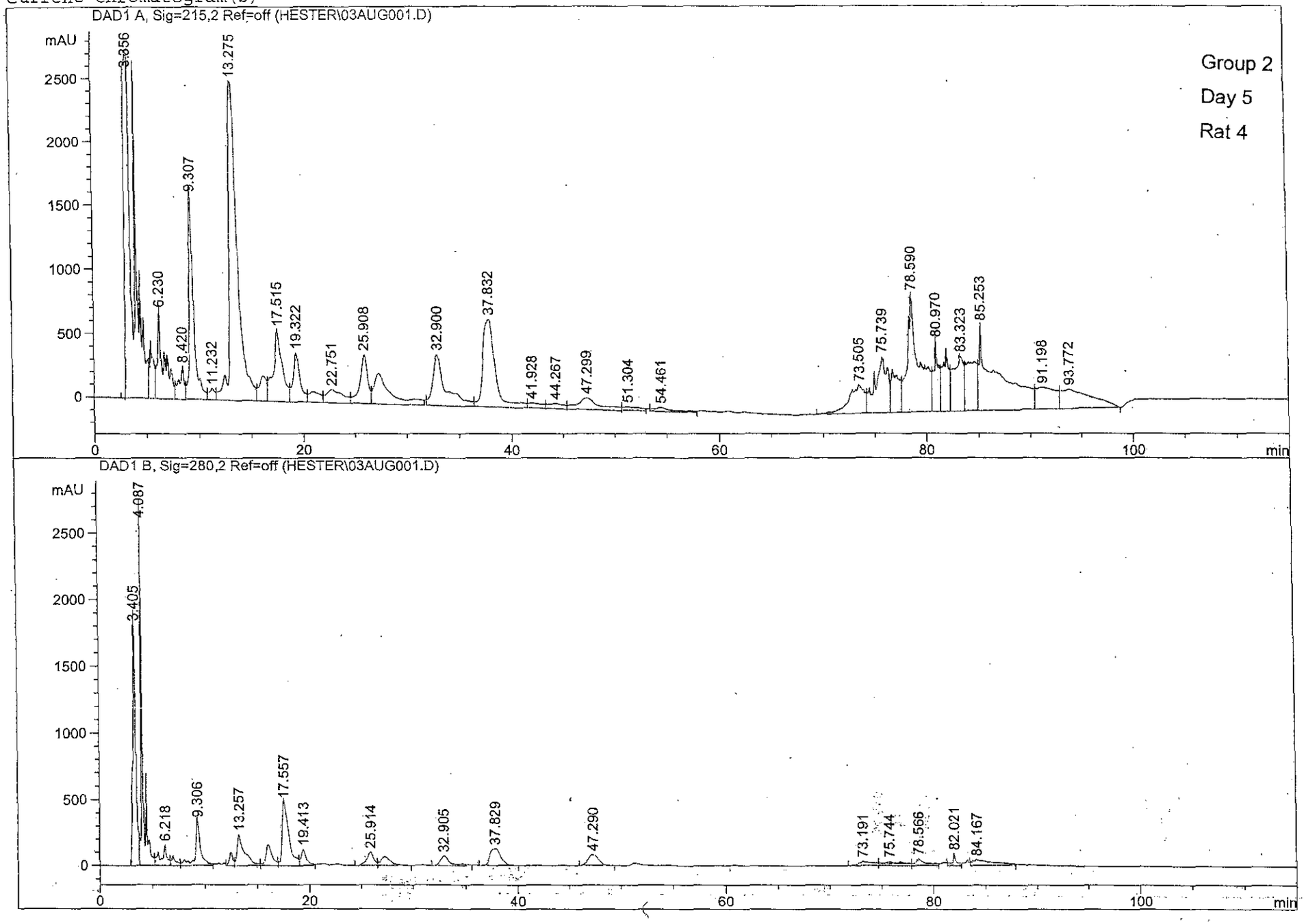

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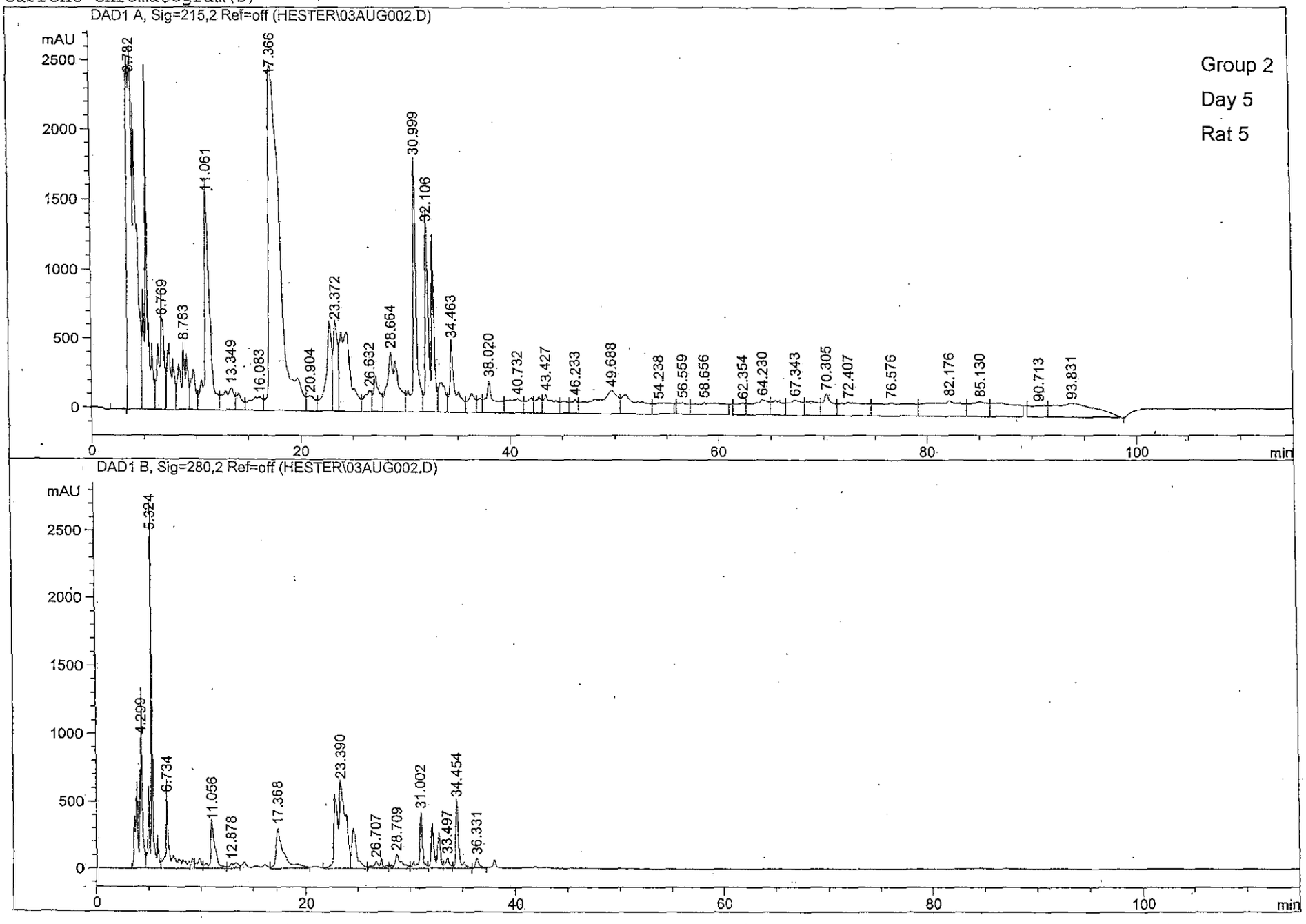

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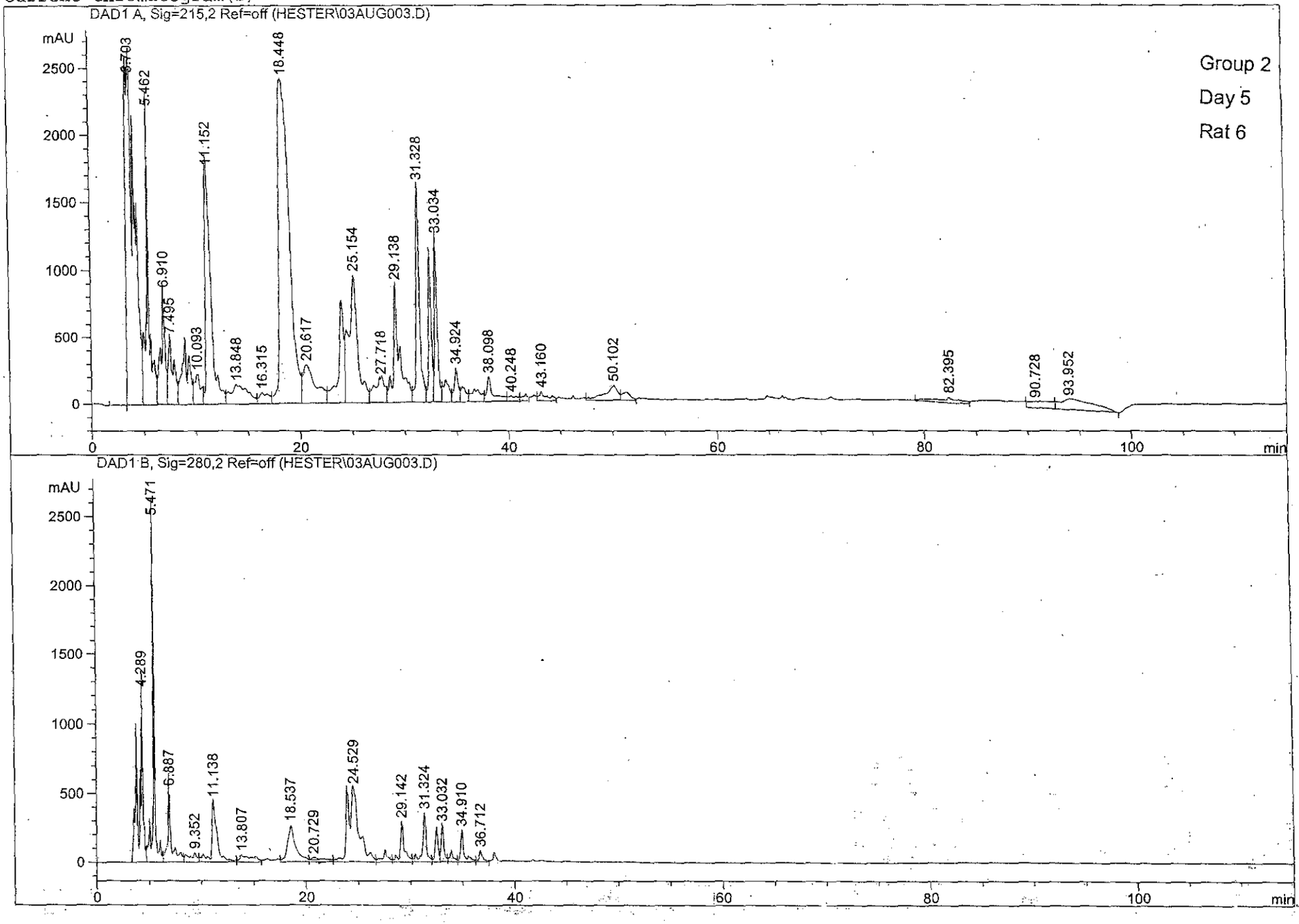

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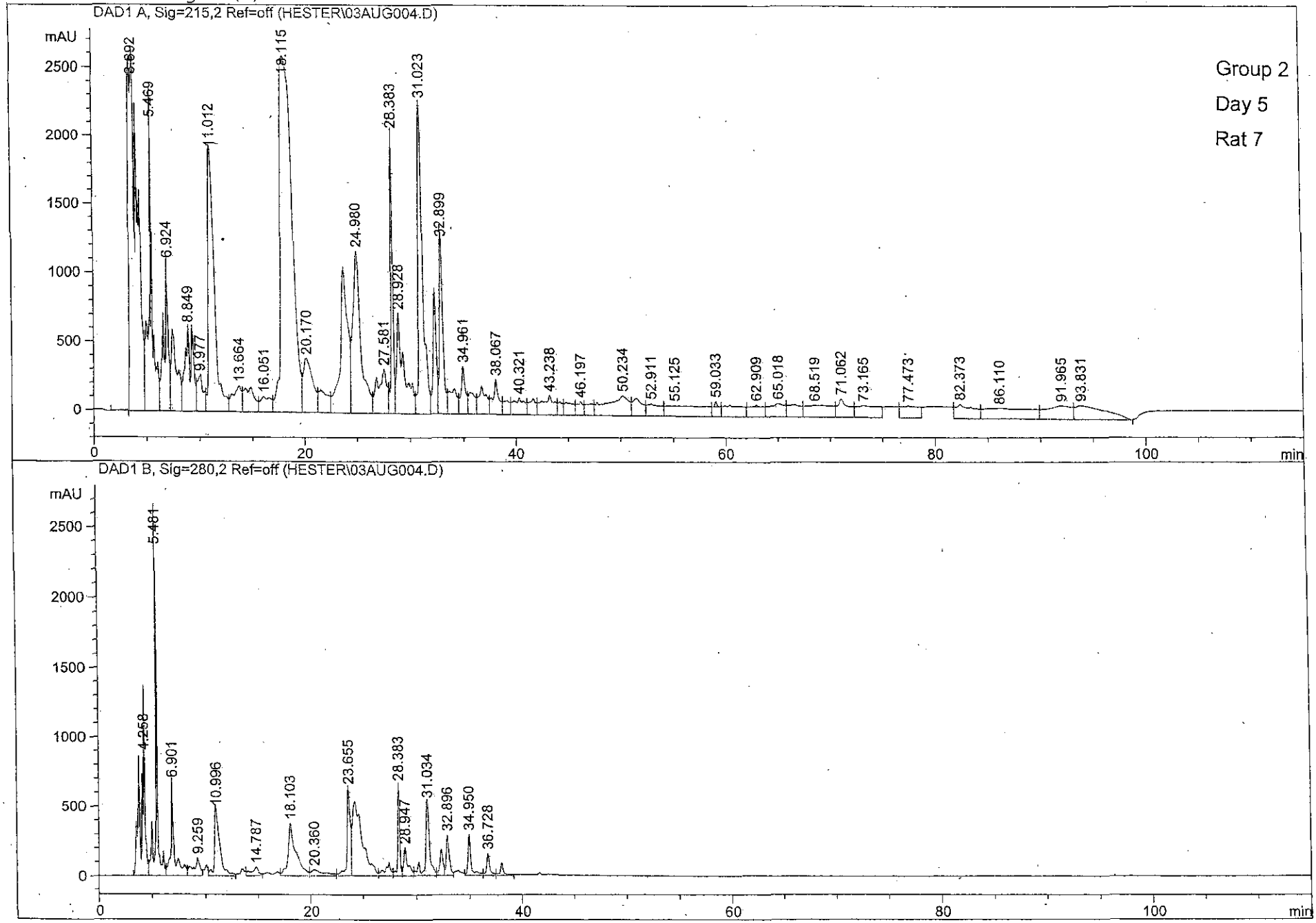
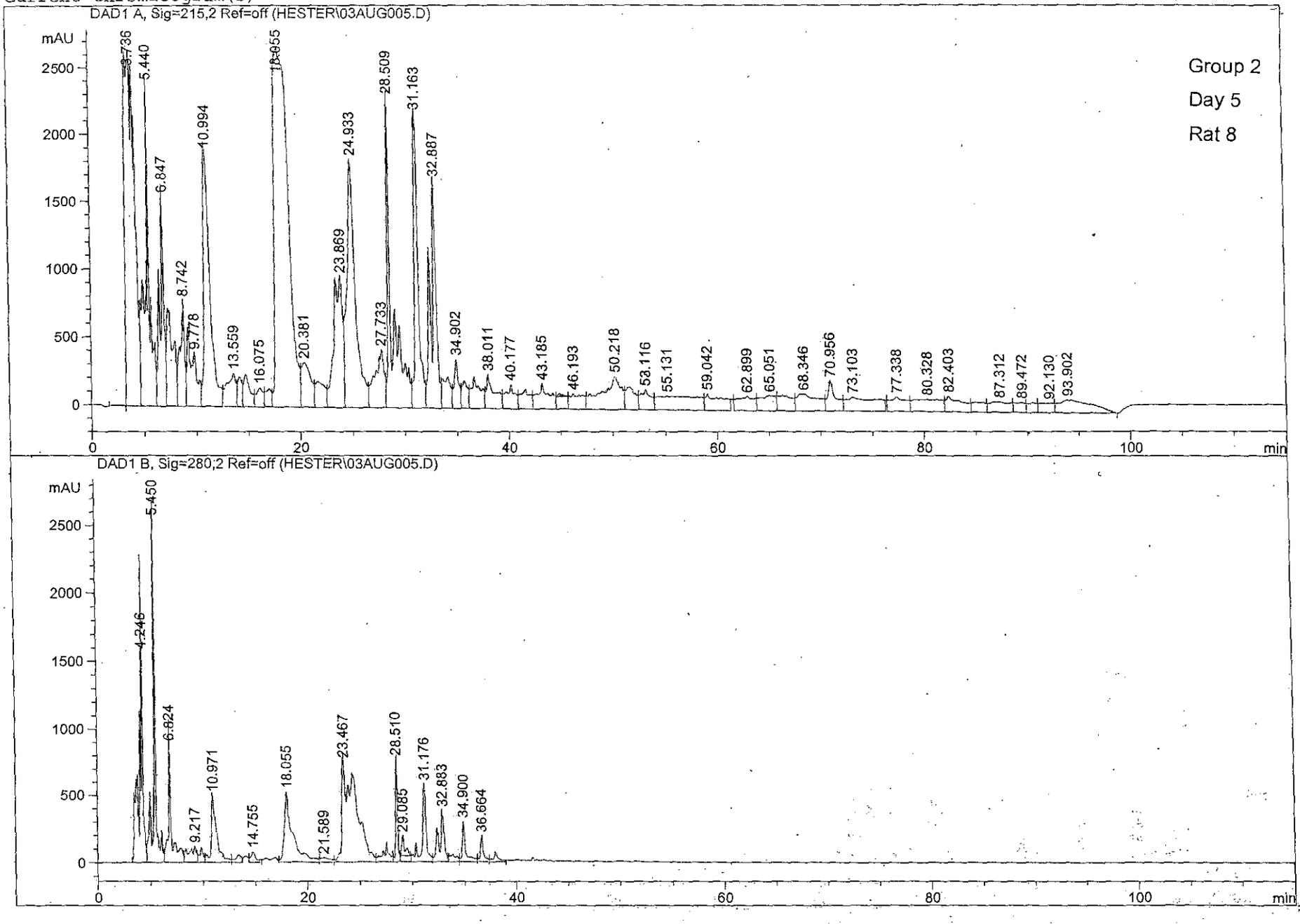
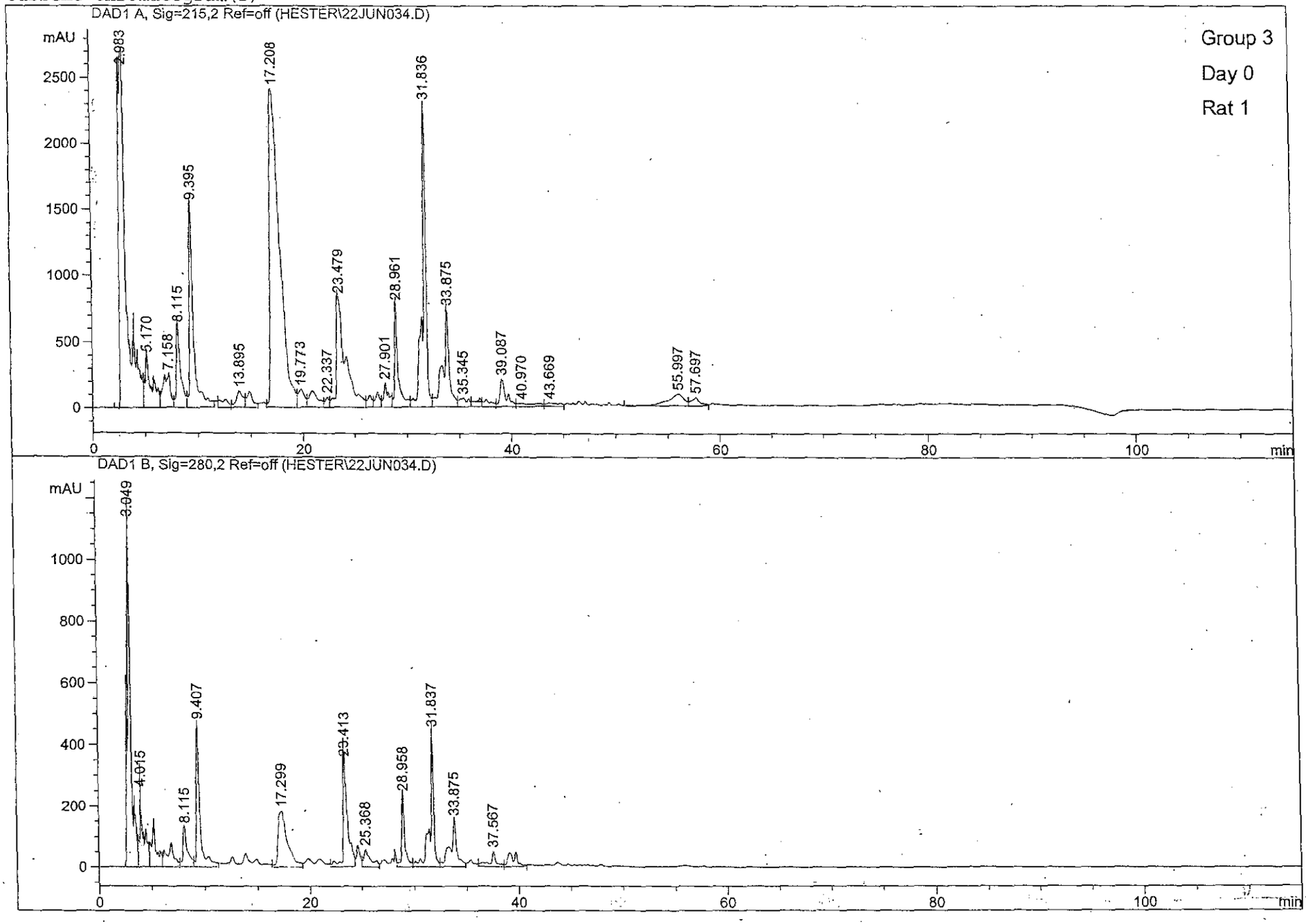


Current Chromatogram(s)



## Normal diet and vehicle (control)

Current Chromatogram(s)



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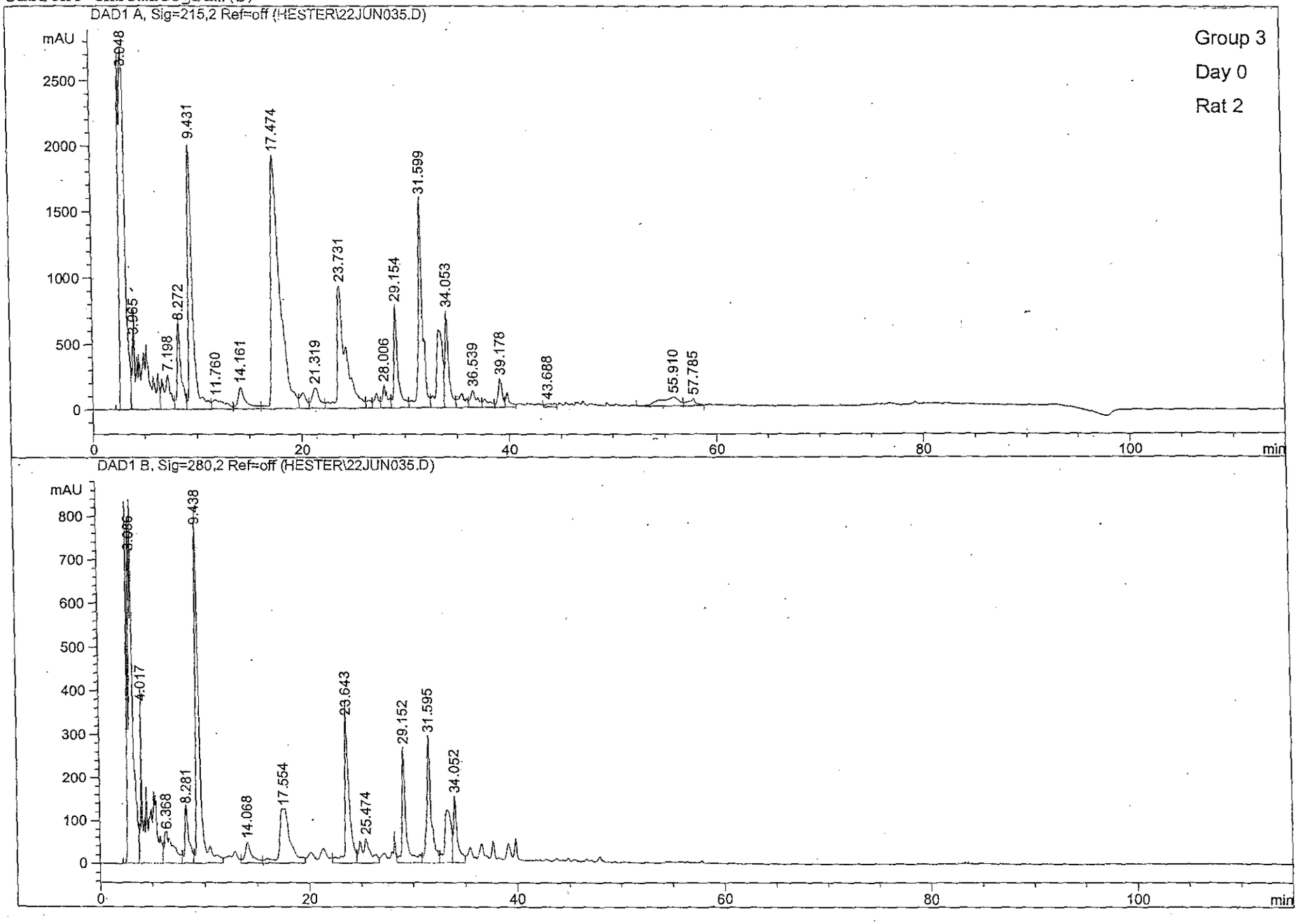

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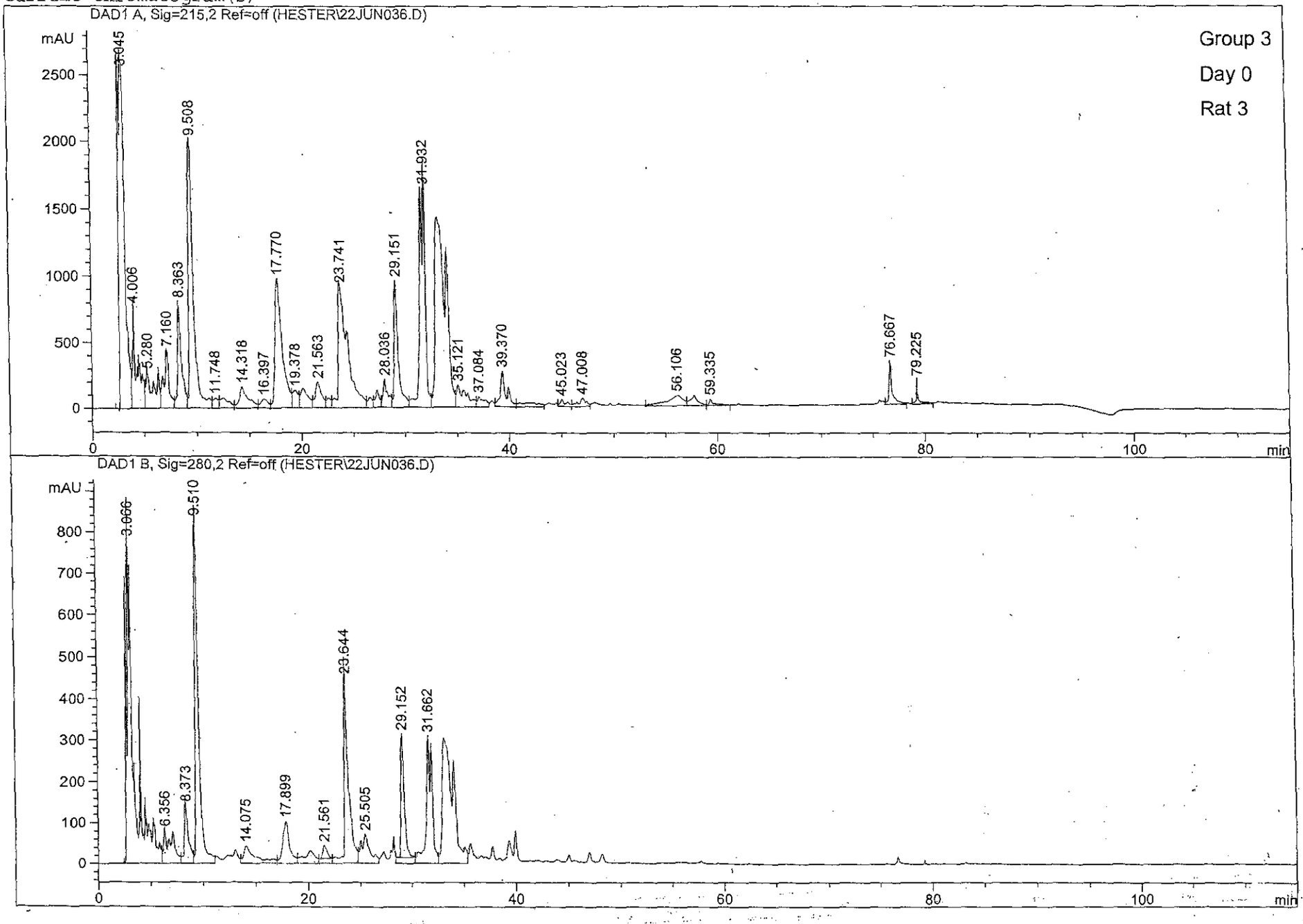

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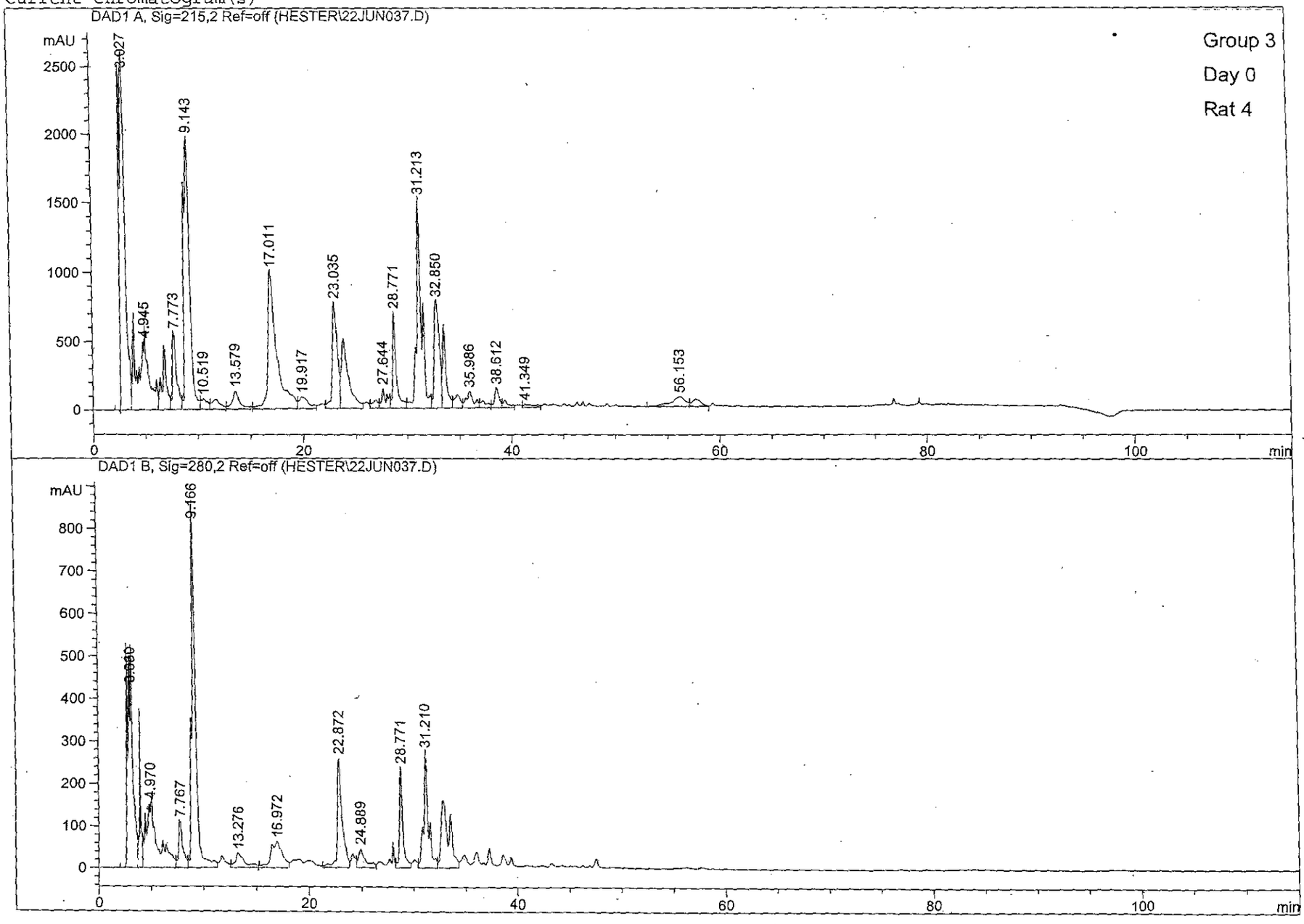

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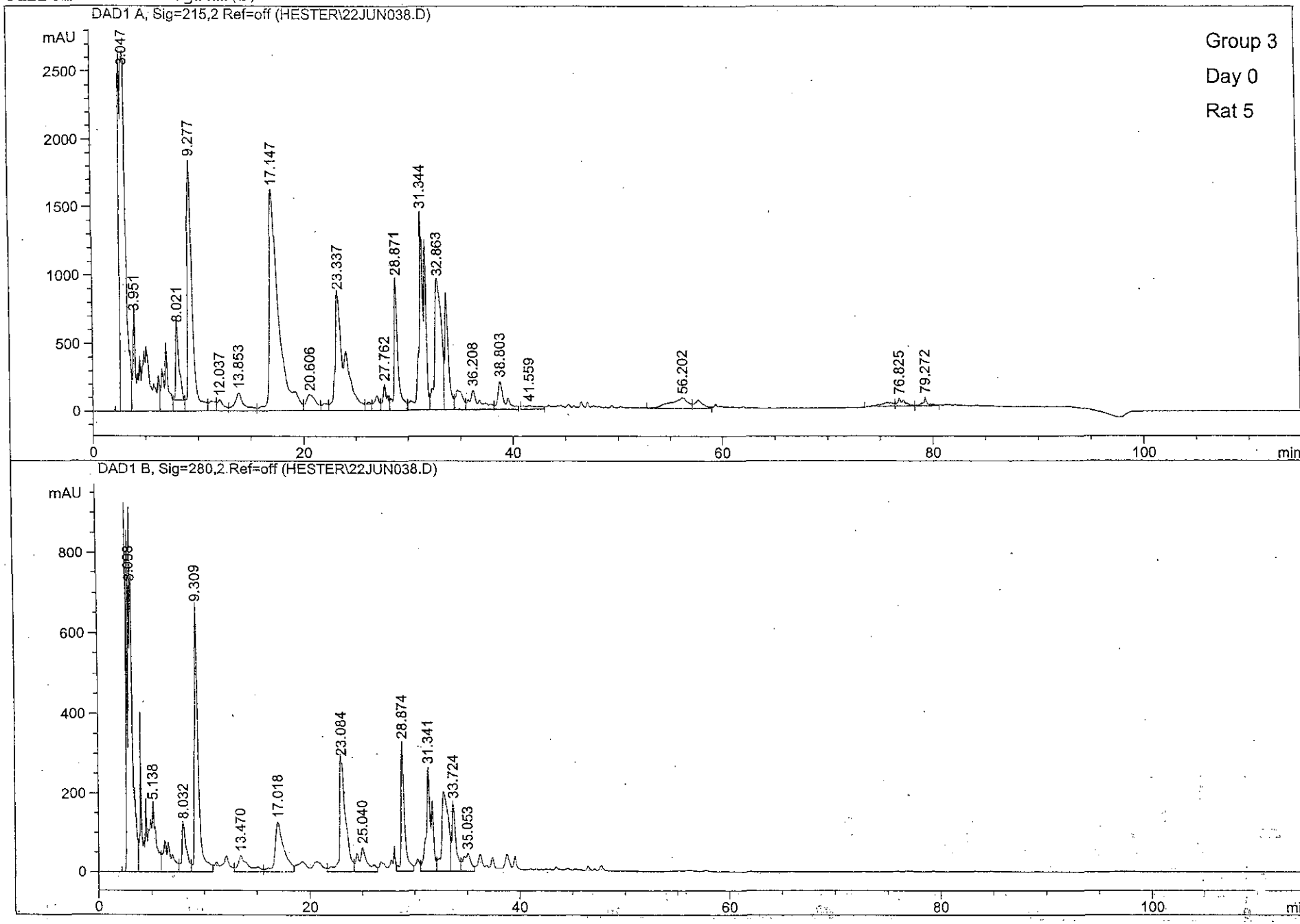

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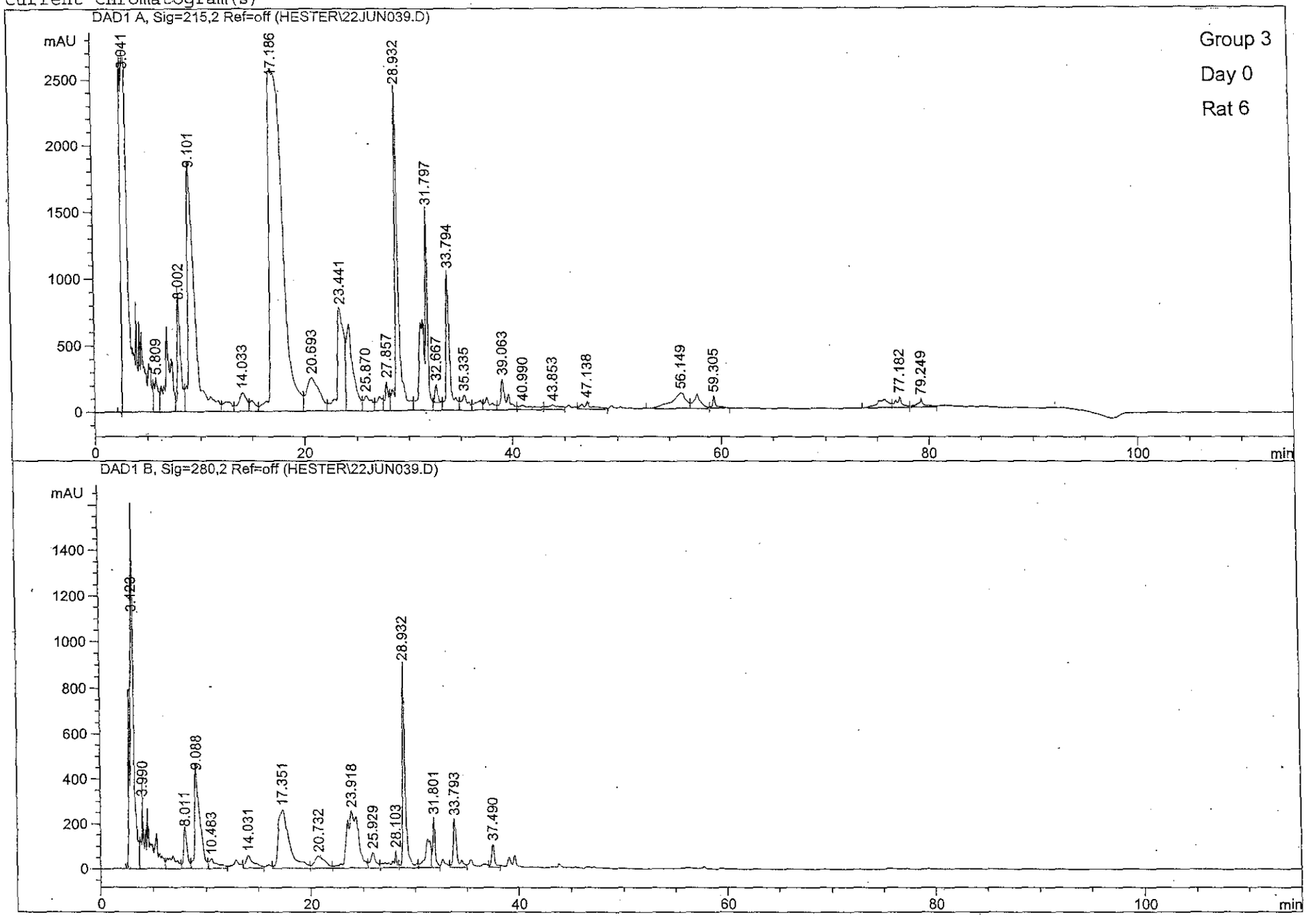

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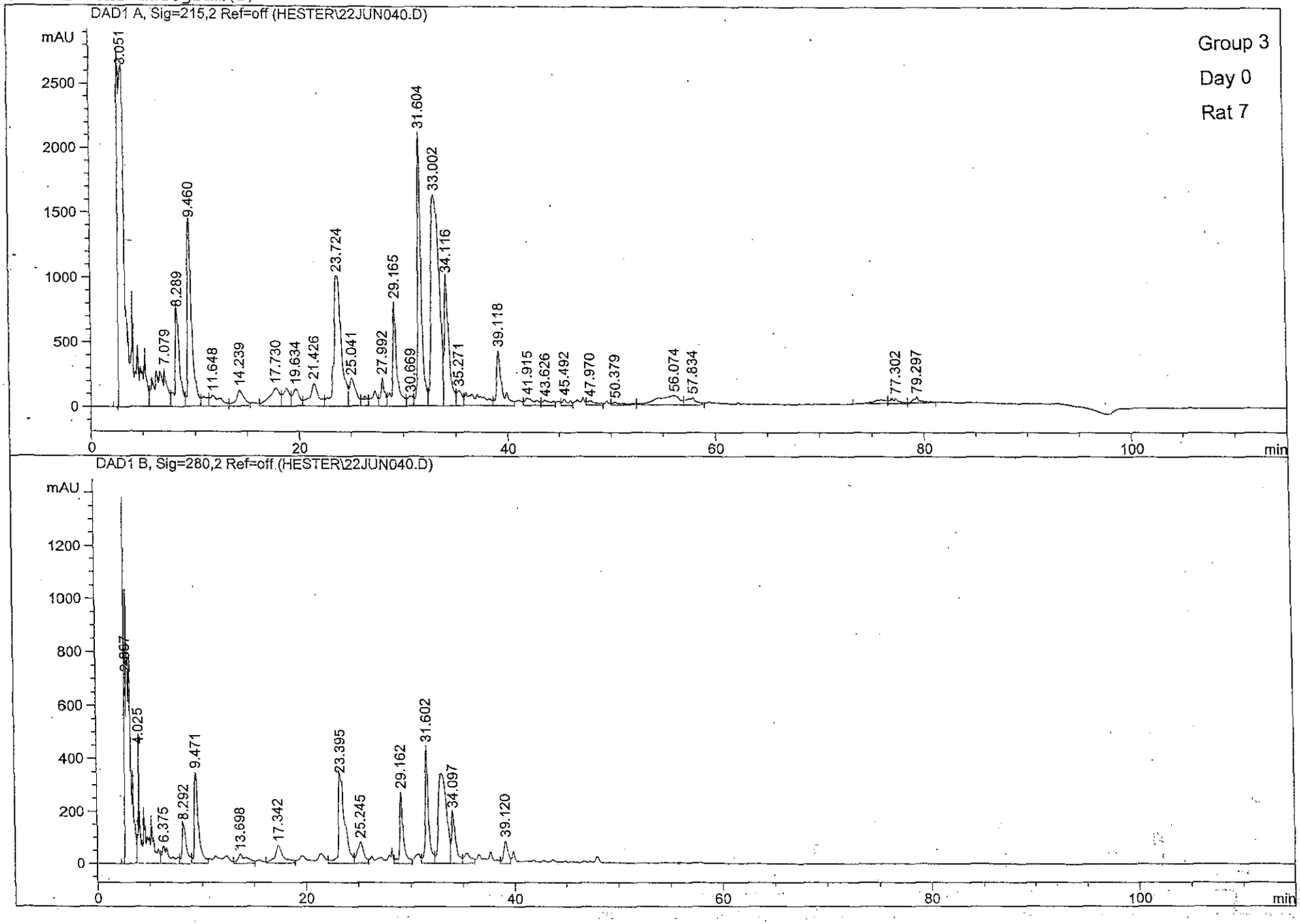

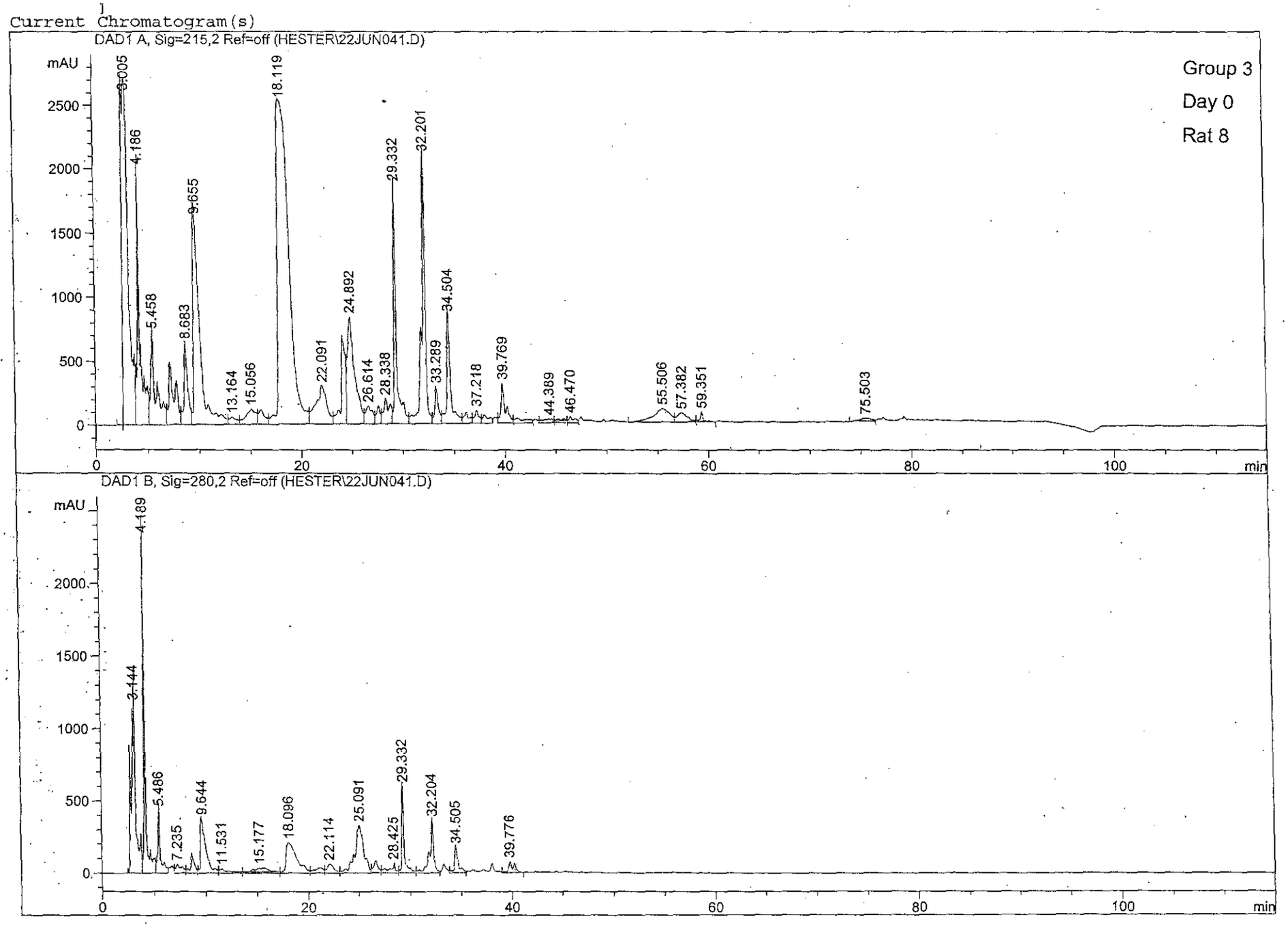
Current Chromatogram(s)
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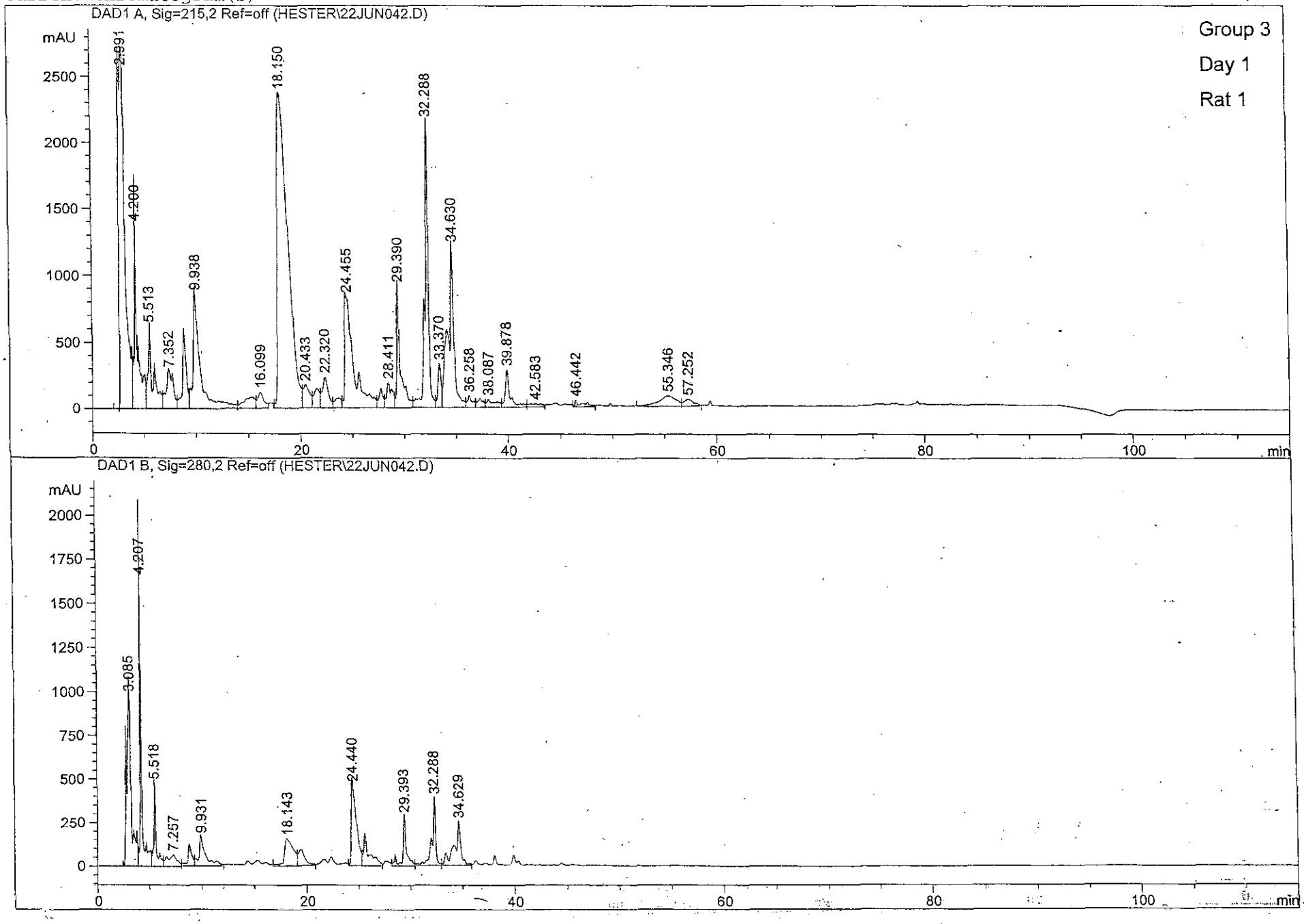
Current Chromatogram(s)



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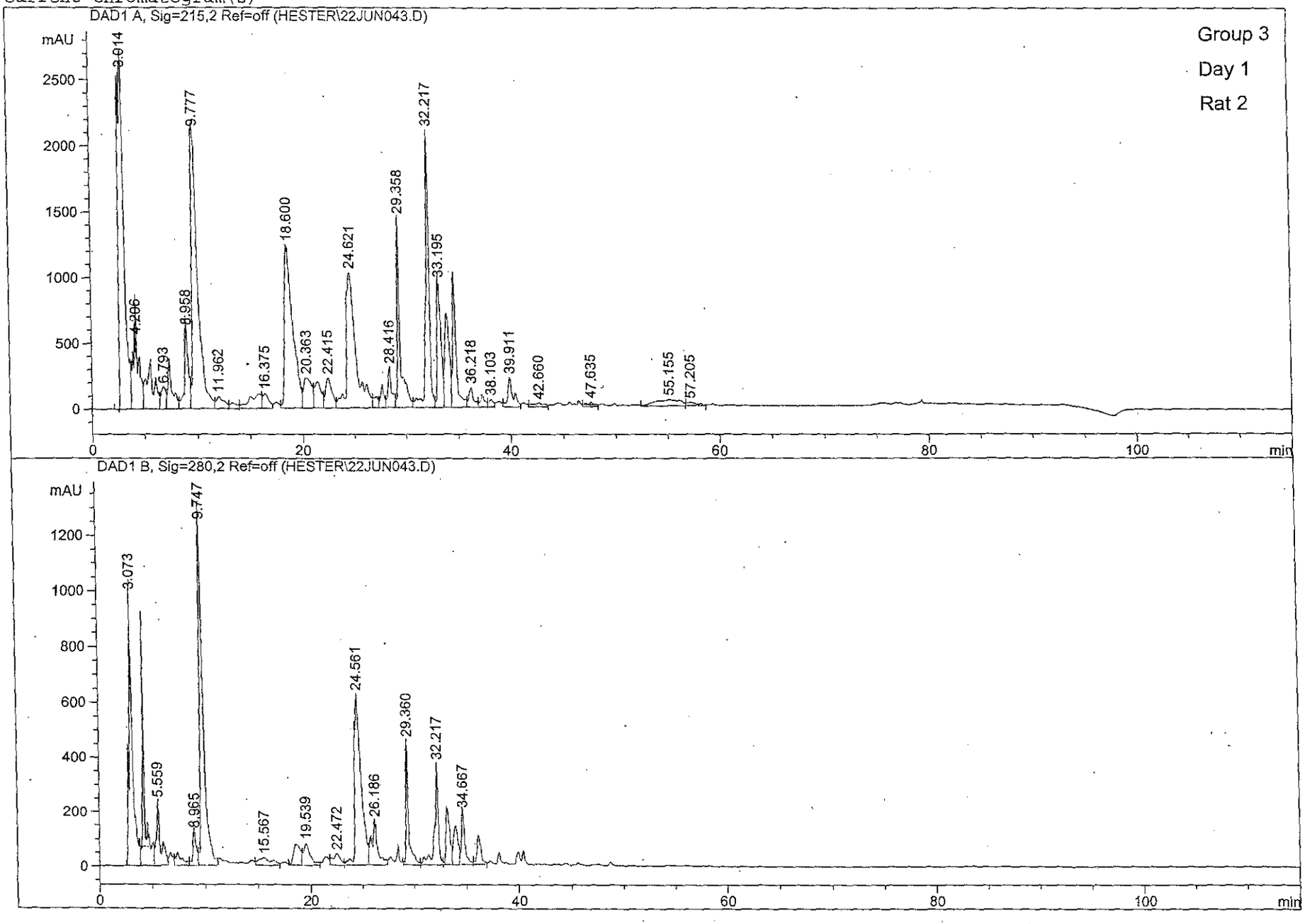

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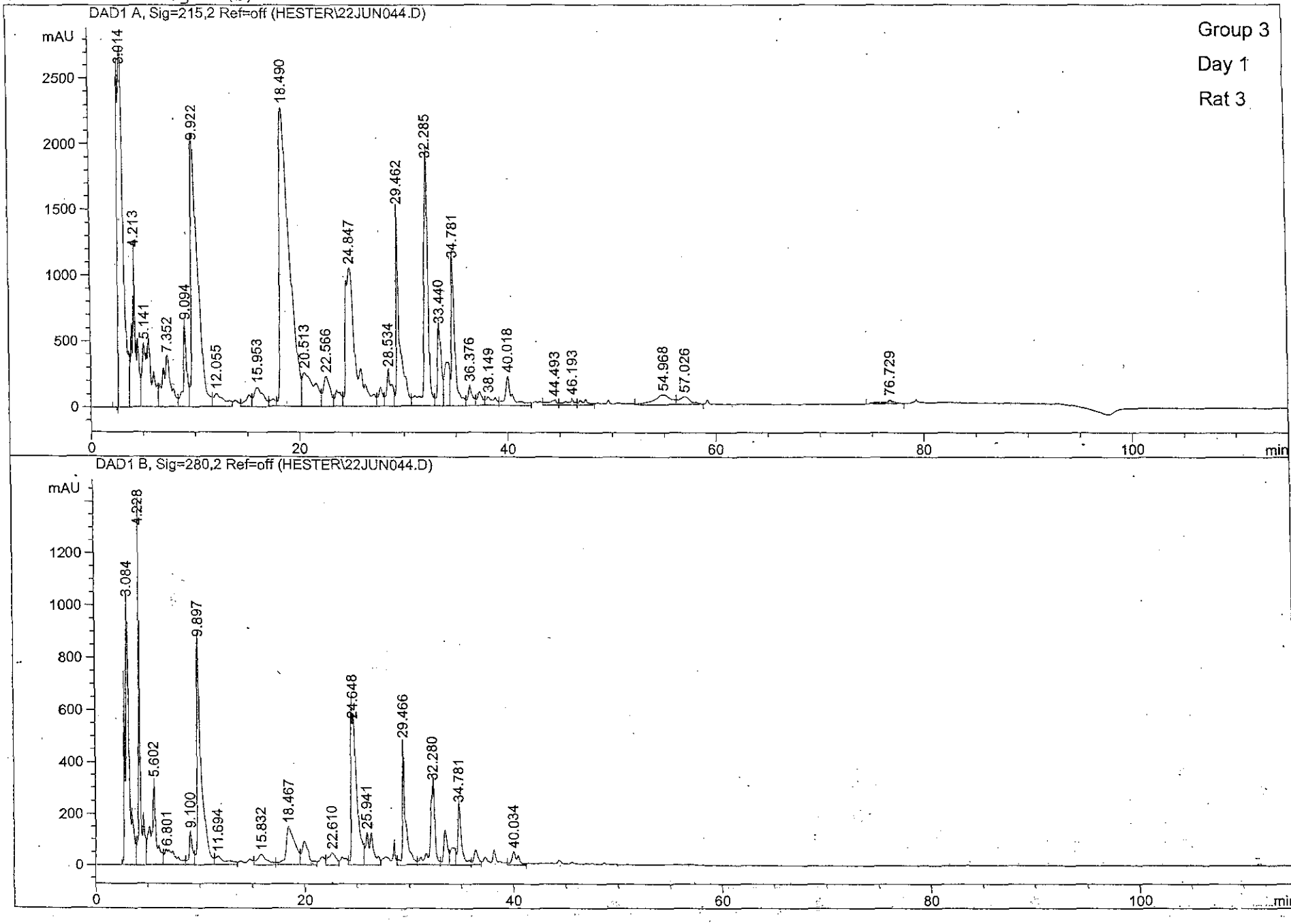

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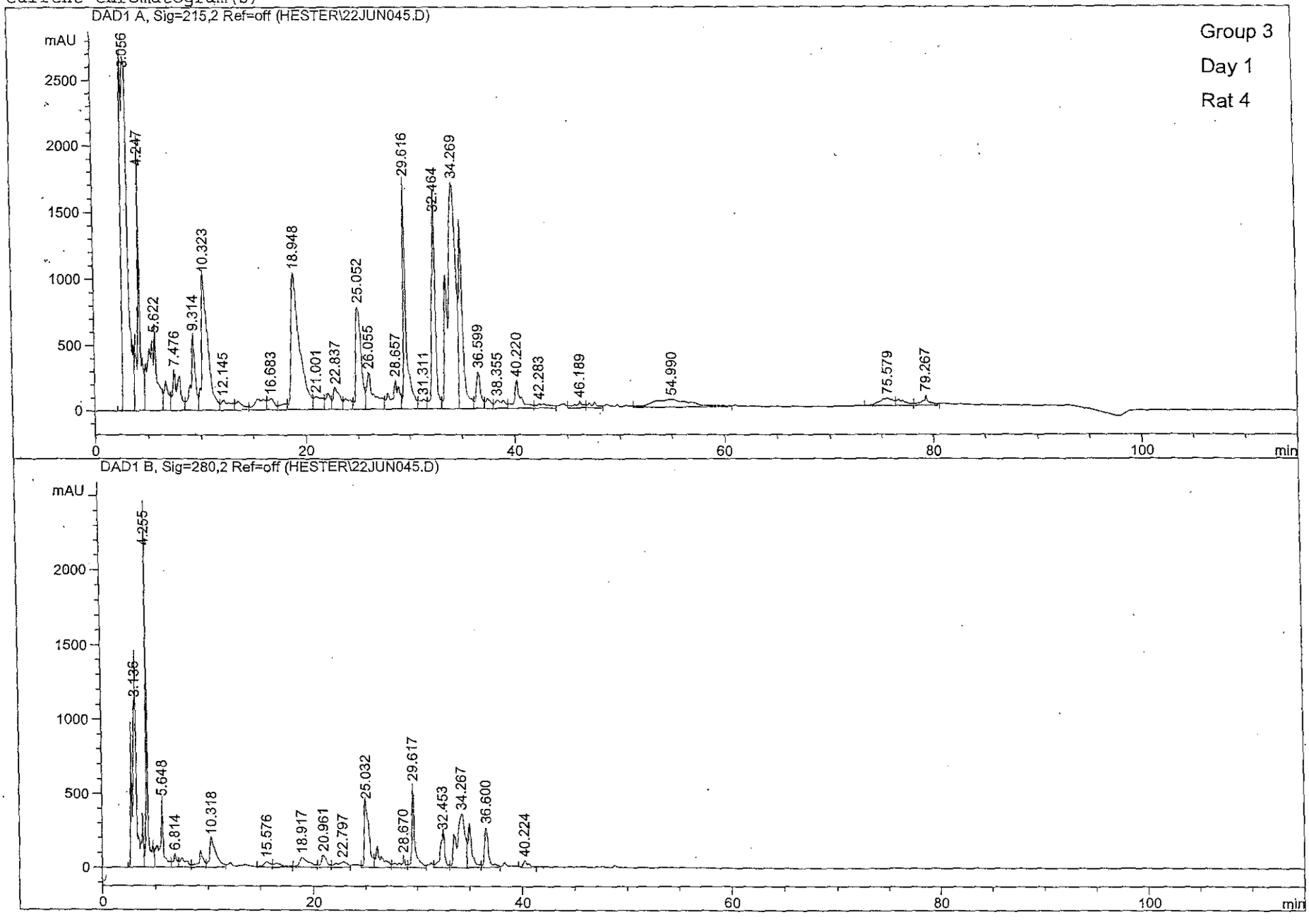

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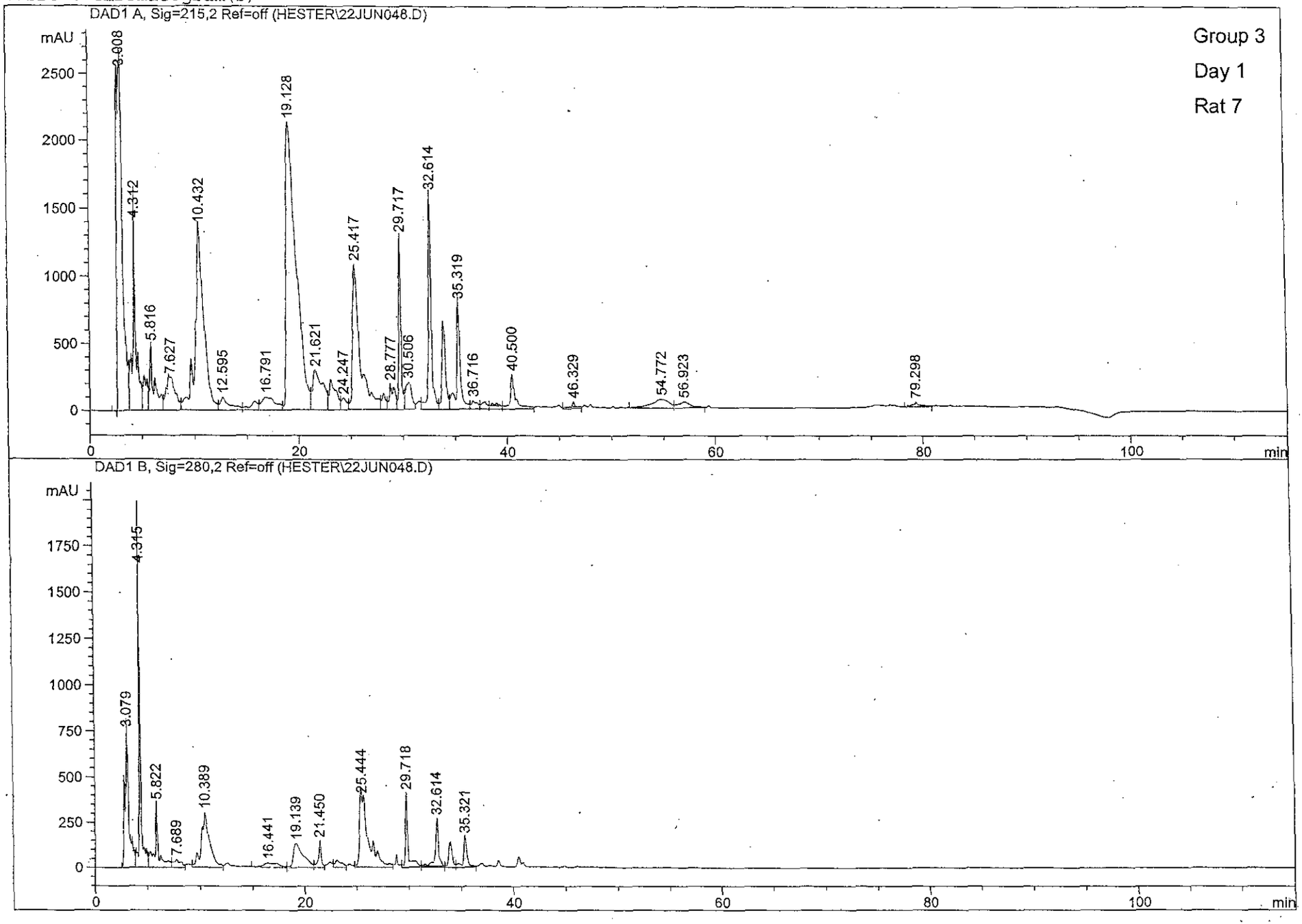

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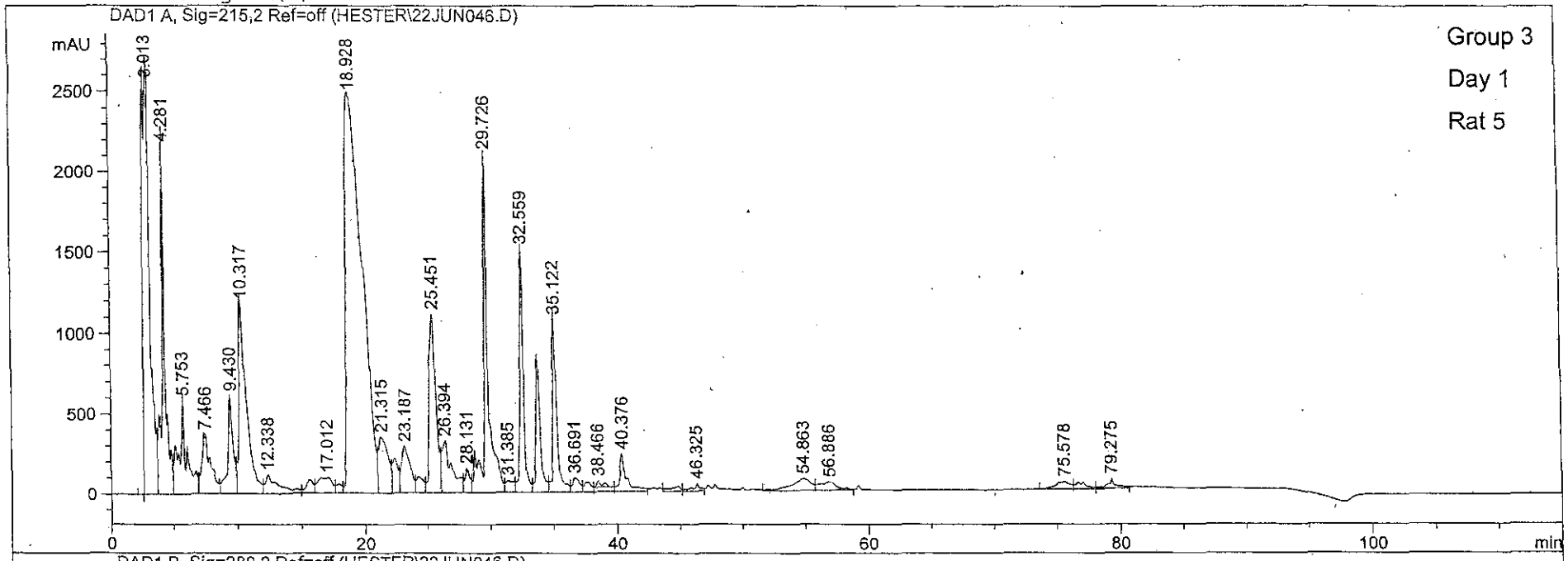


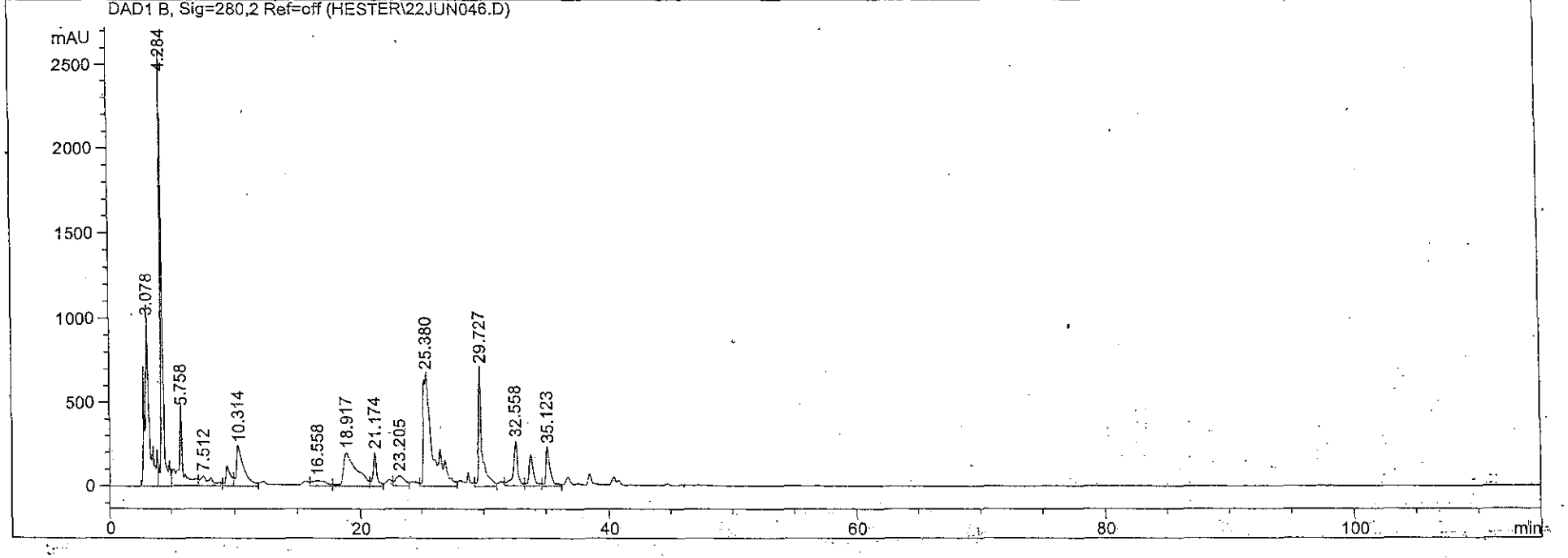

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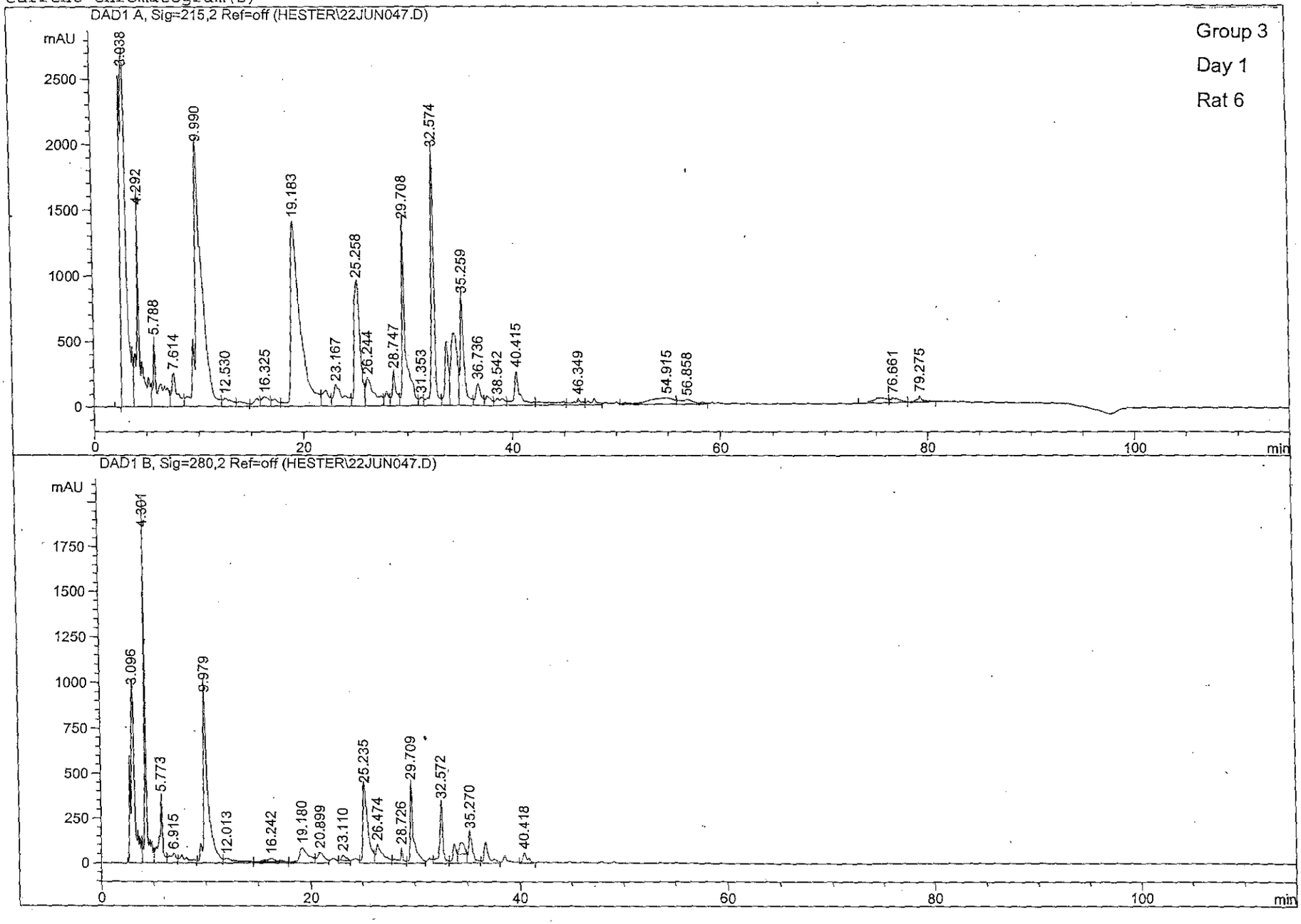

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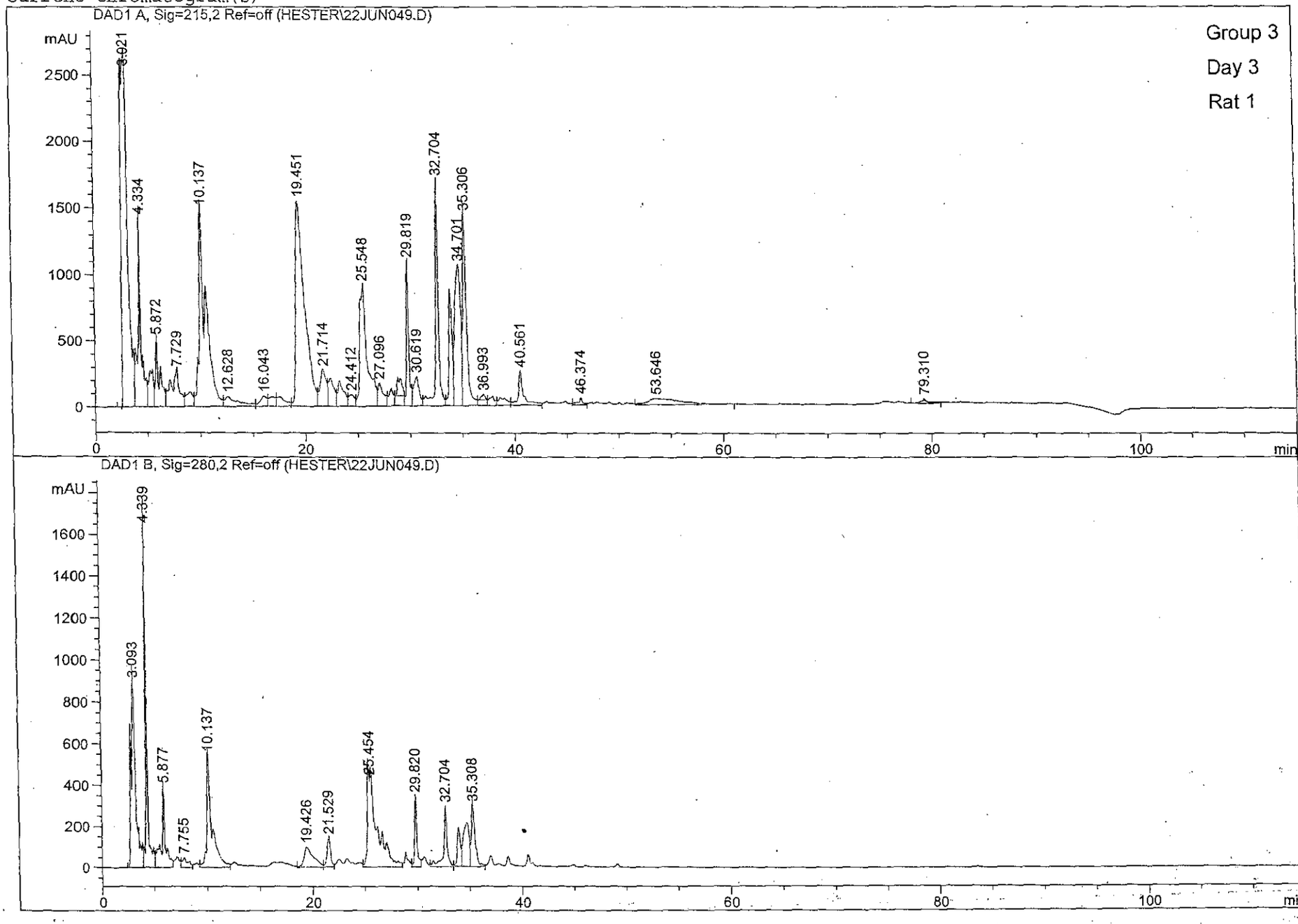

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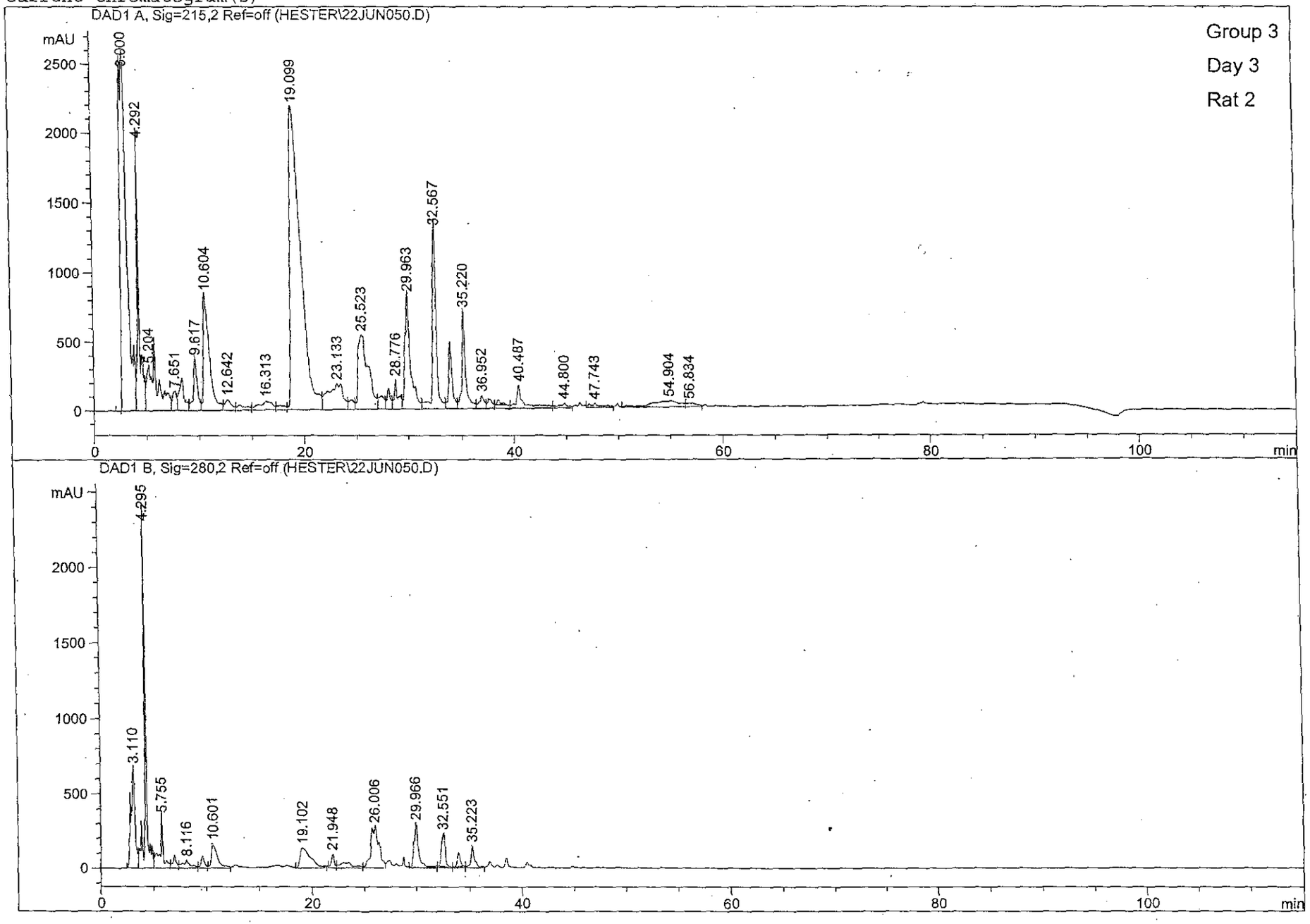

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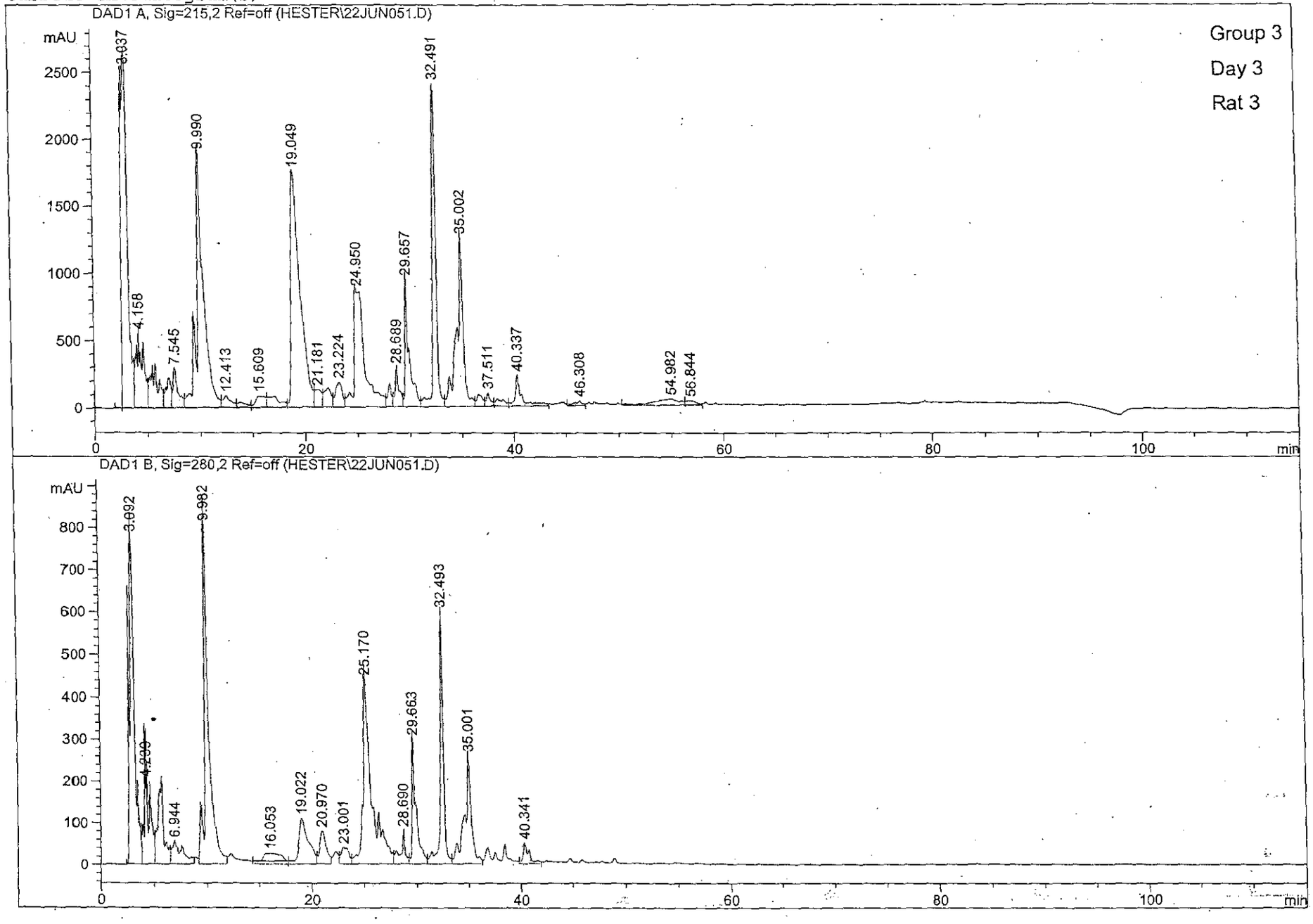

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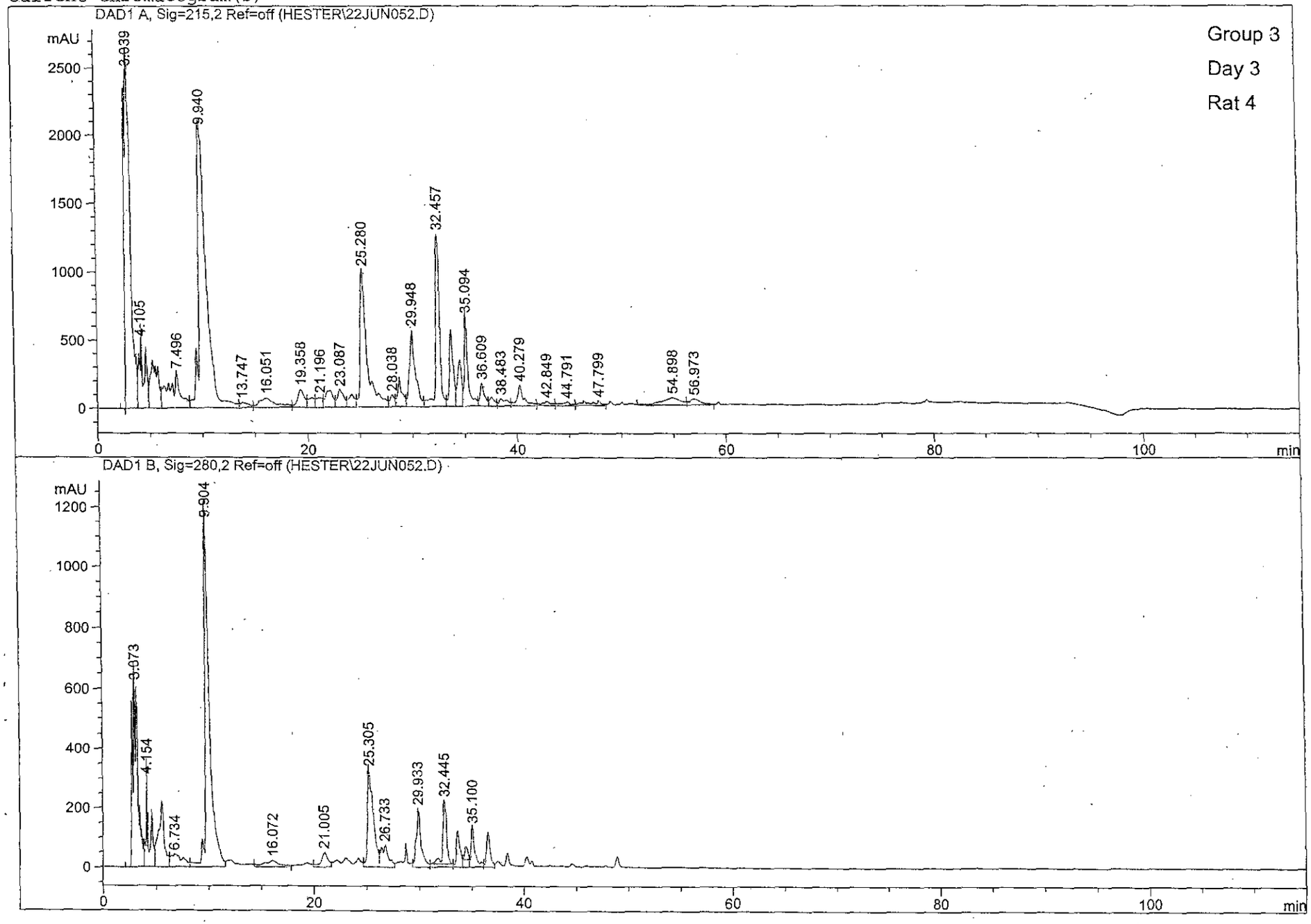

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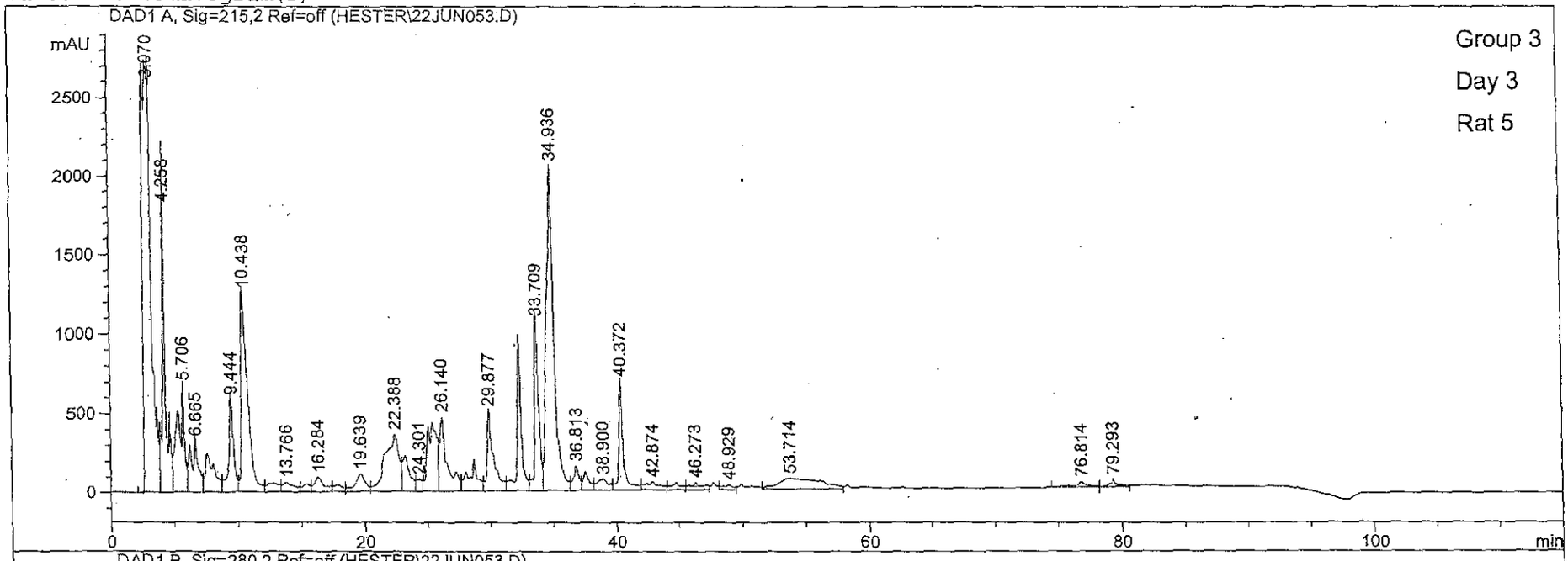


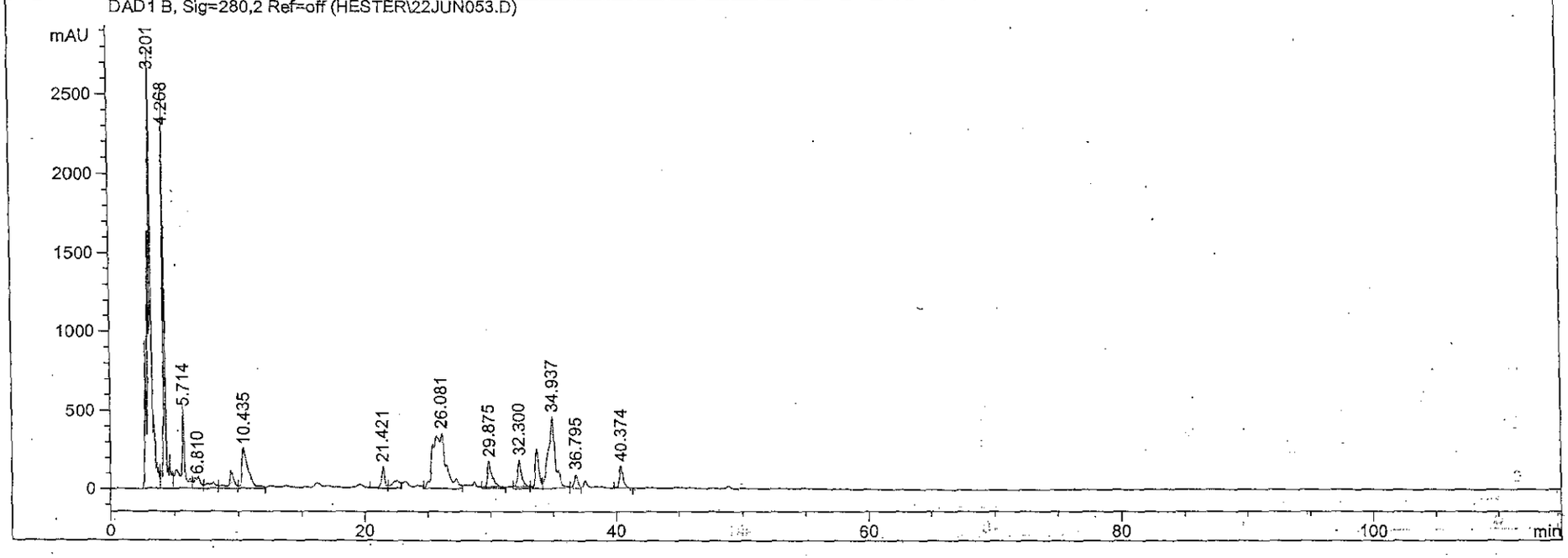

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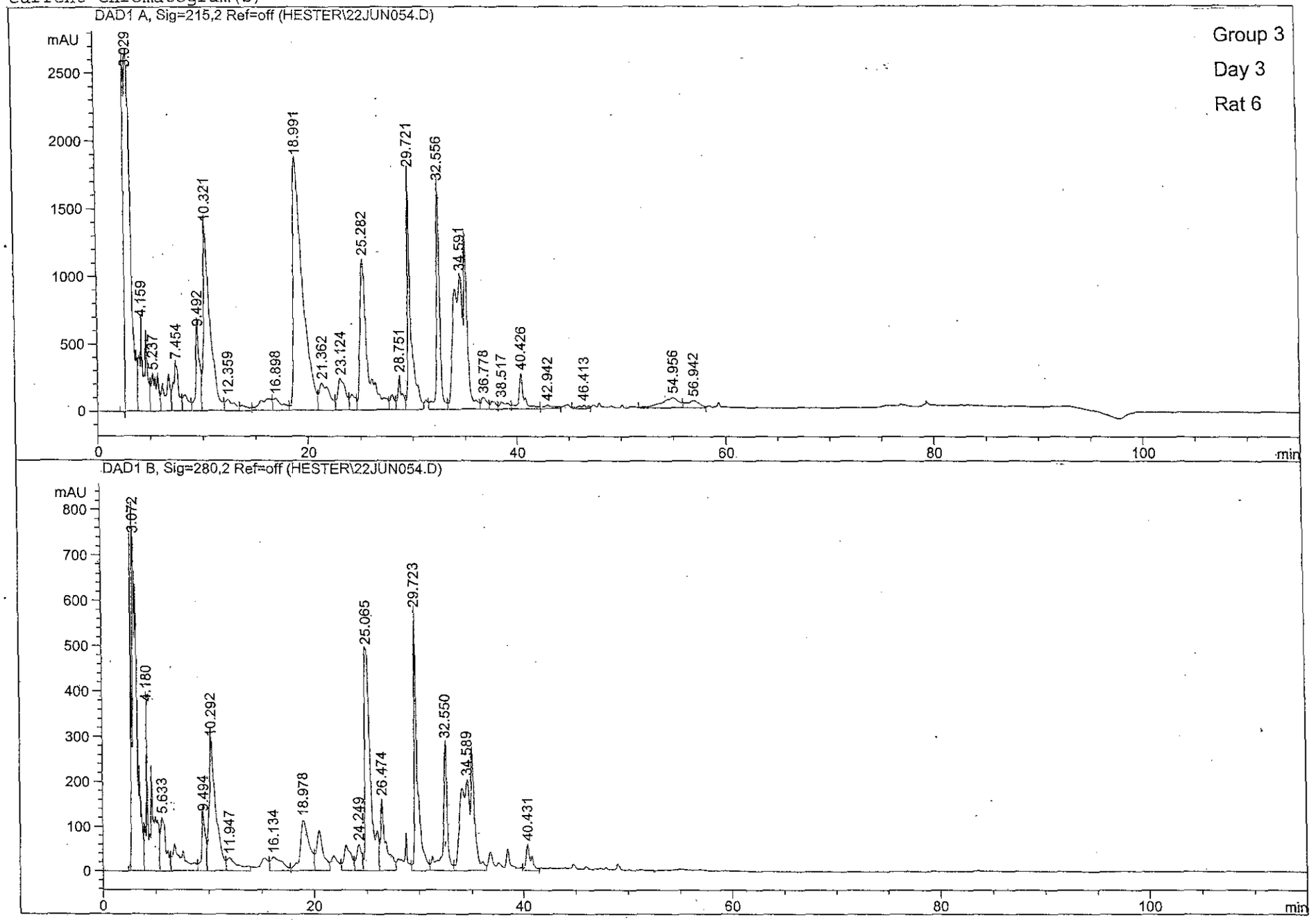

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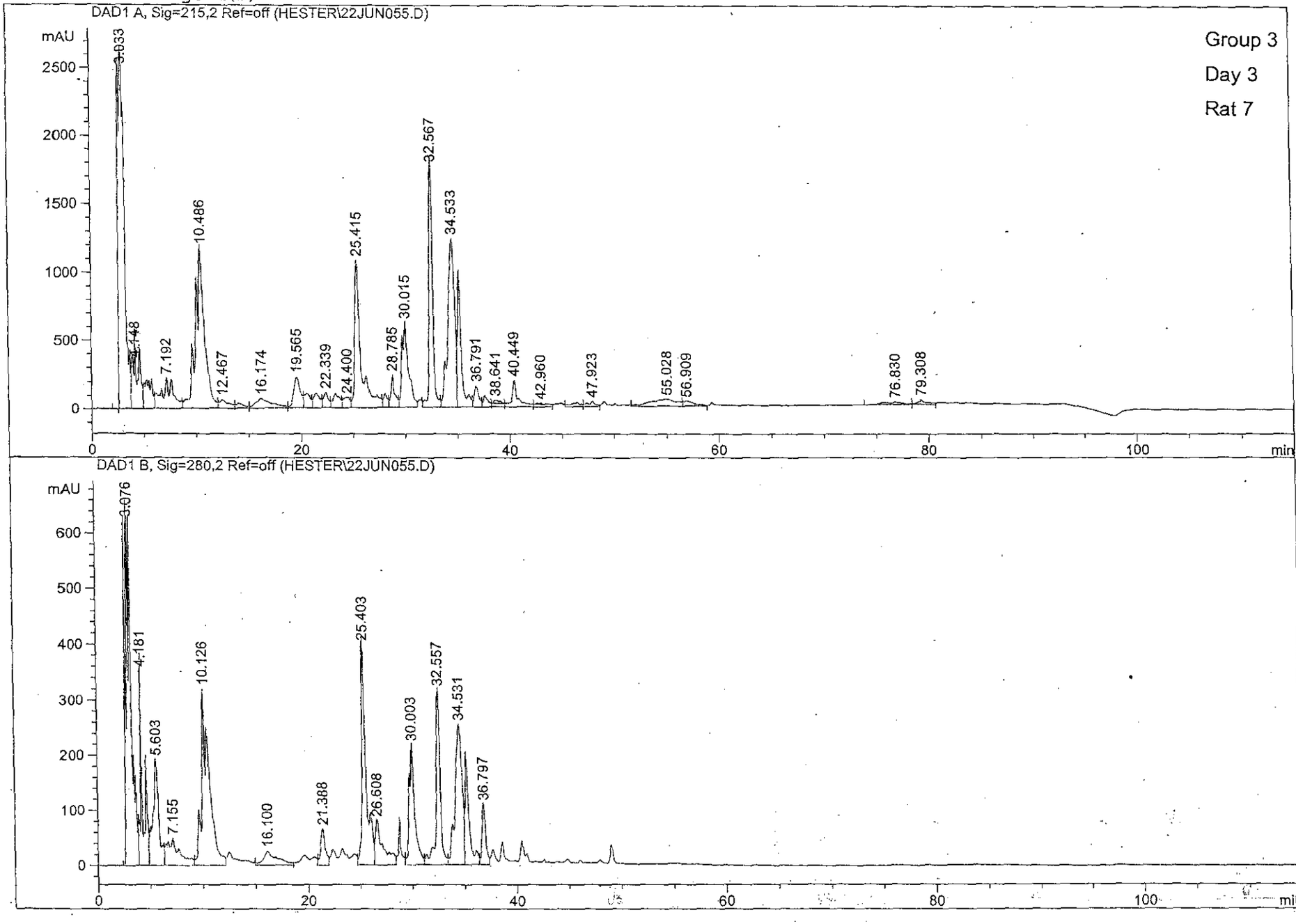

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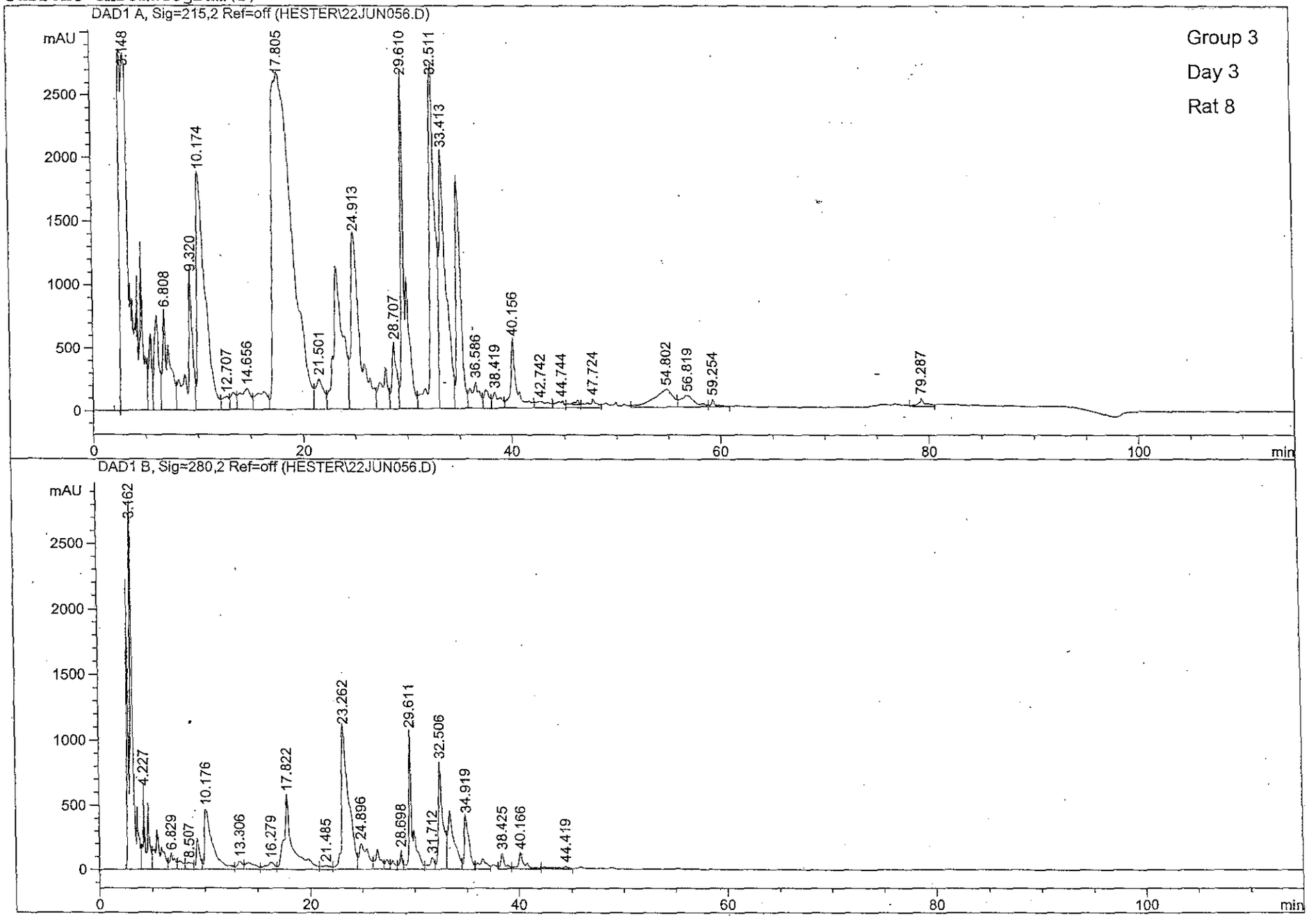

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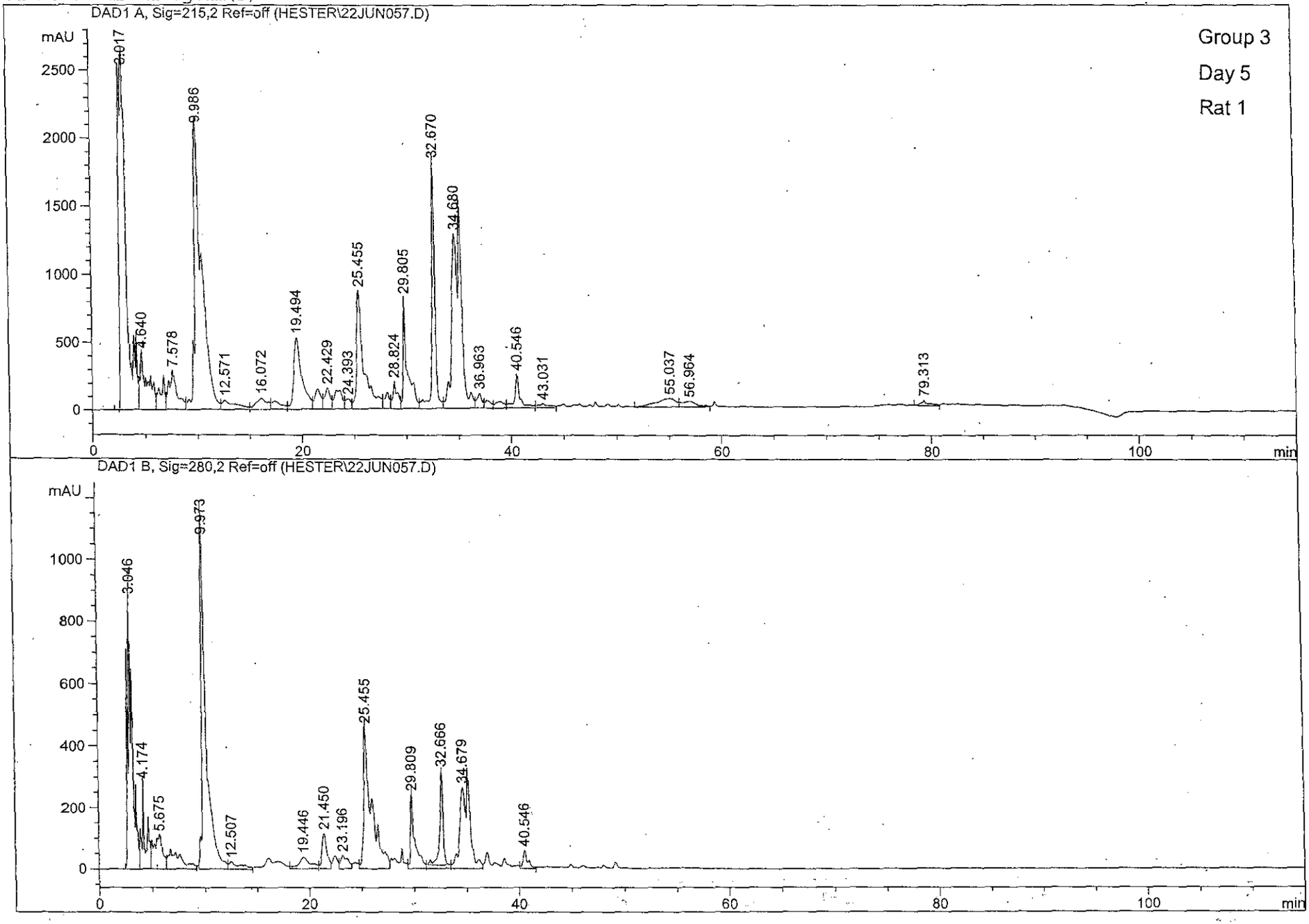

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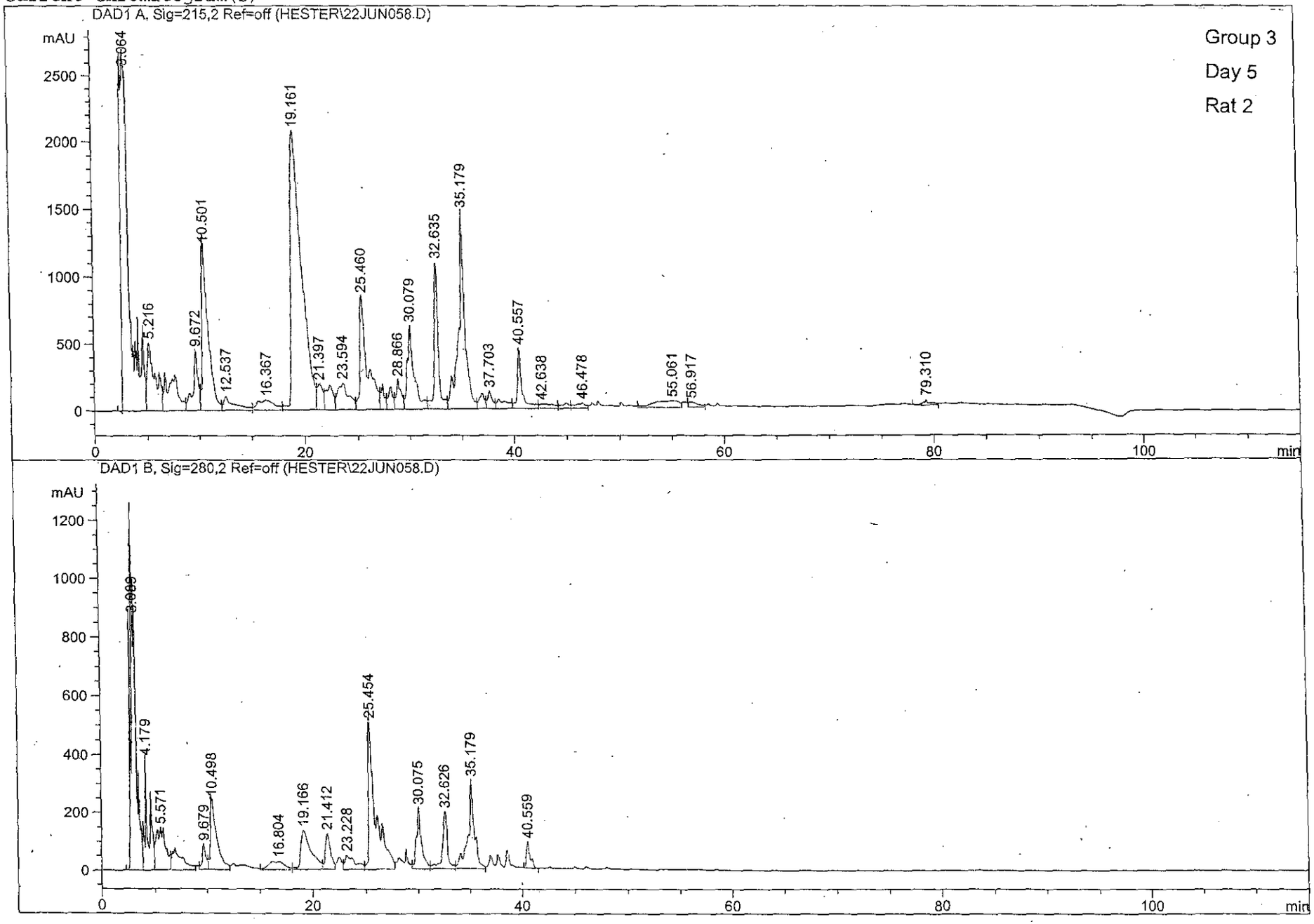

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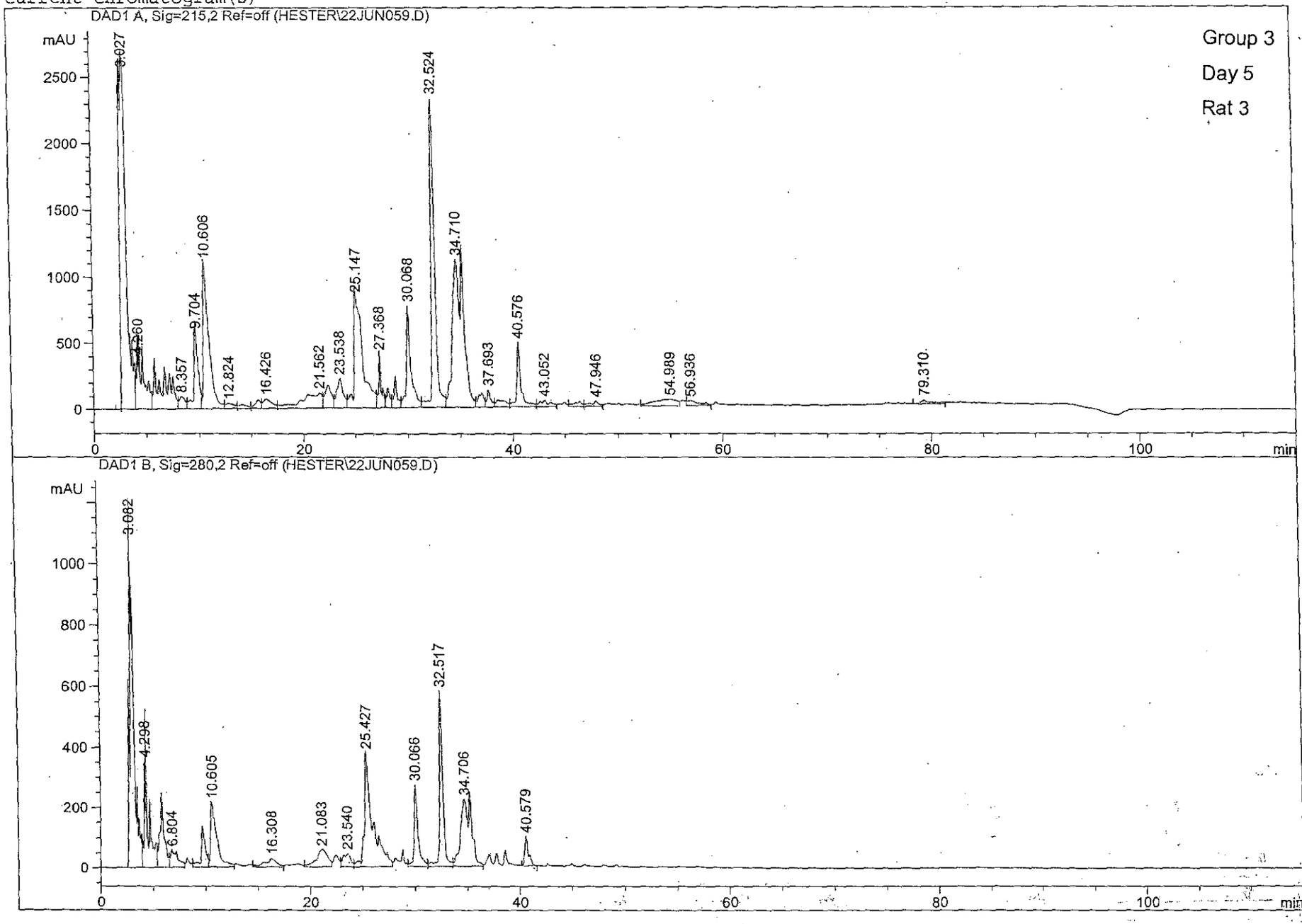

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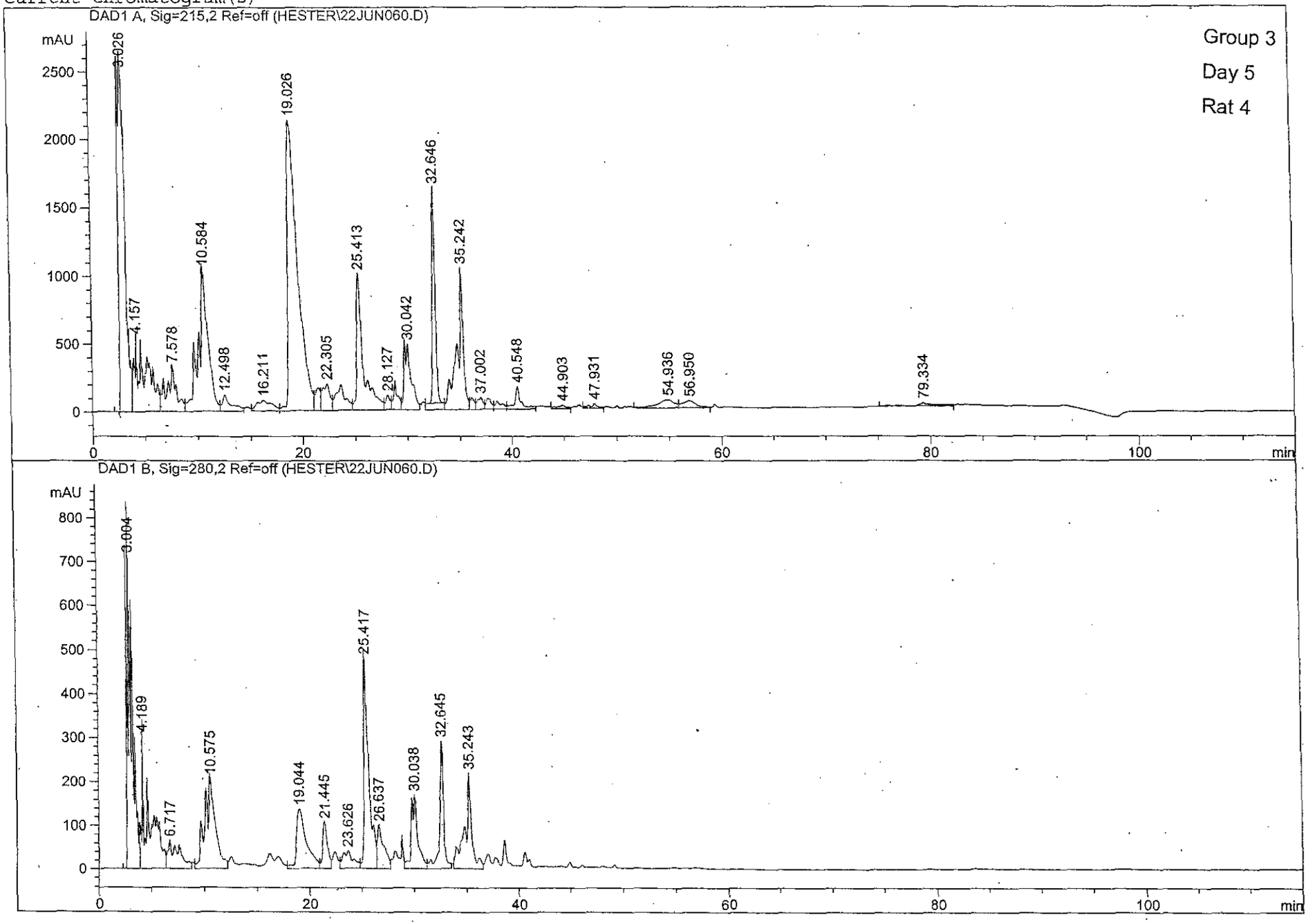

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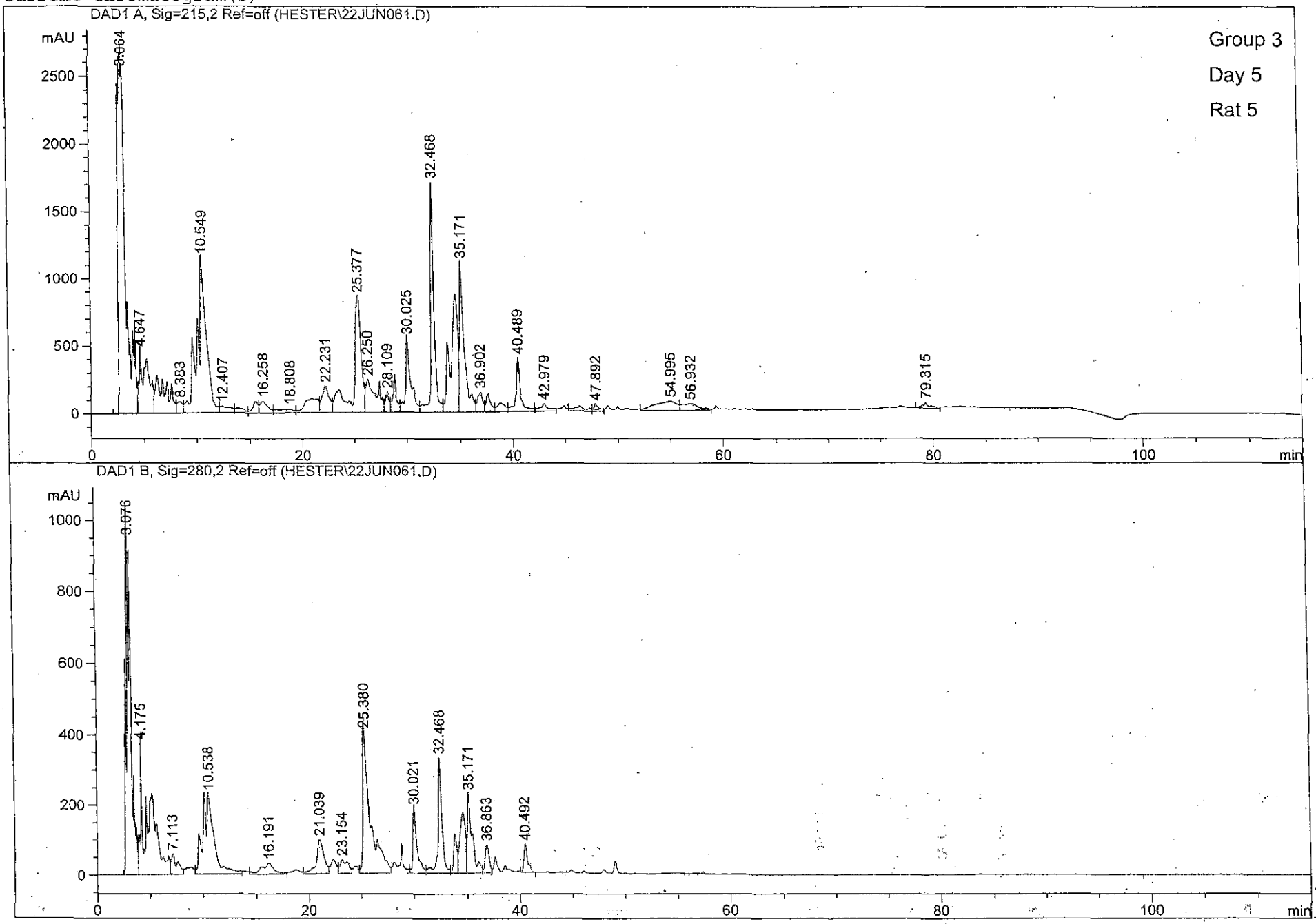

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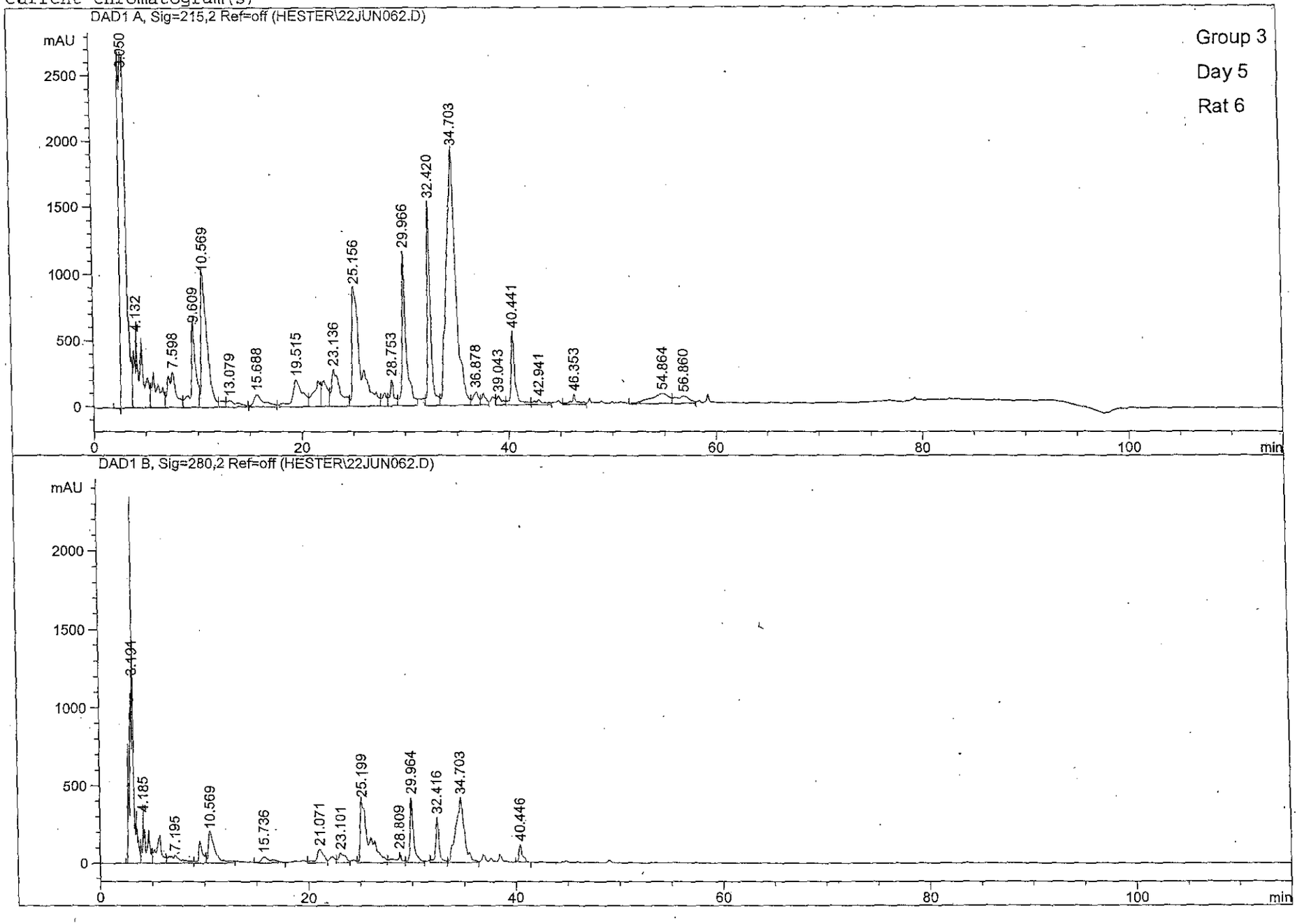

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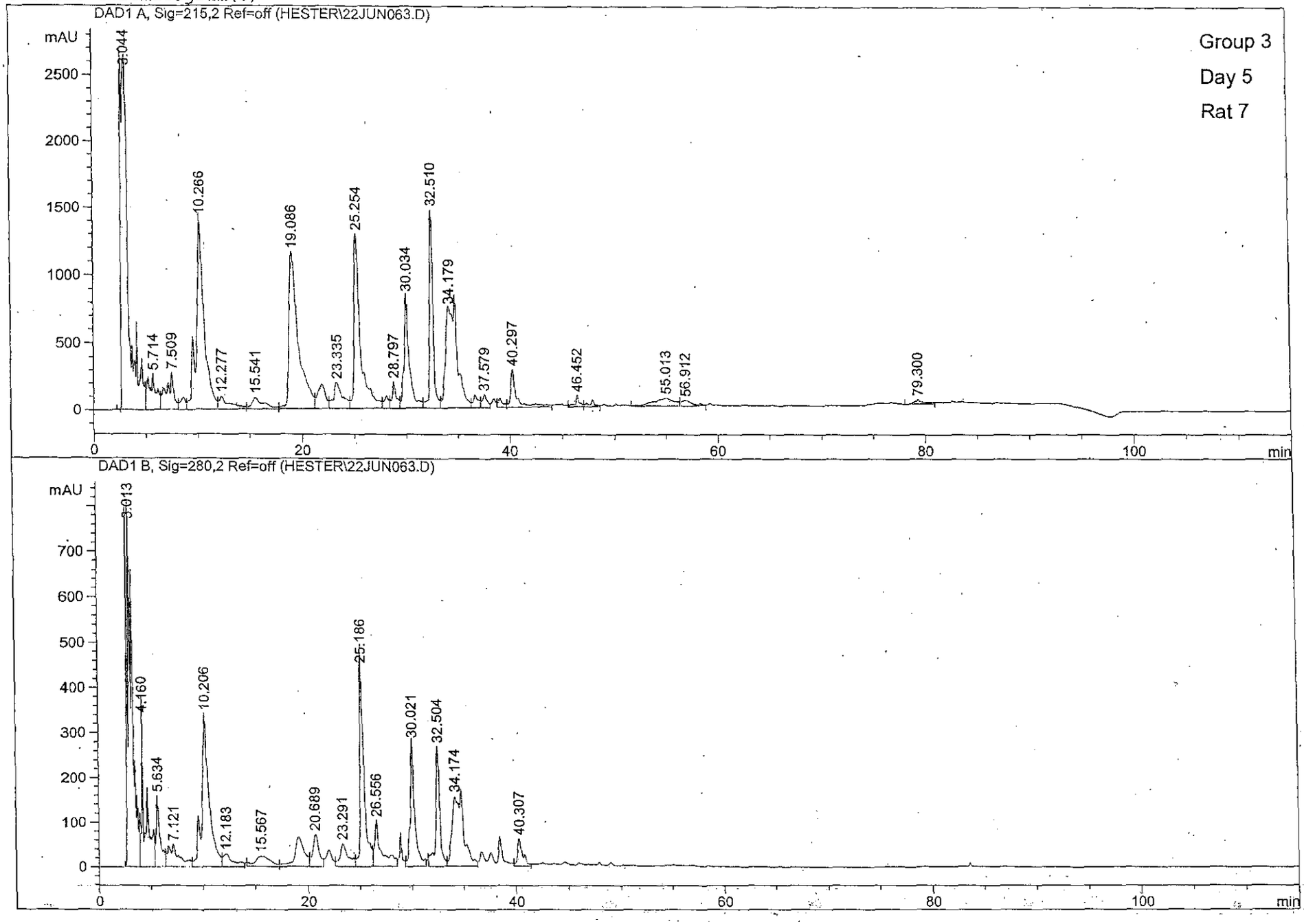

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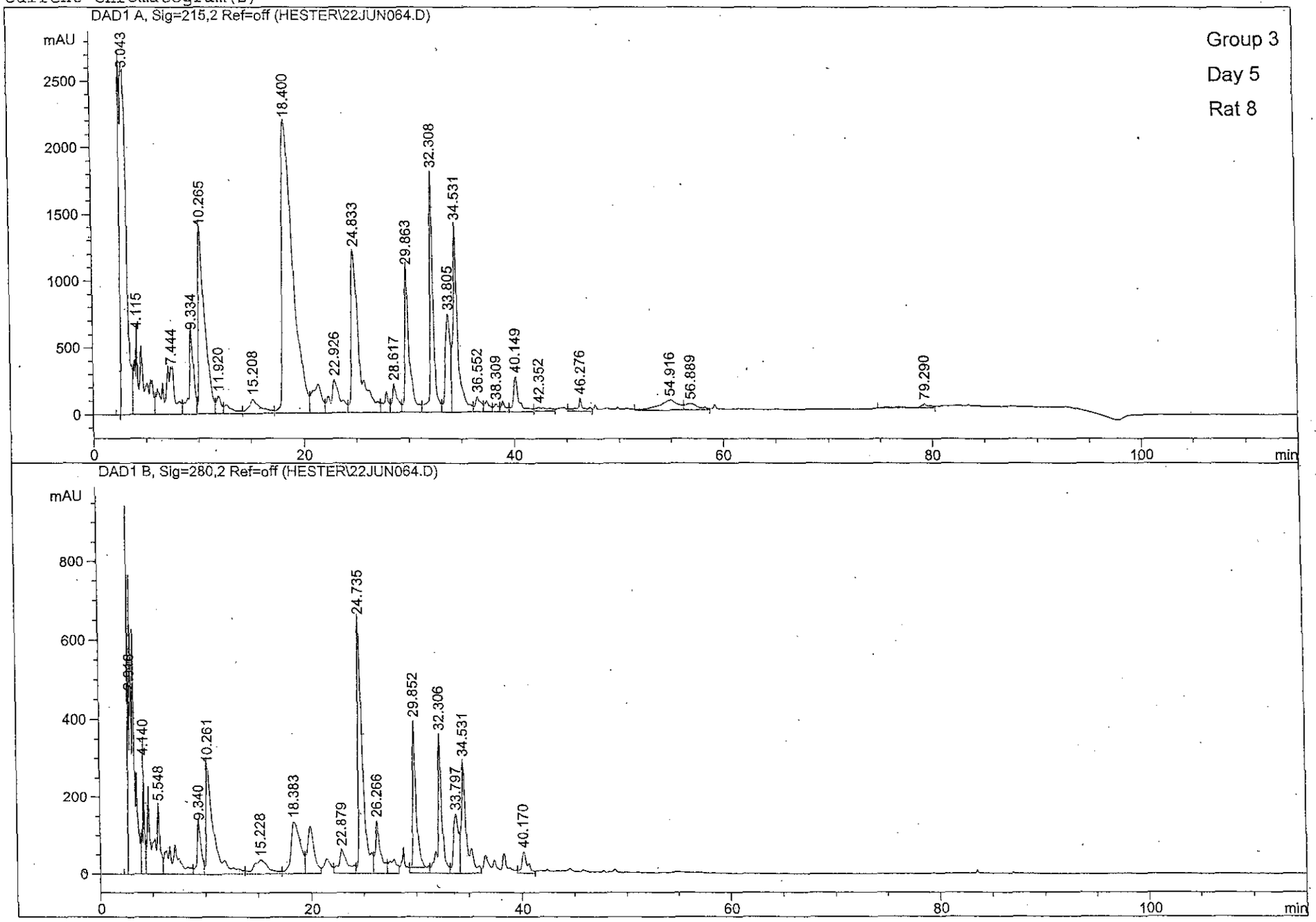
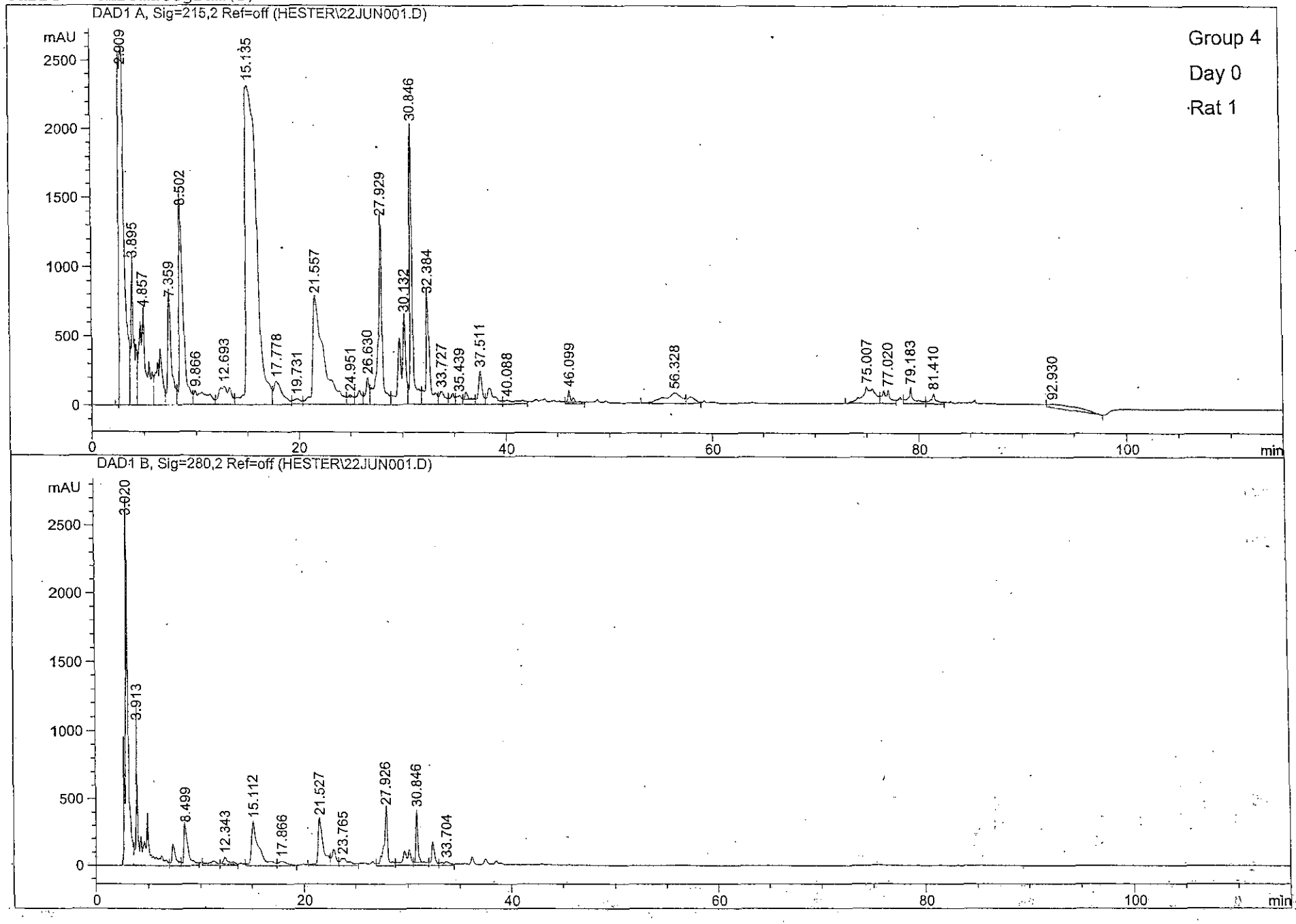


## Normal diet and cyclosporine

Current Chromatogram(s)



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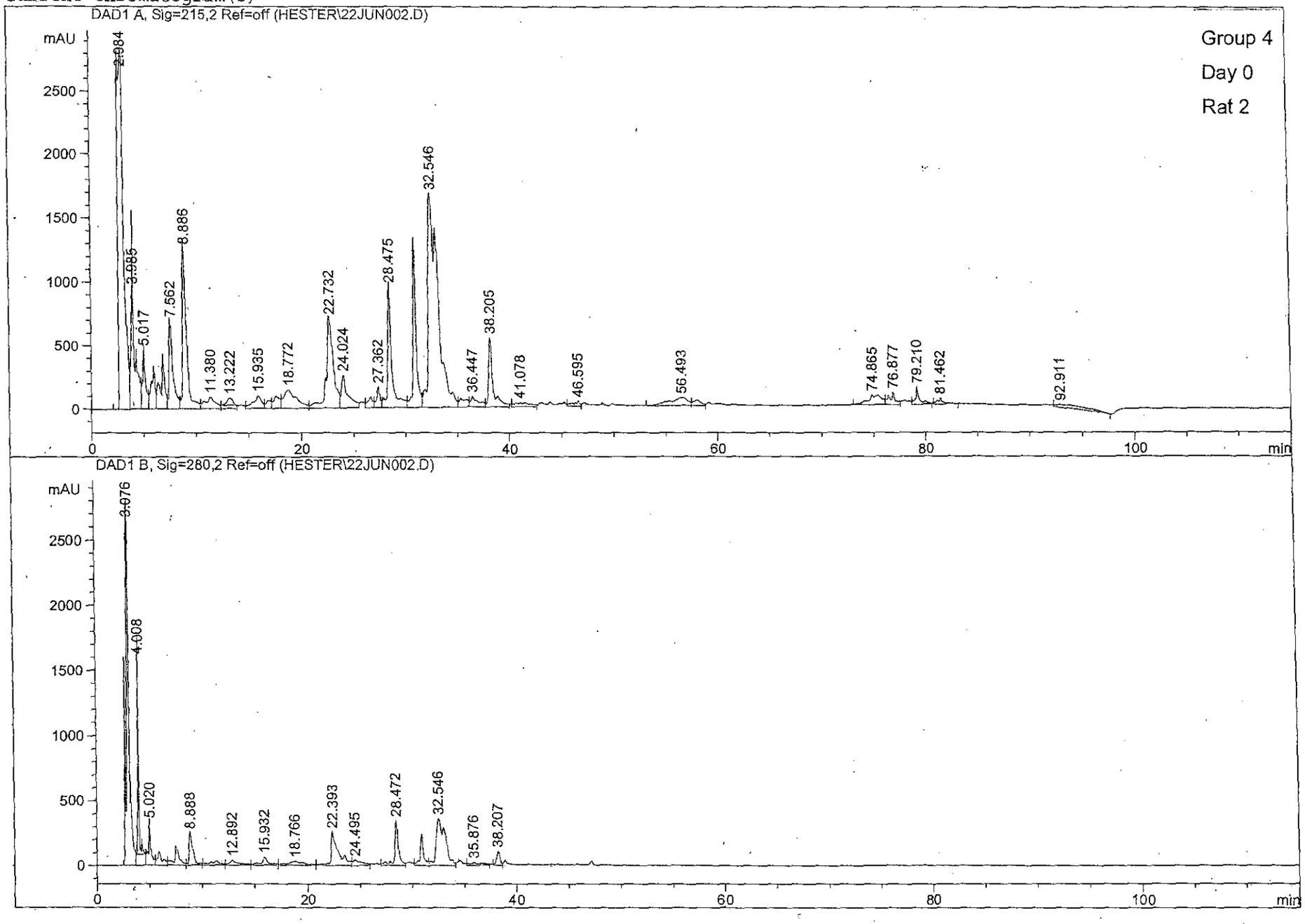

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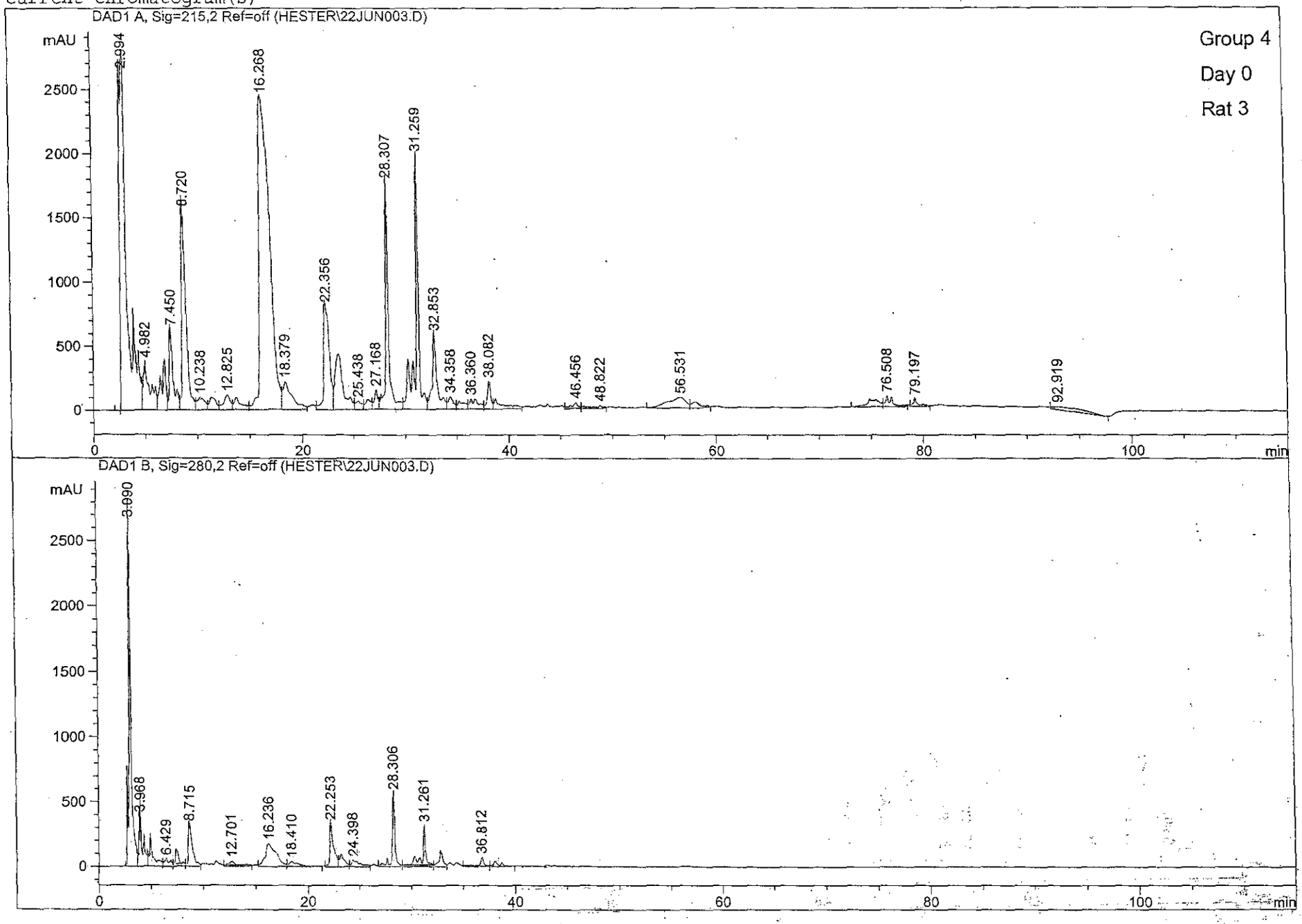

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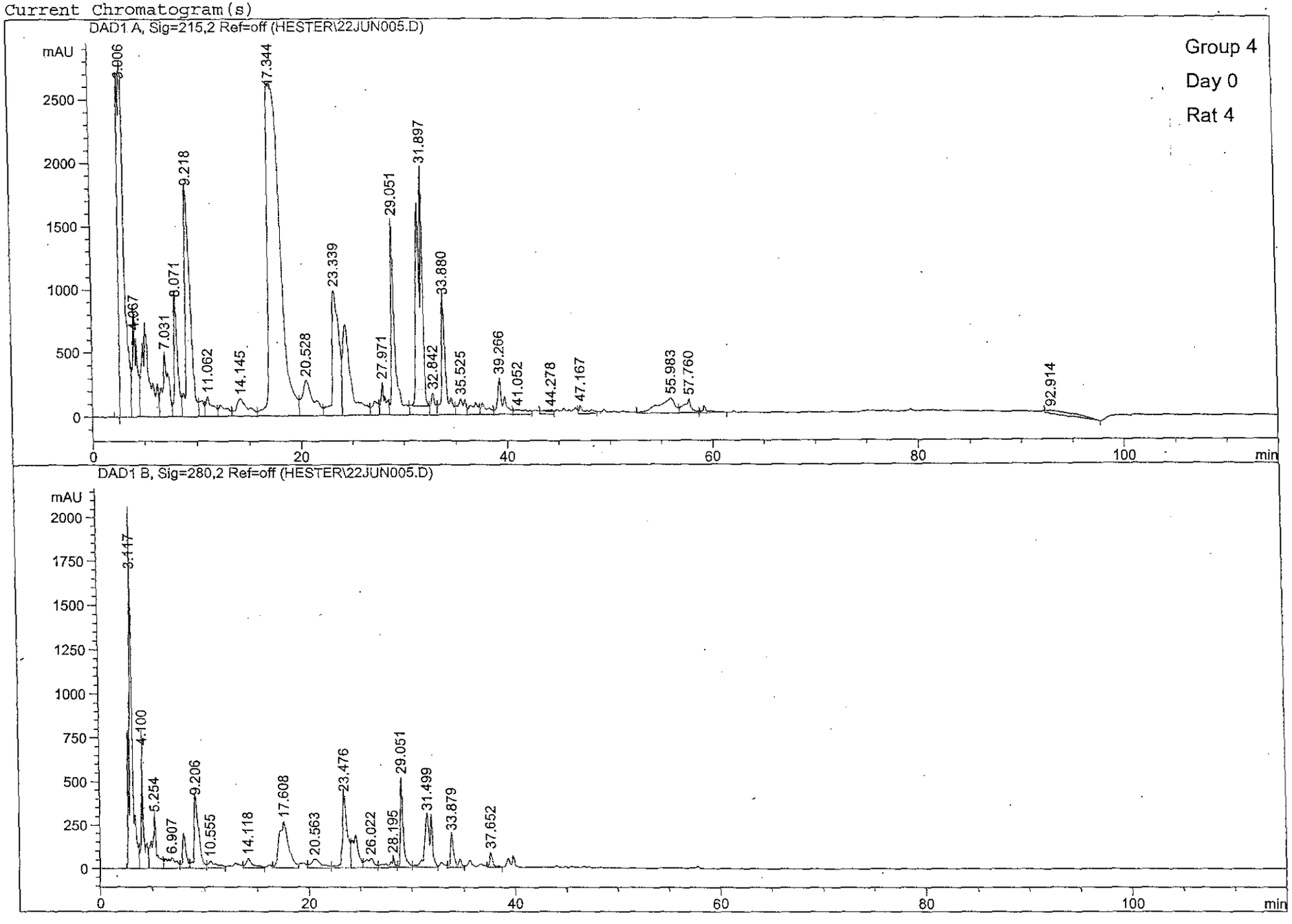

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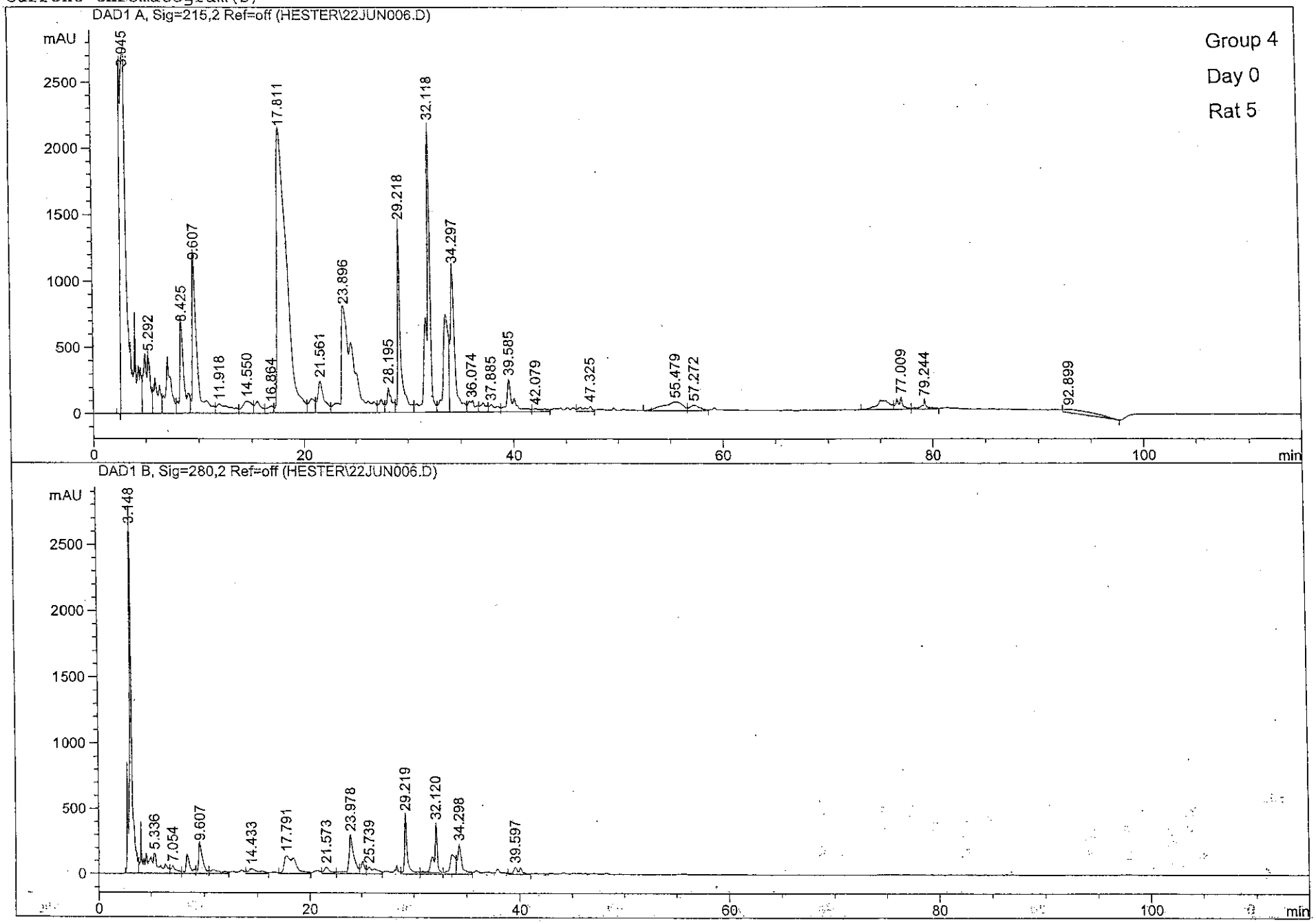

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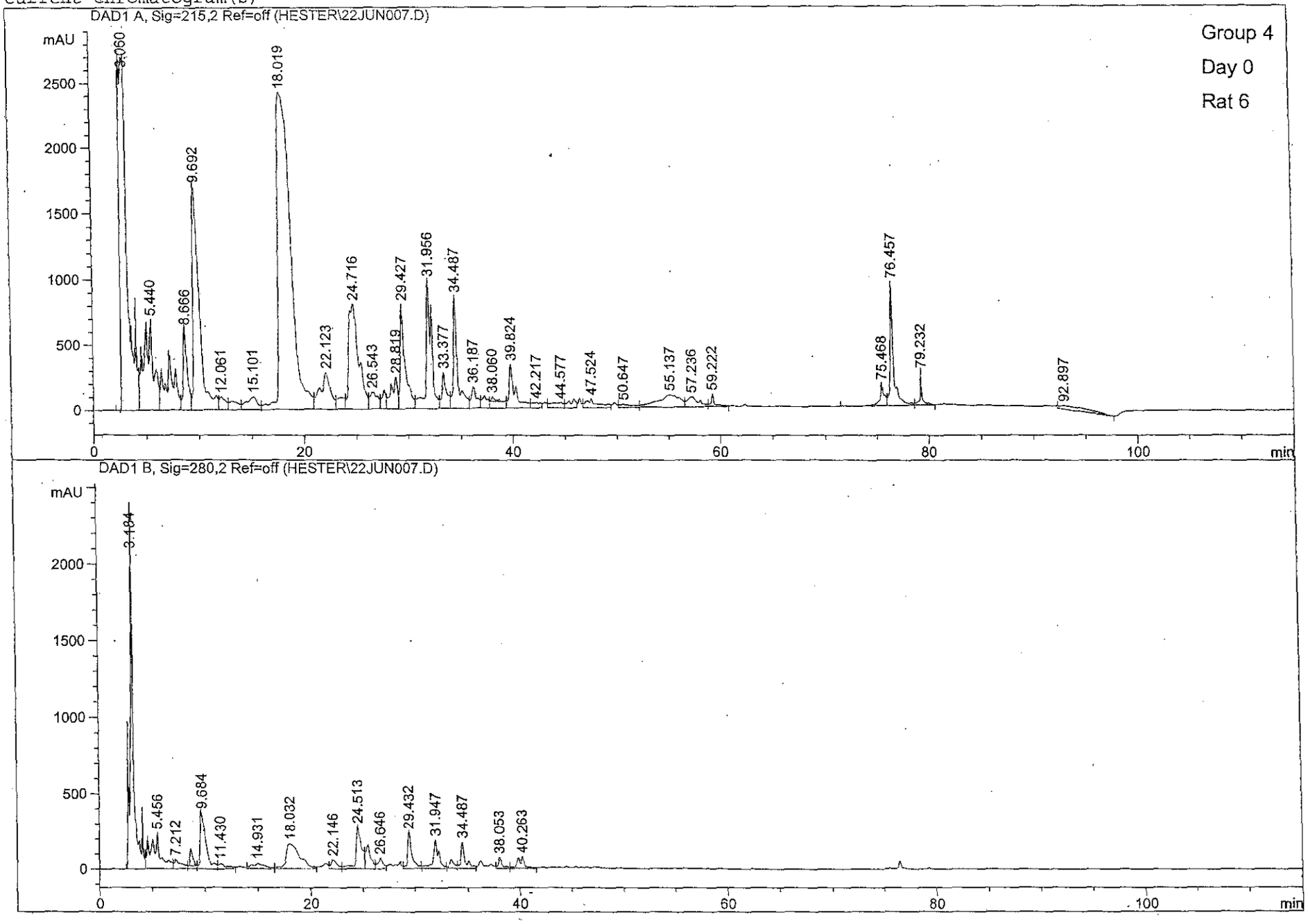

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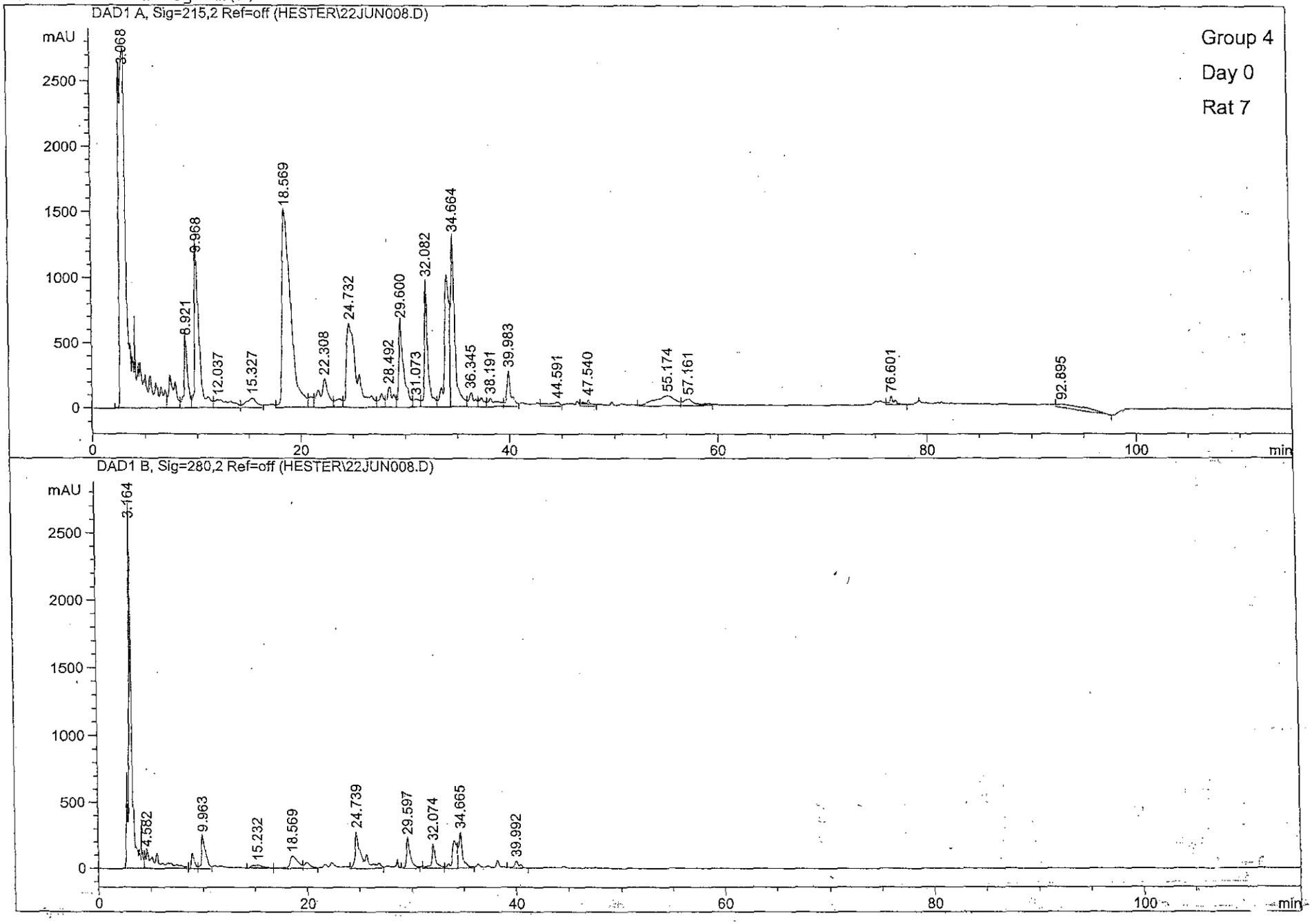

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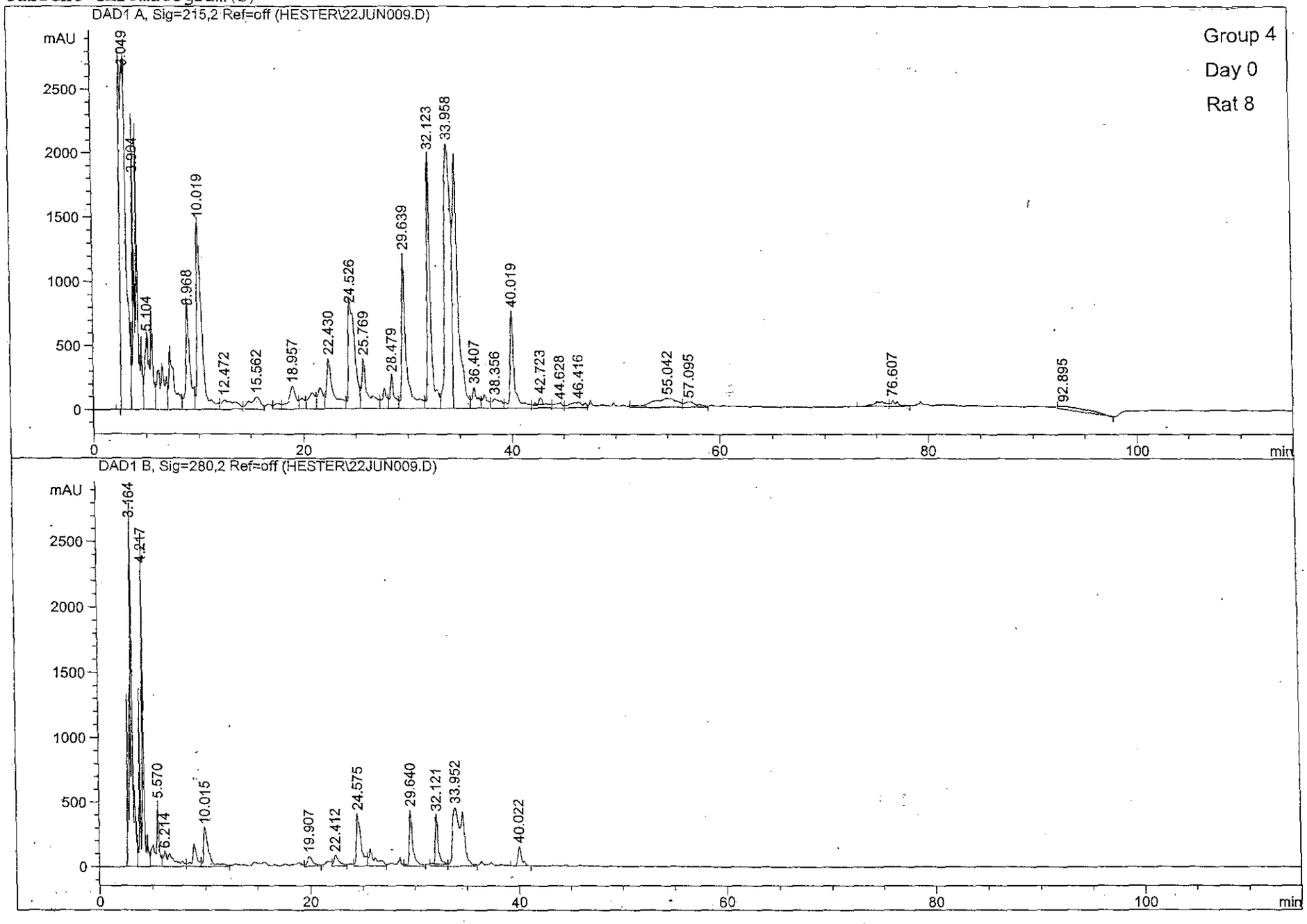

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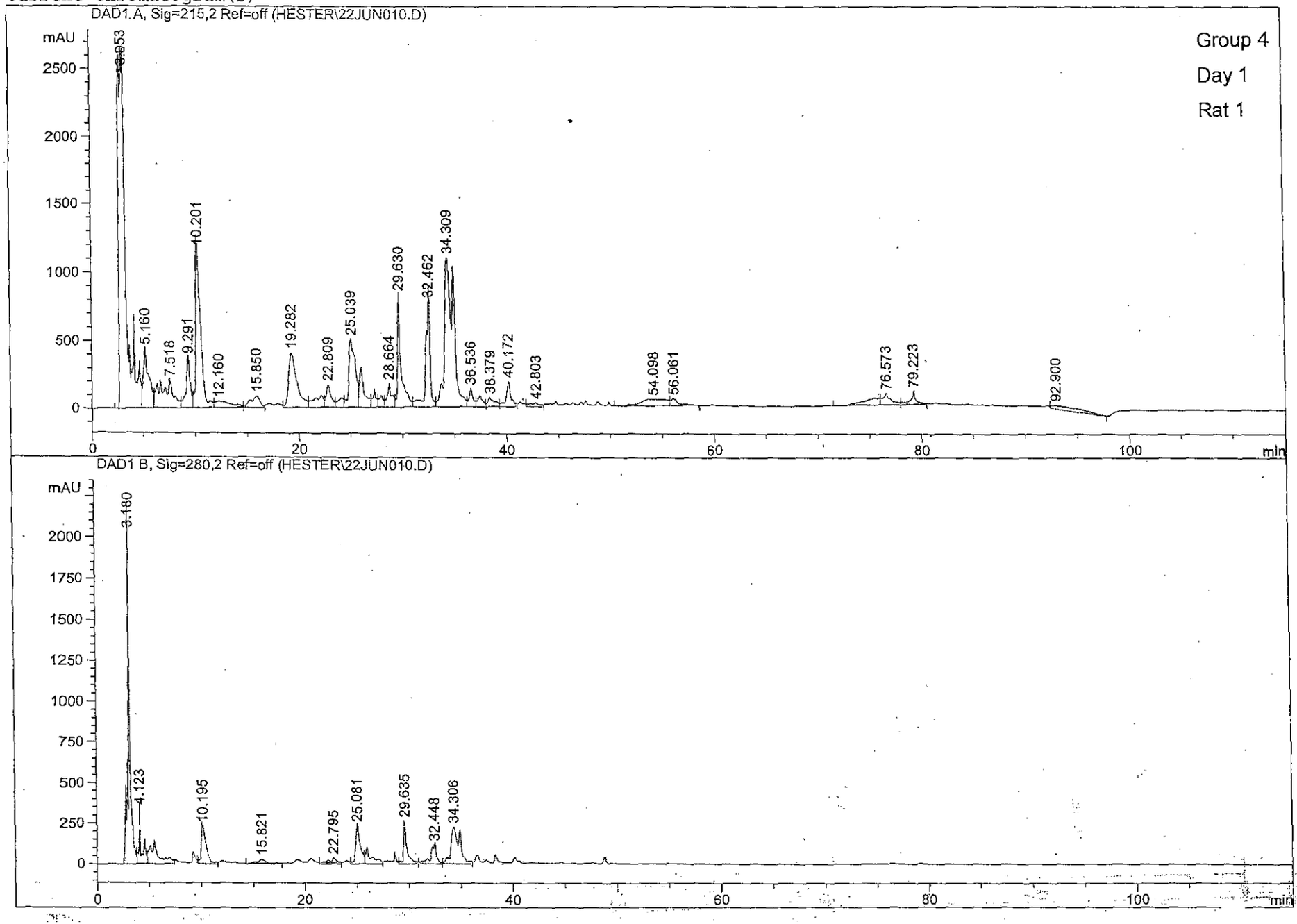

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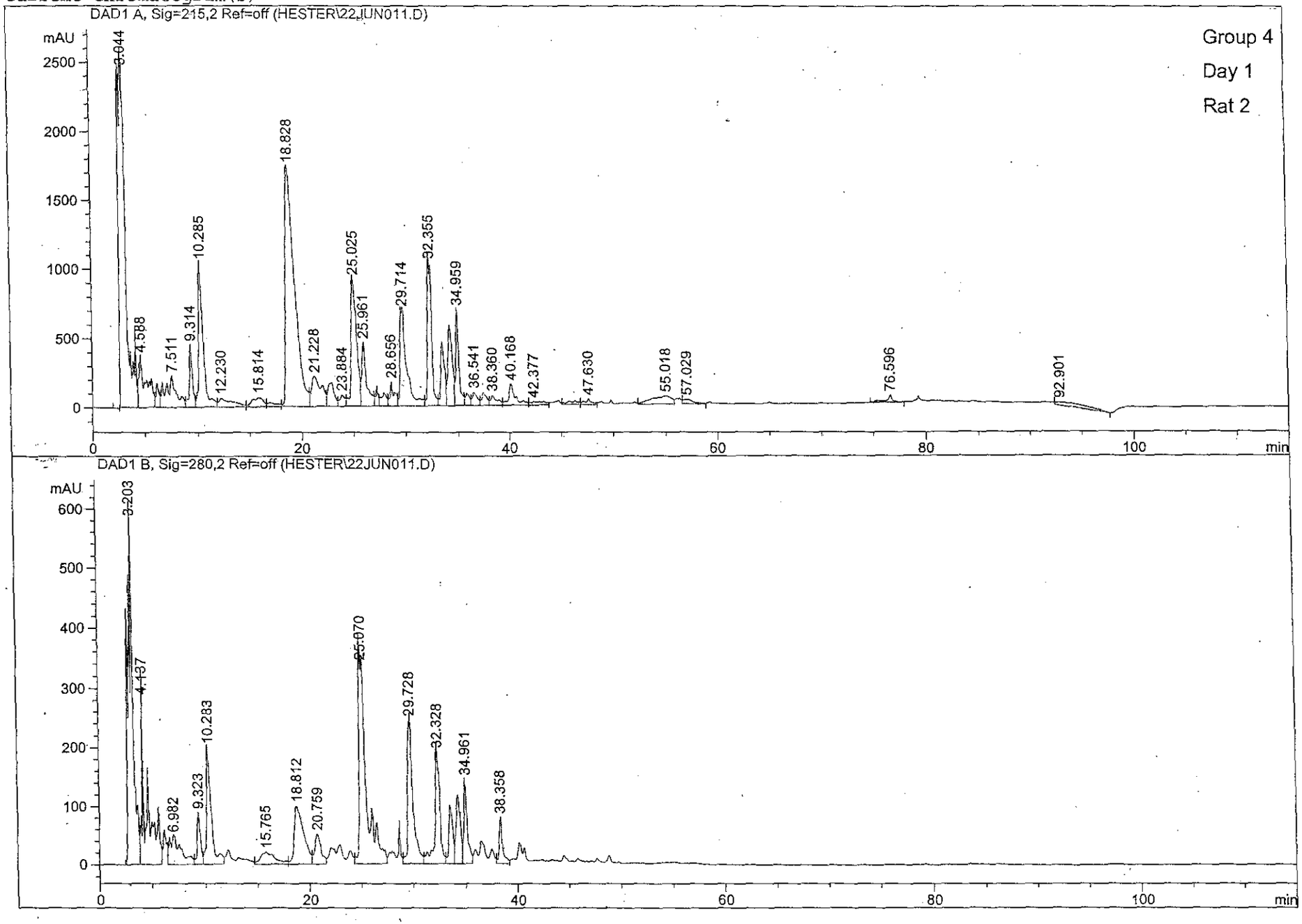

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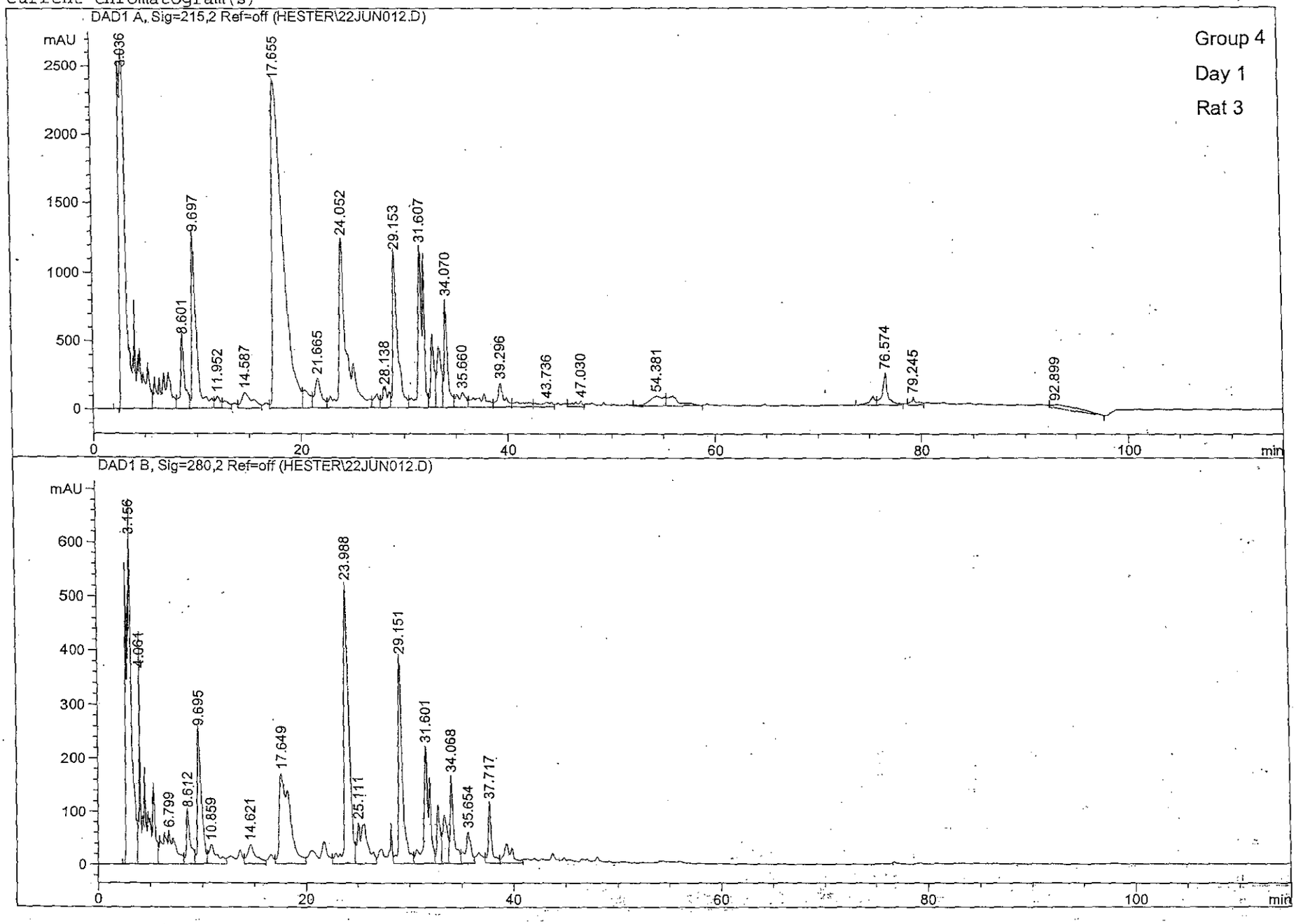

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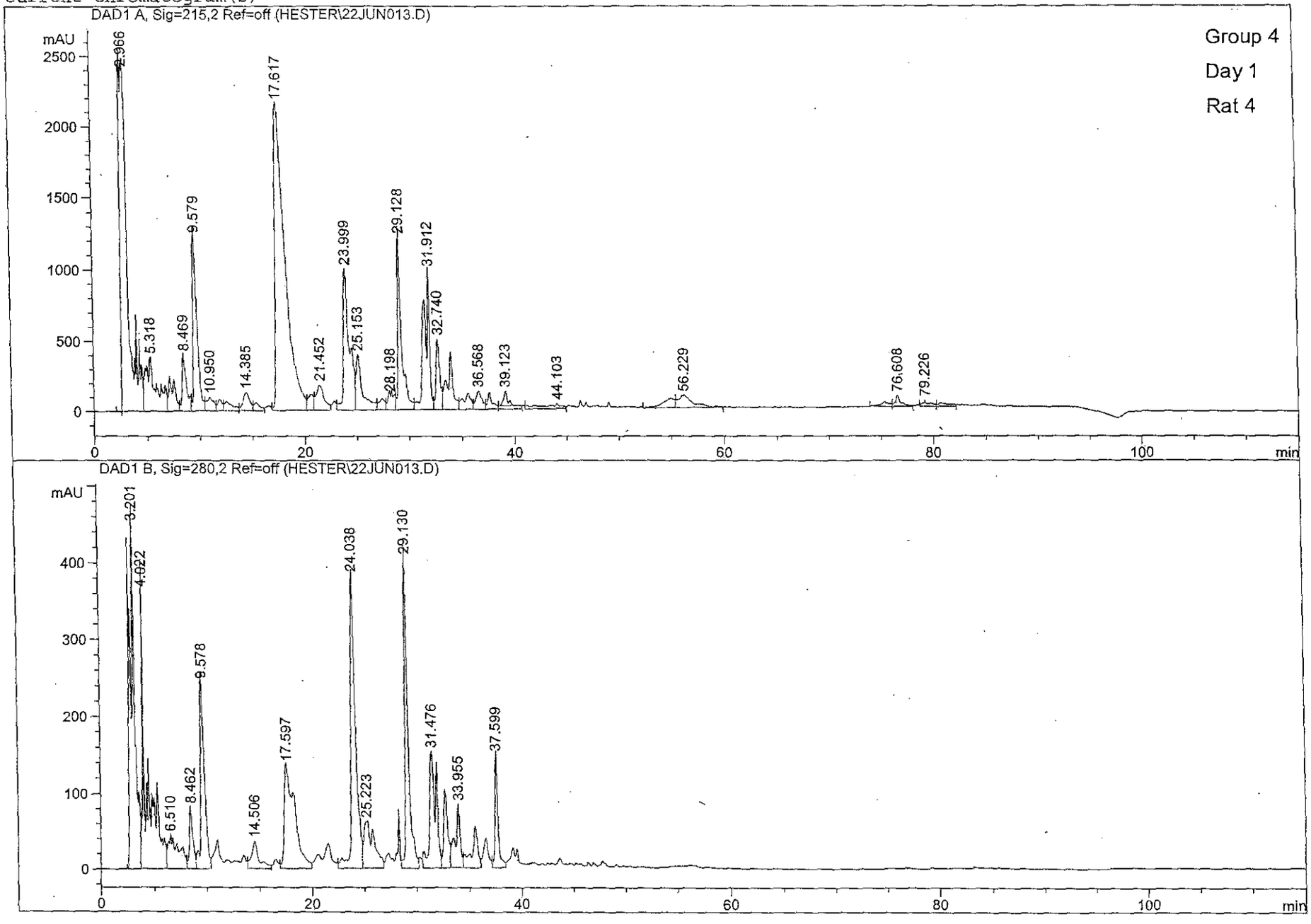

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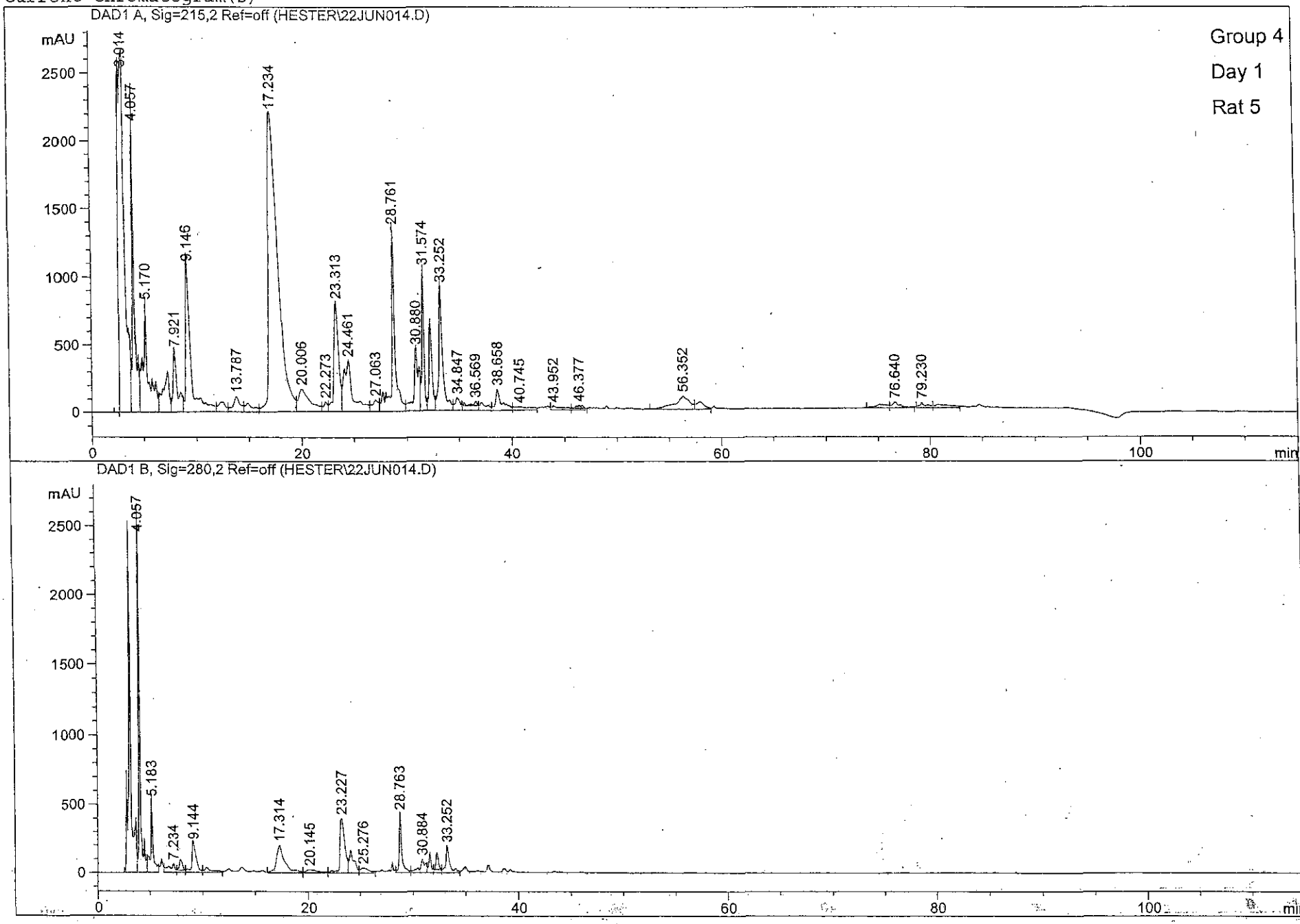

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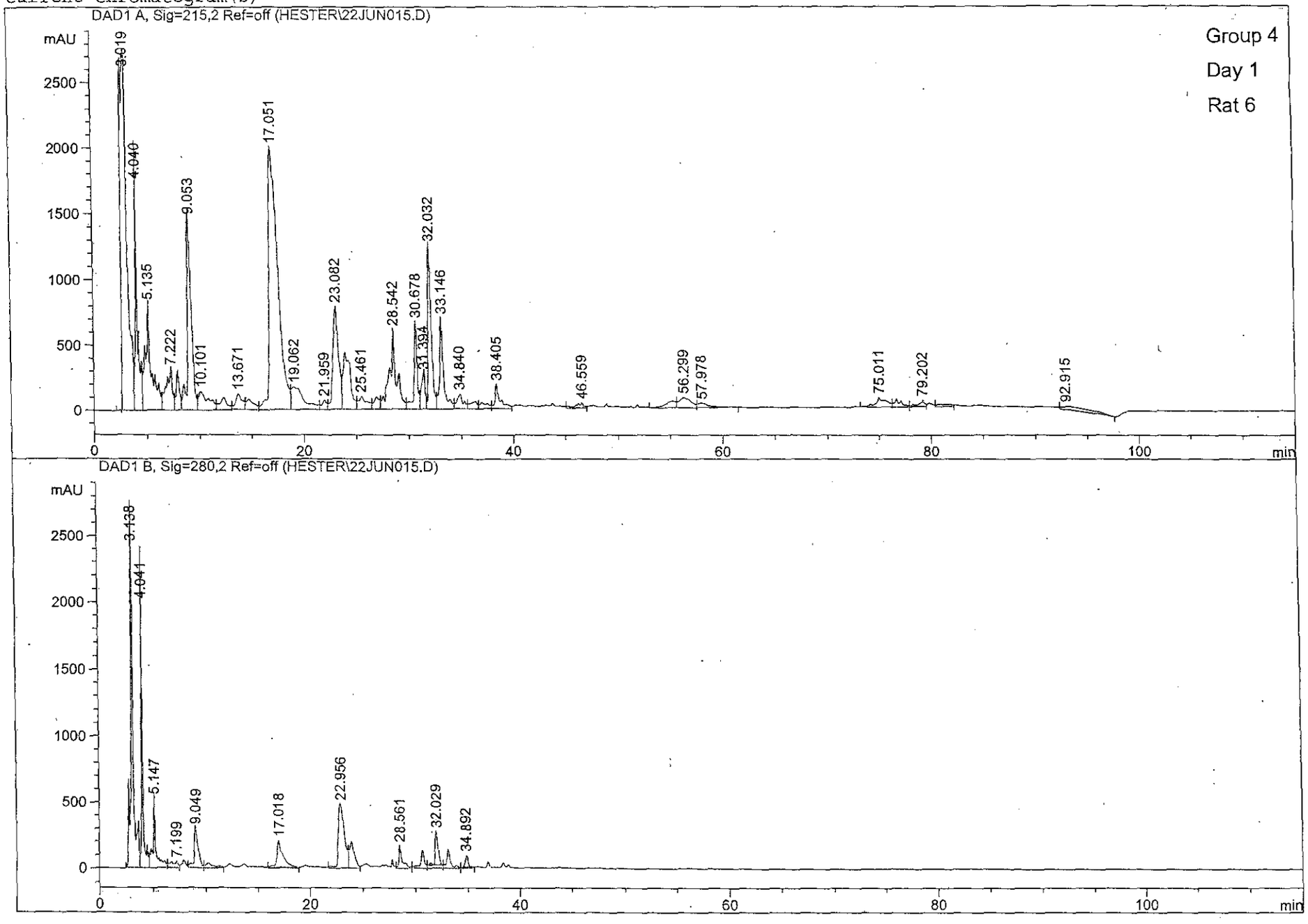

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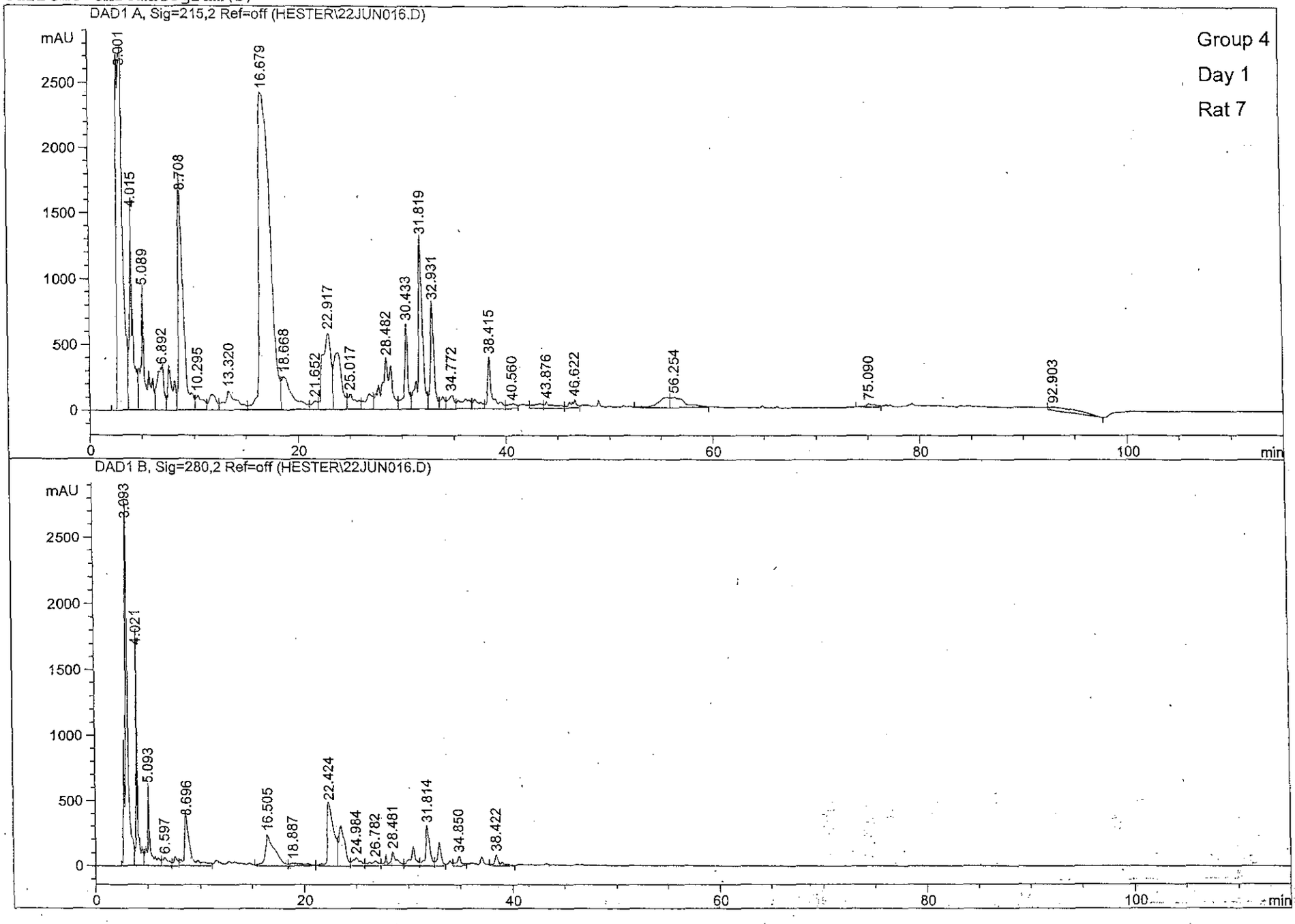

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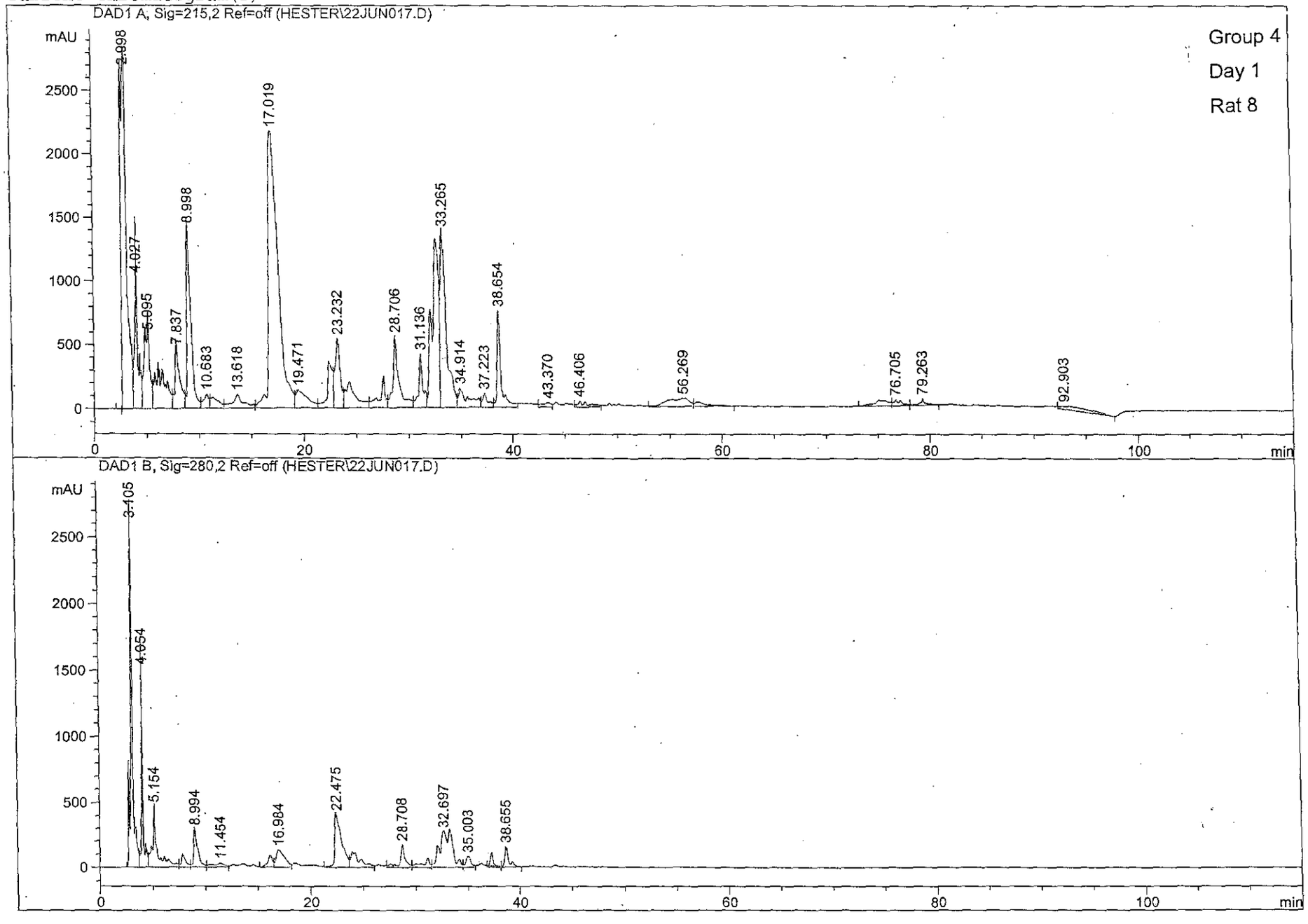

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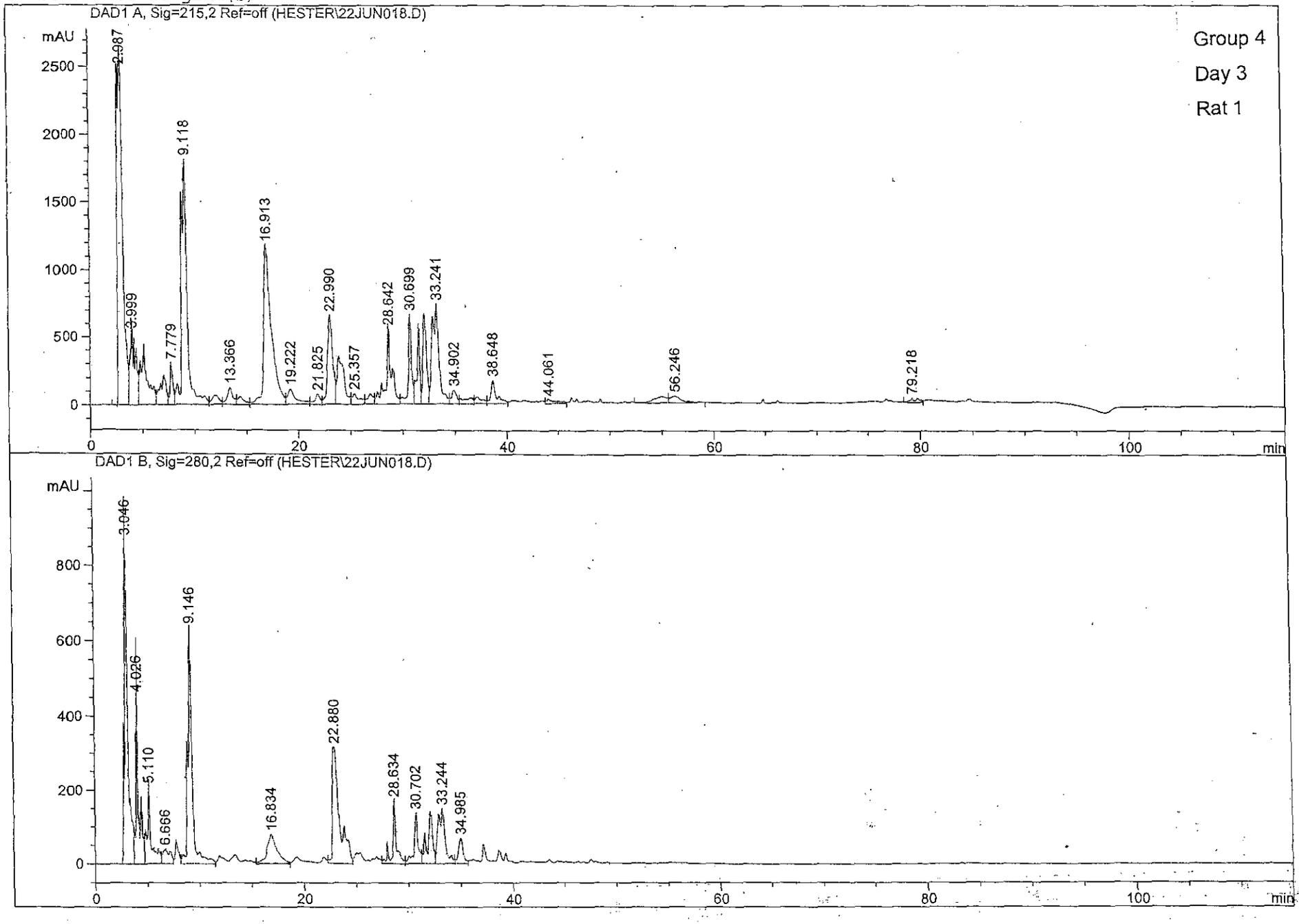

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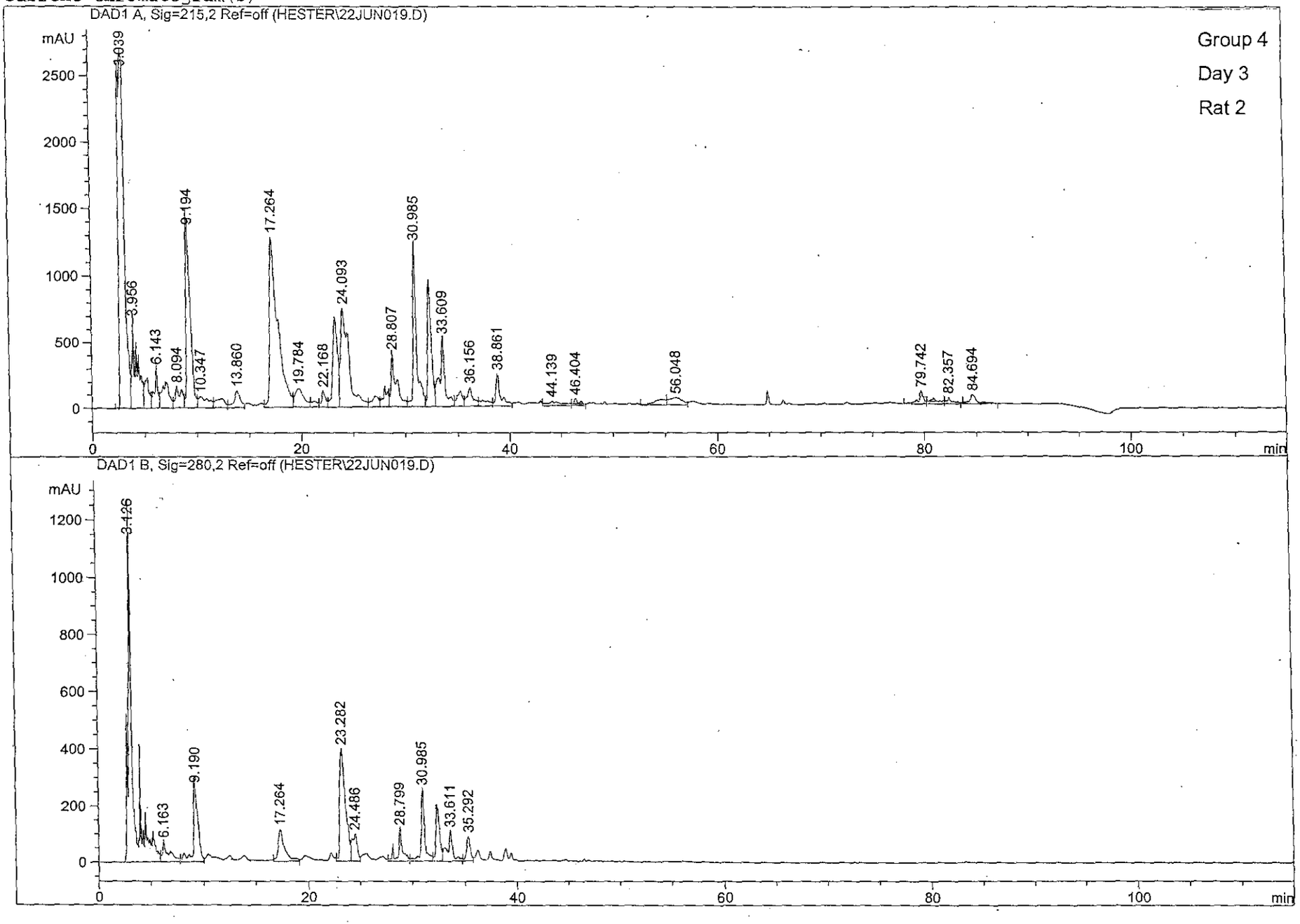

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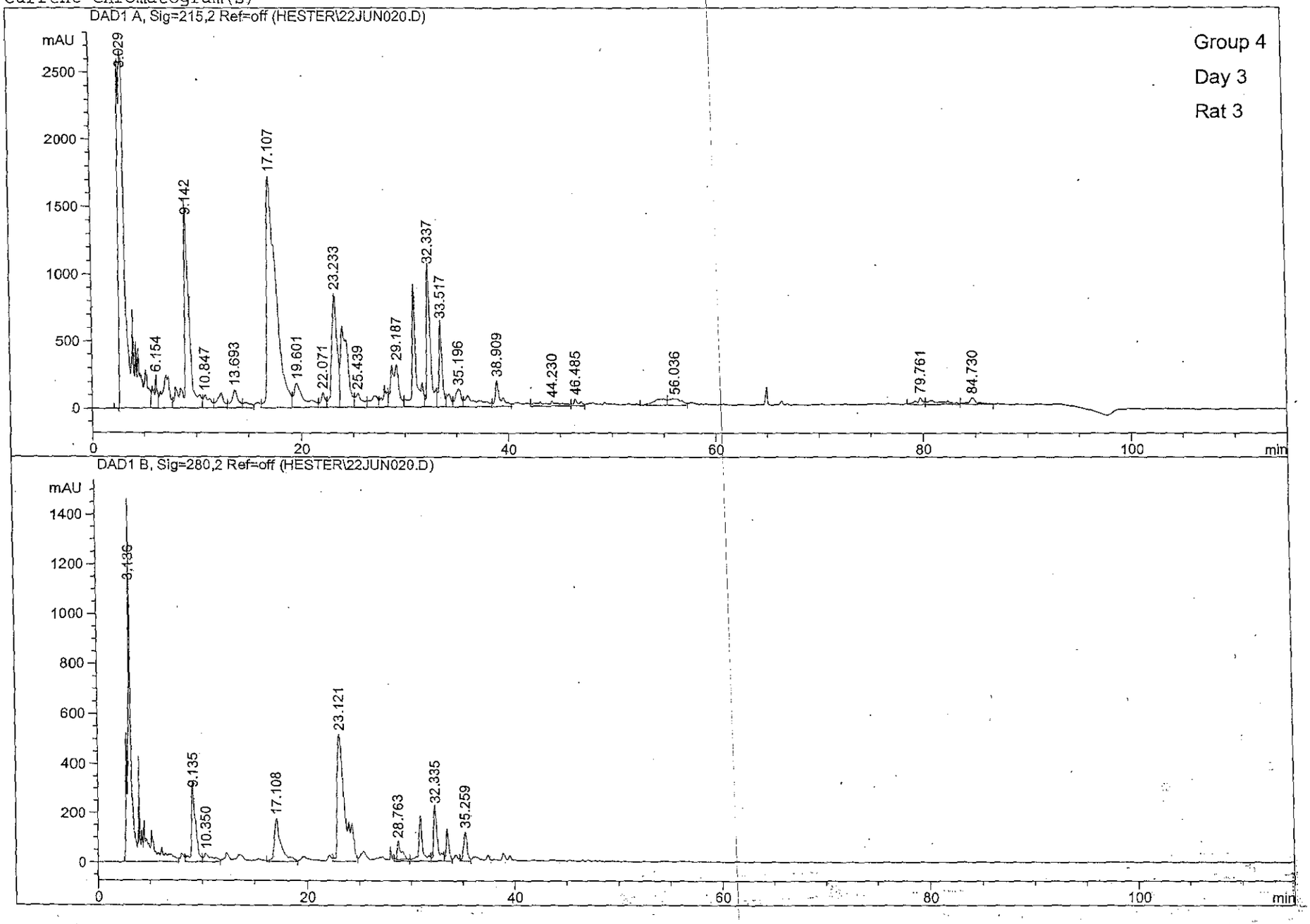

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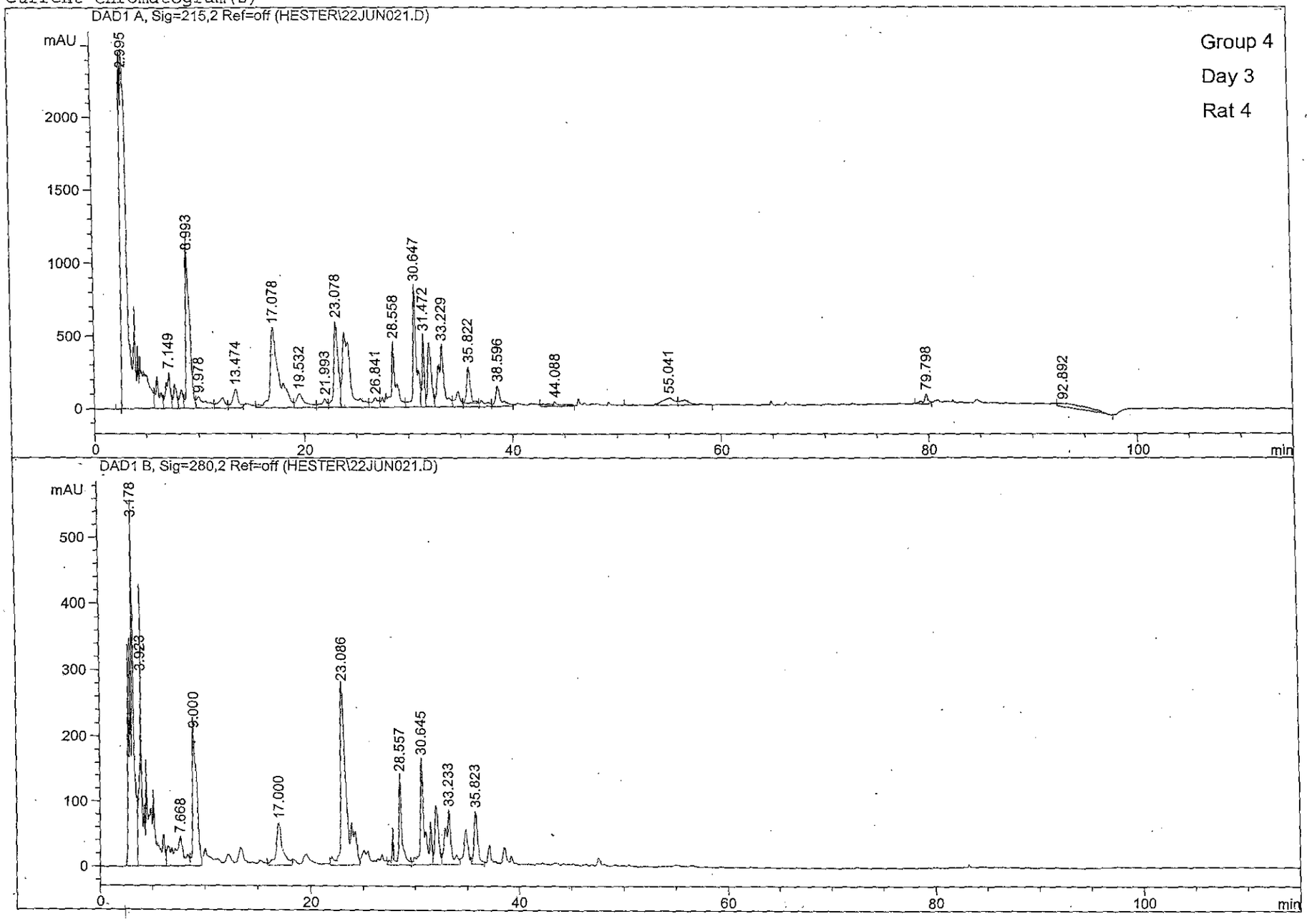

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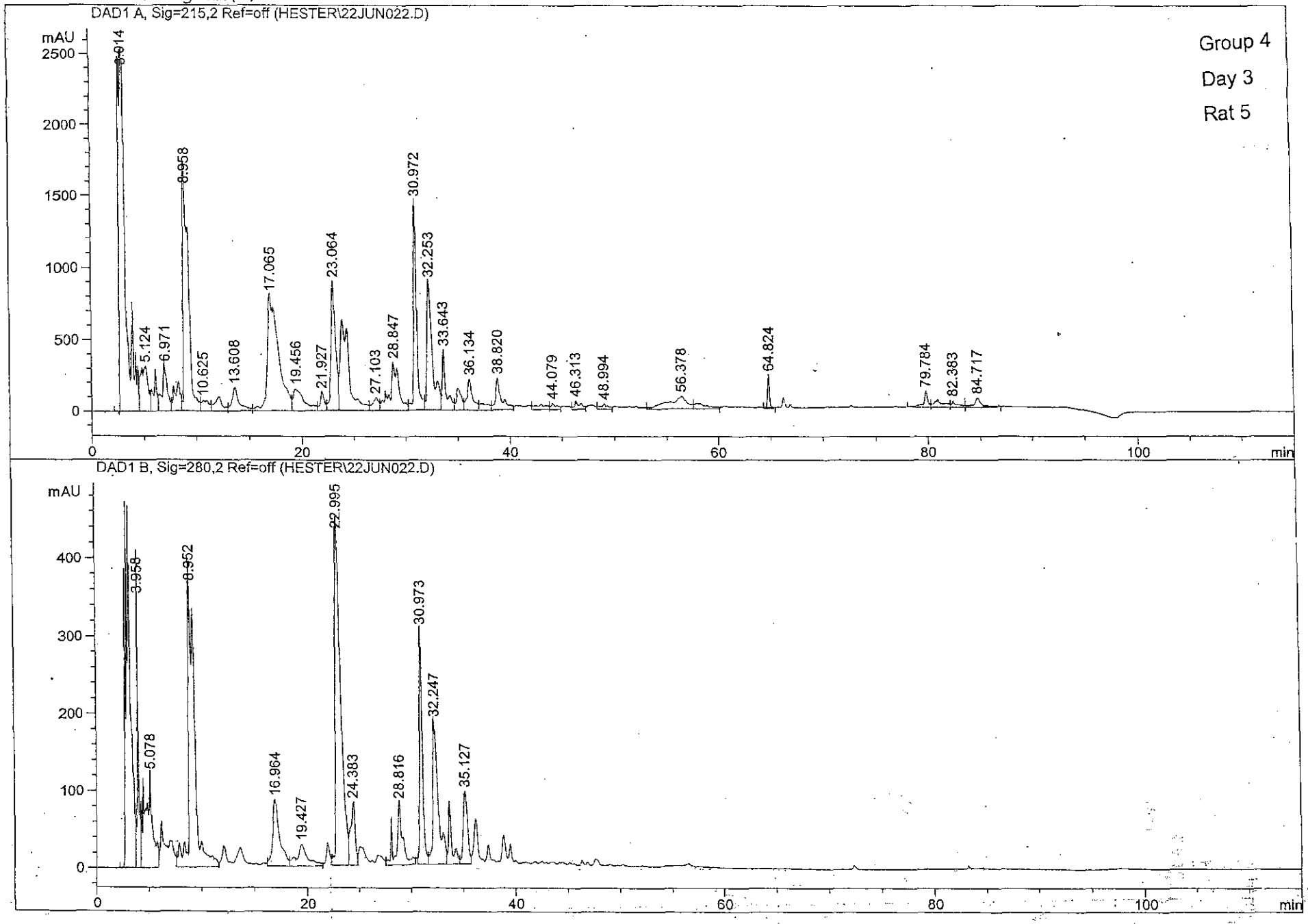
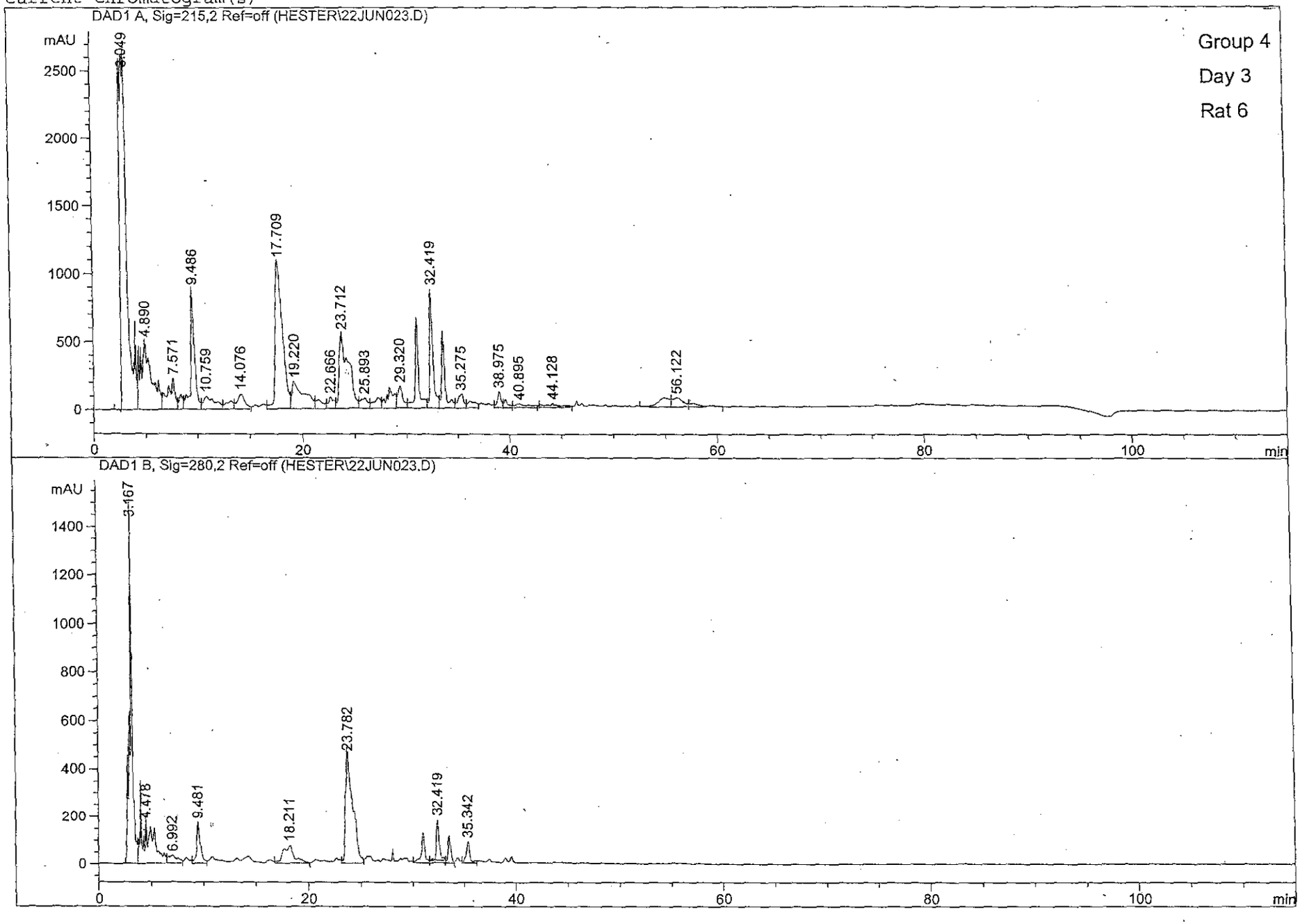


Current Chromatogram(s)



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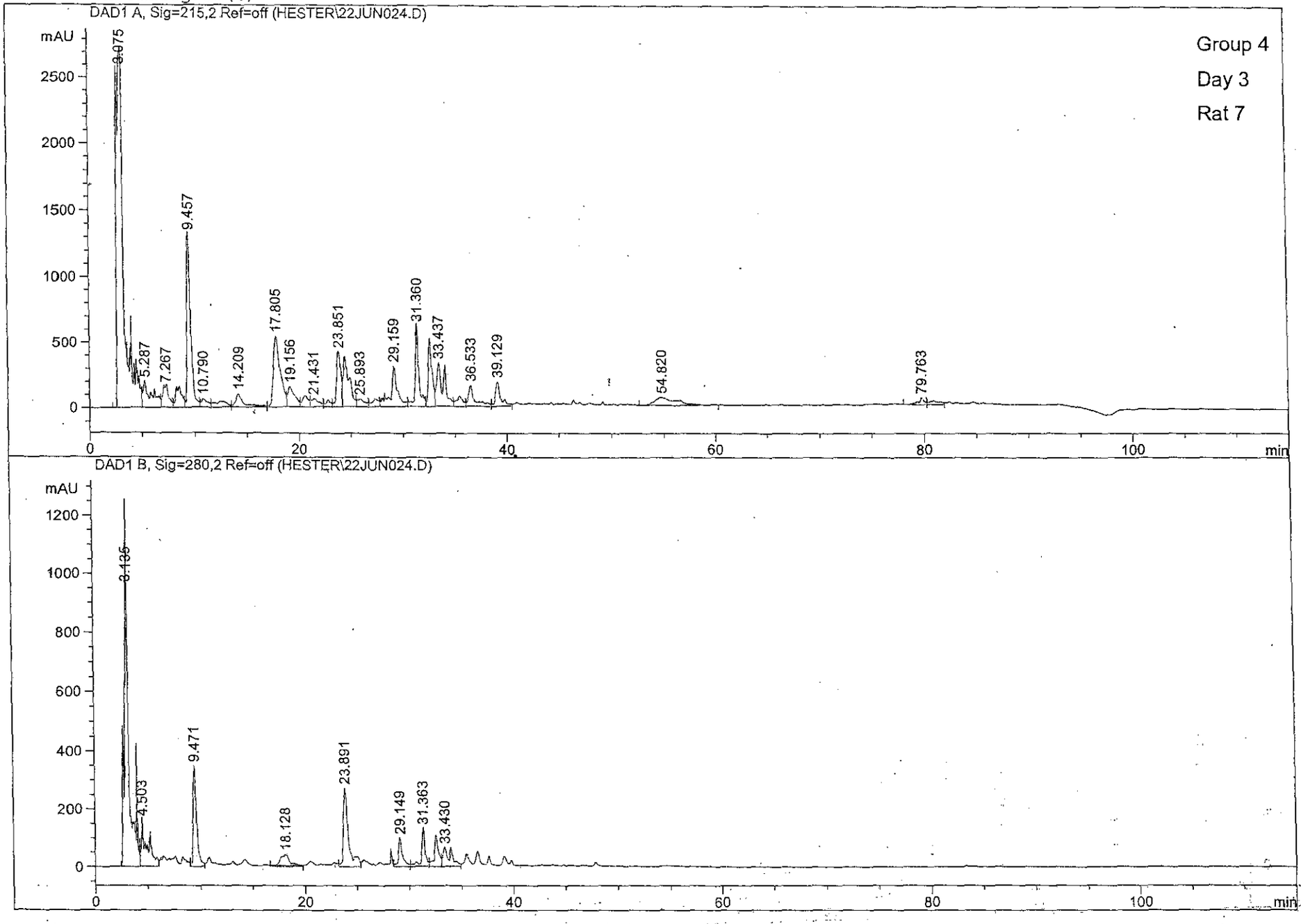

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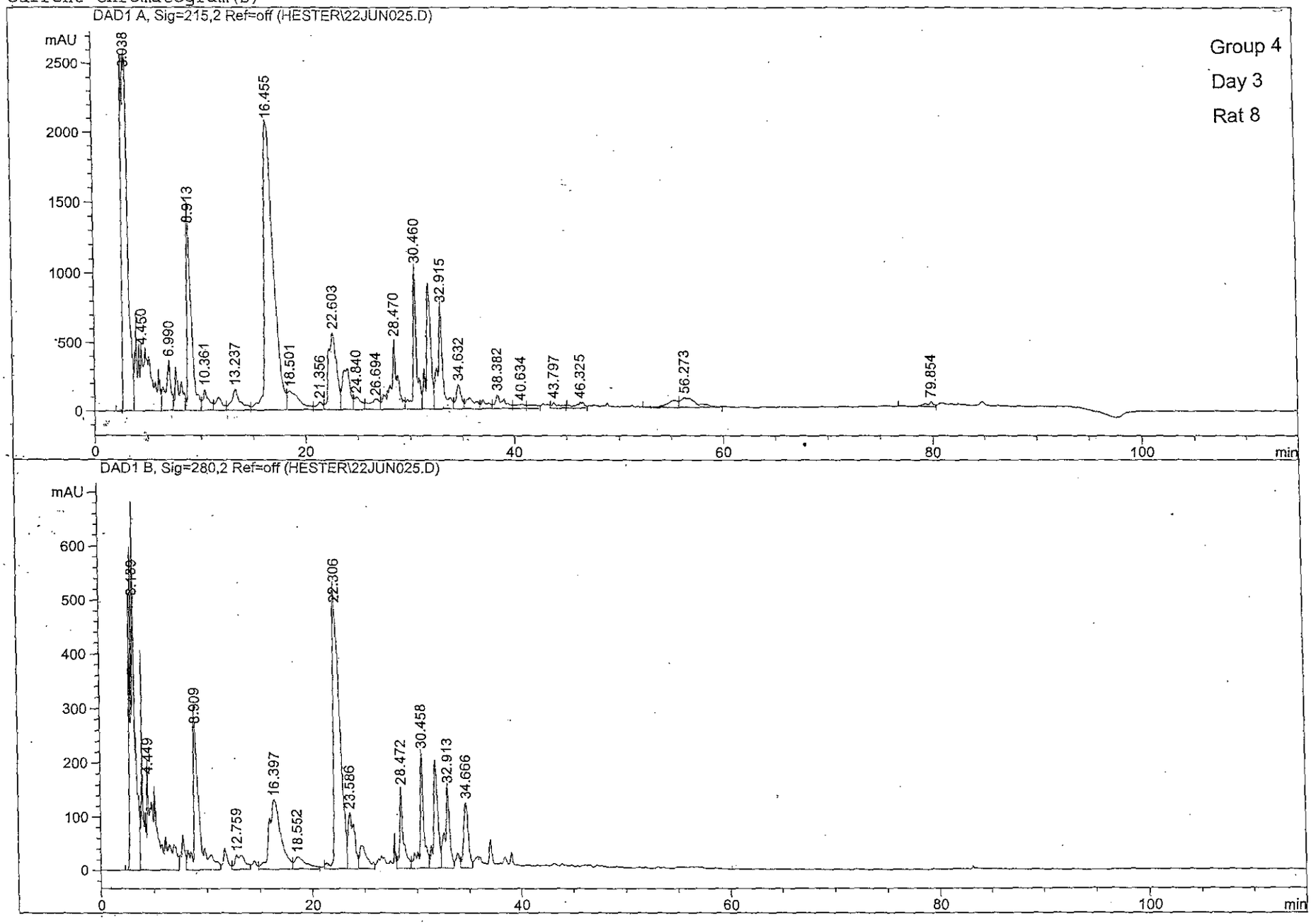

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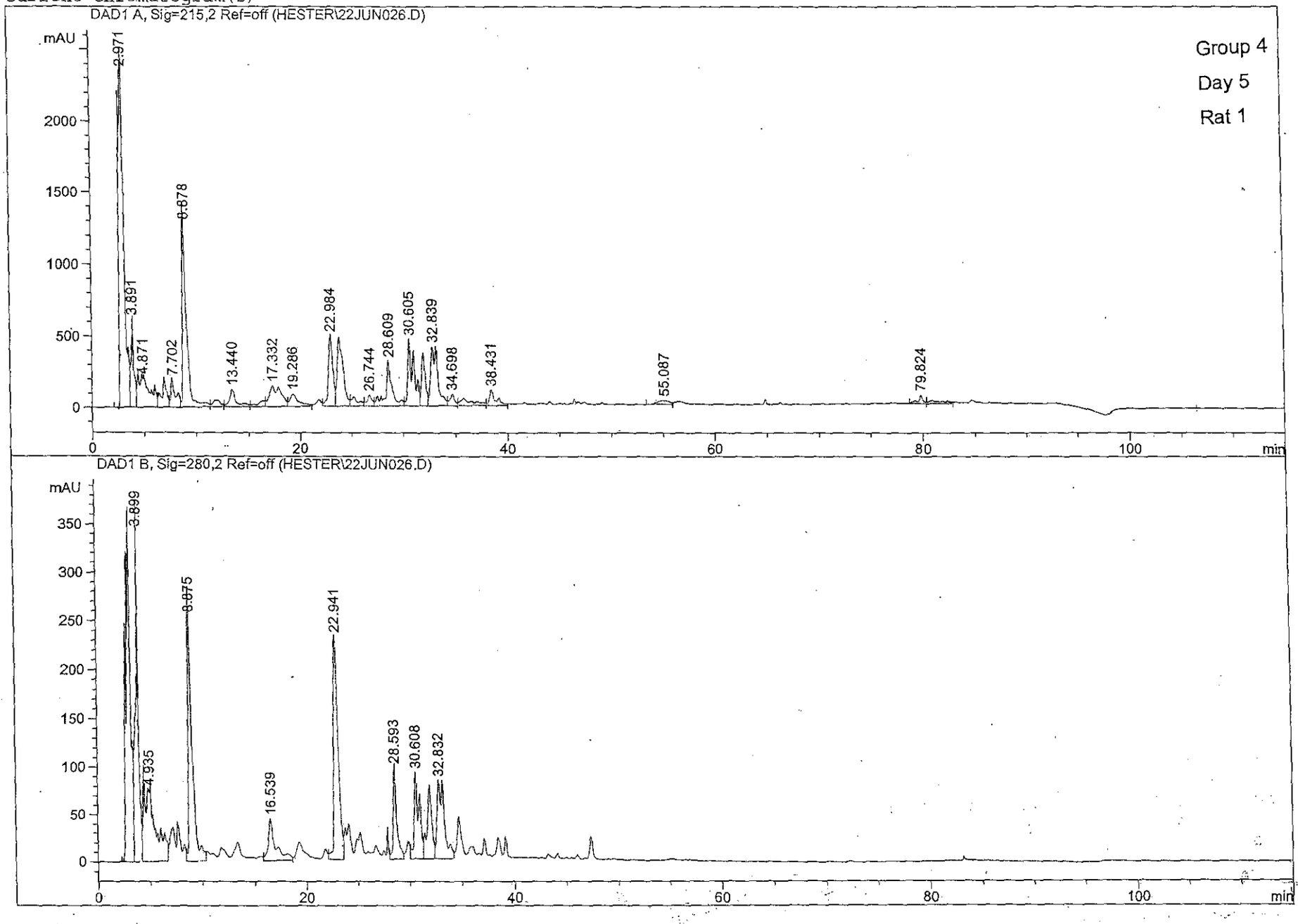

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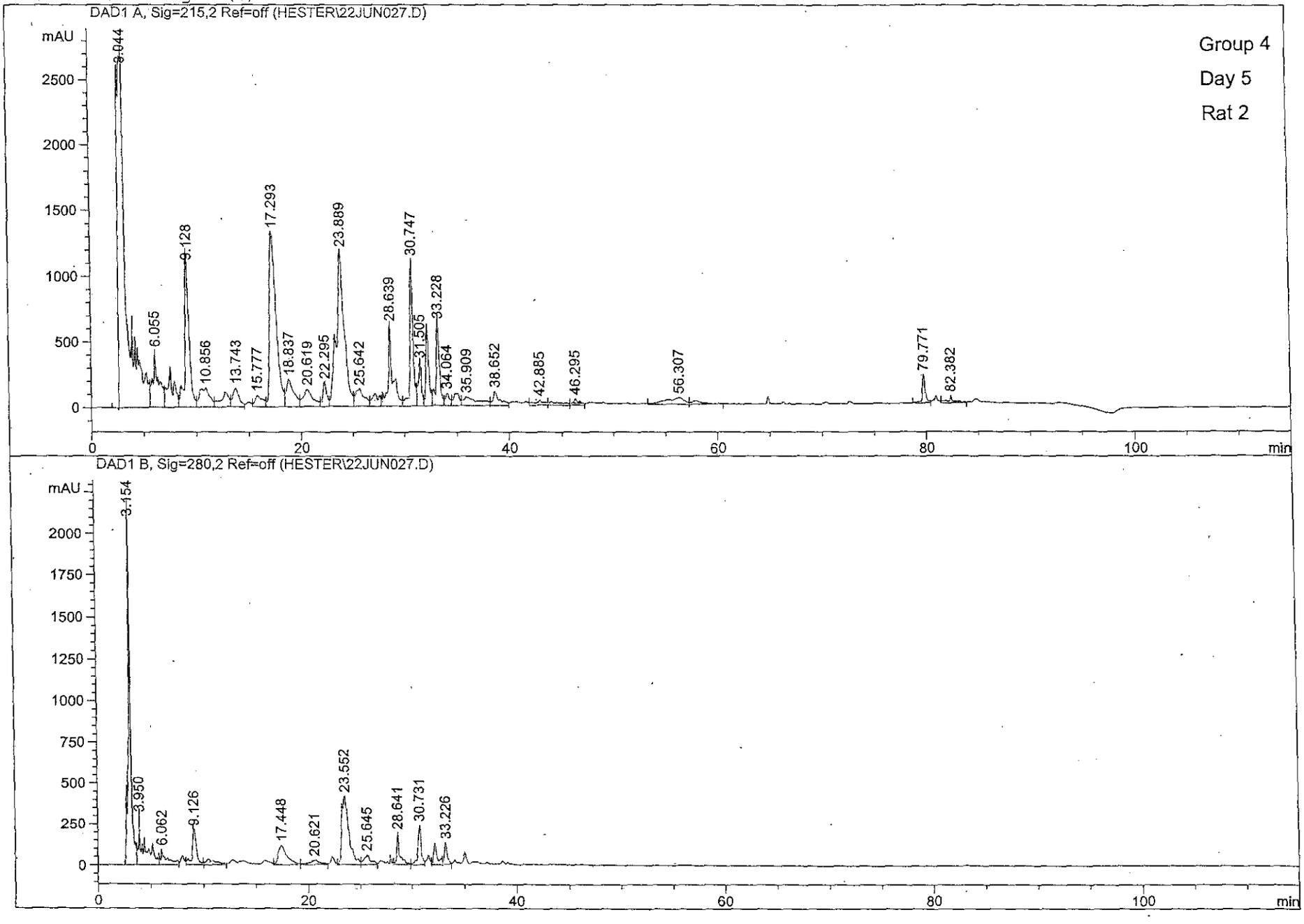

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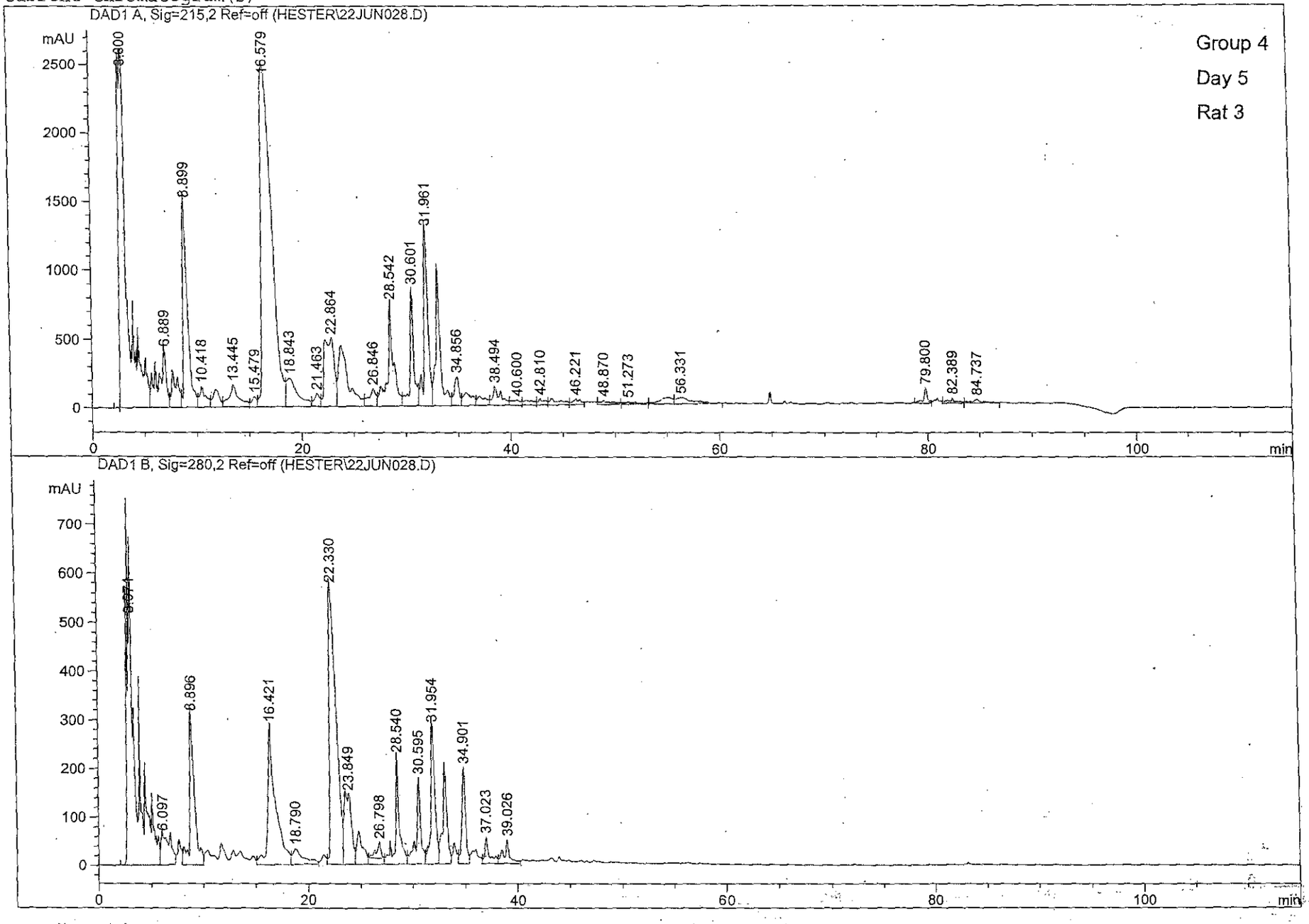

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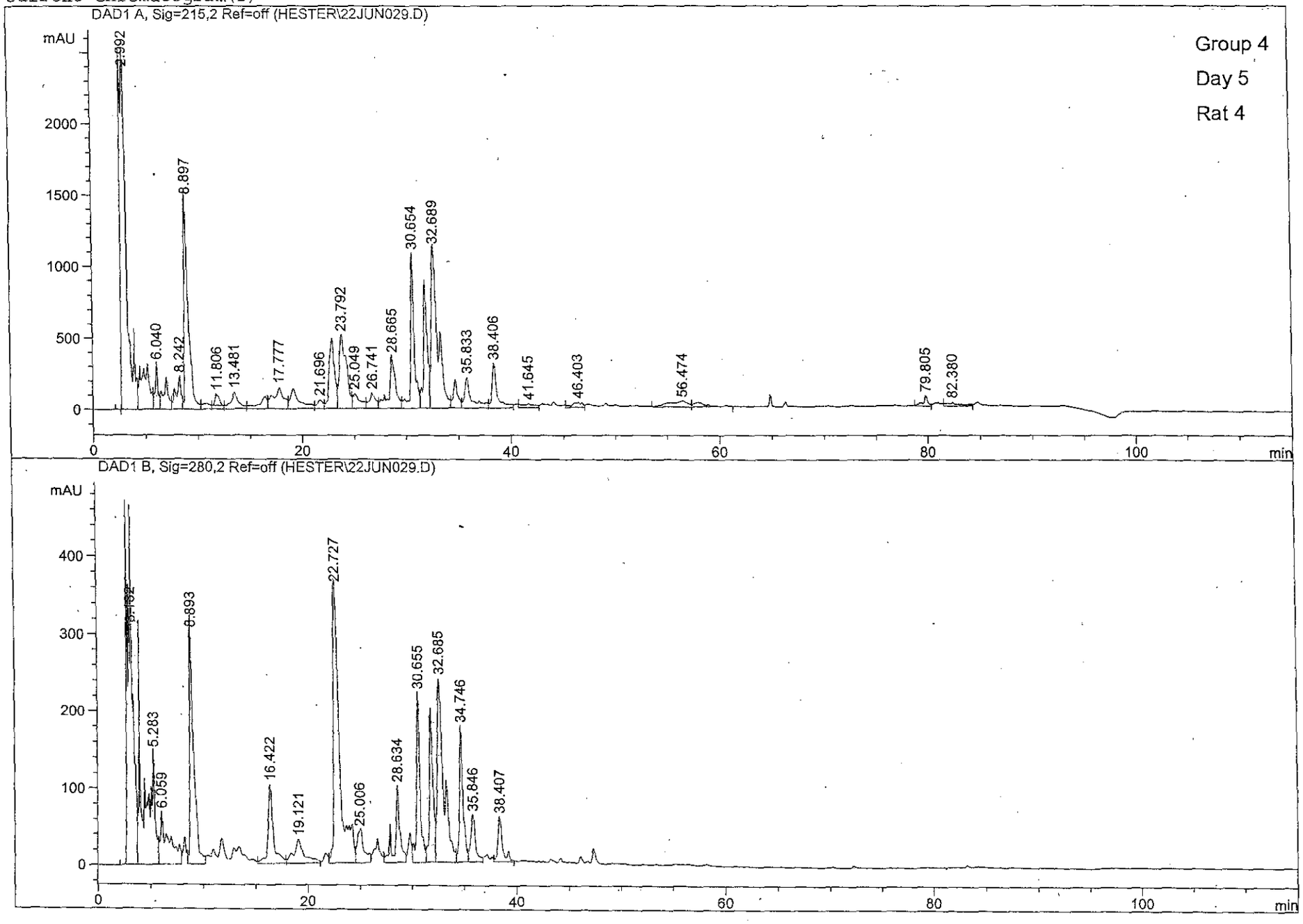

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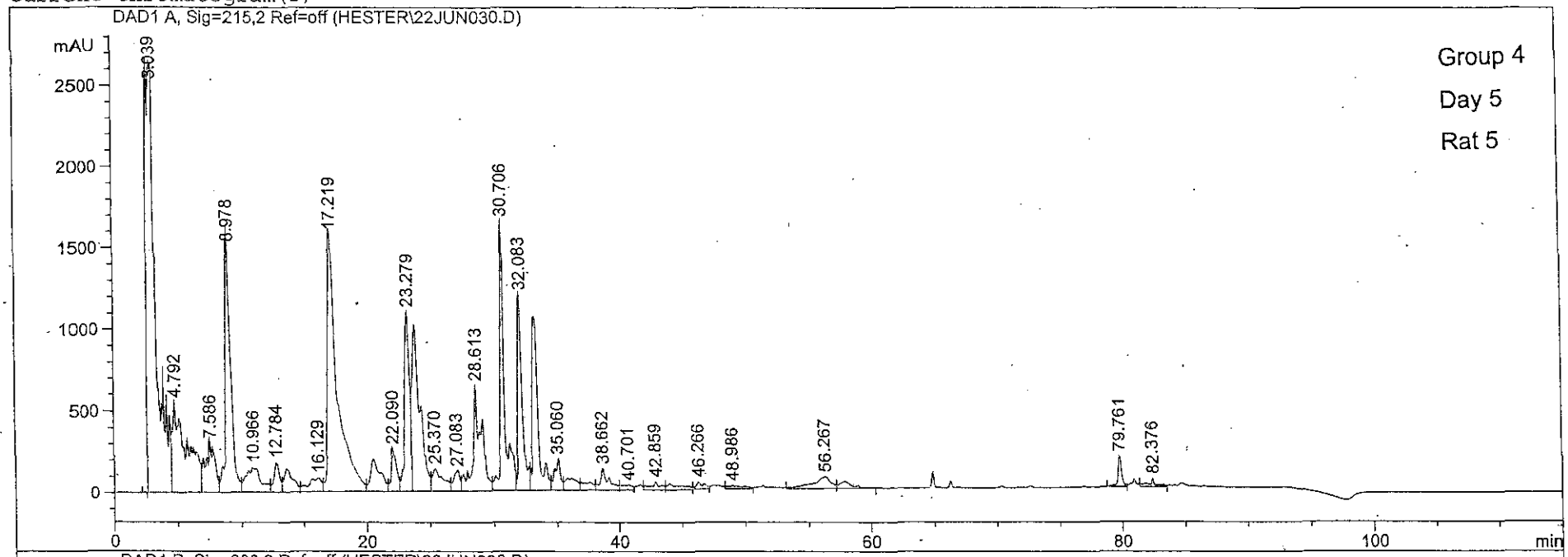


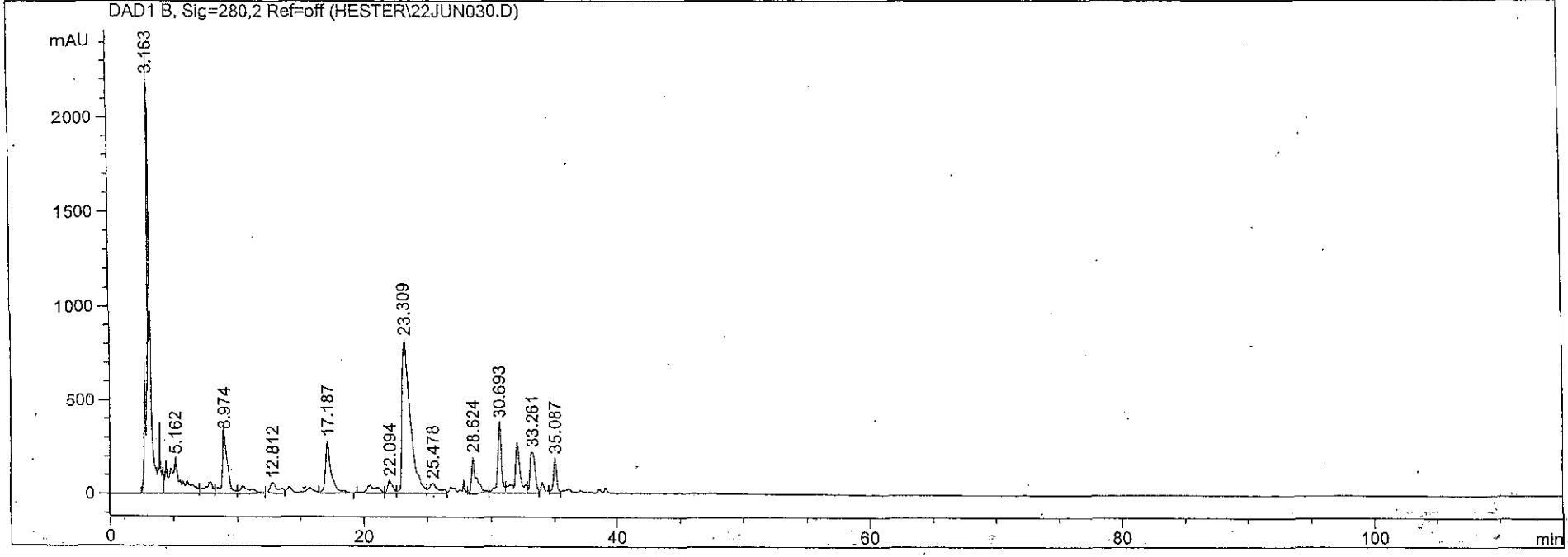

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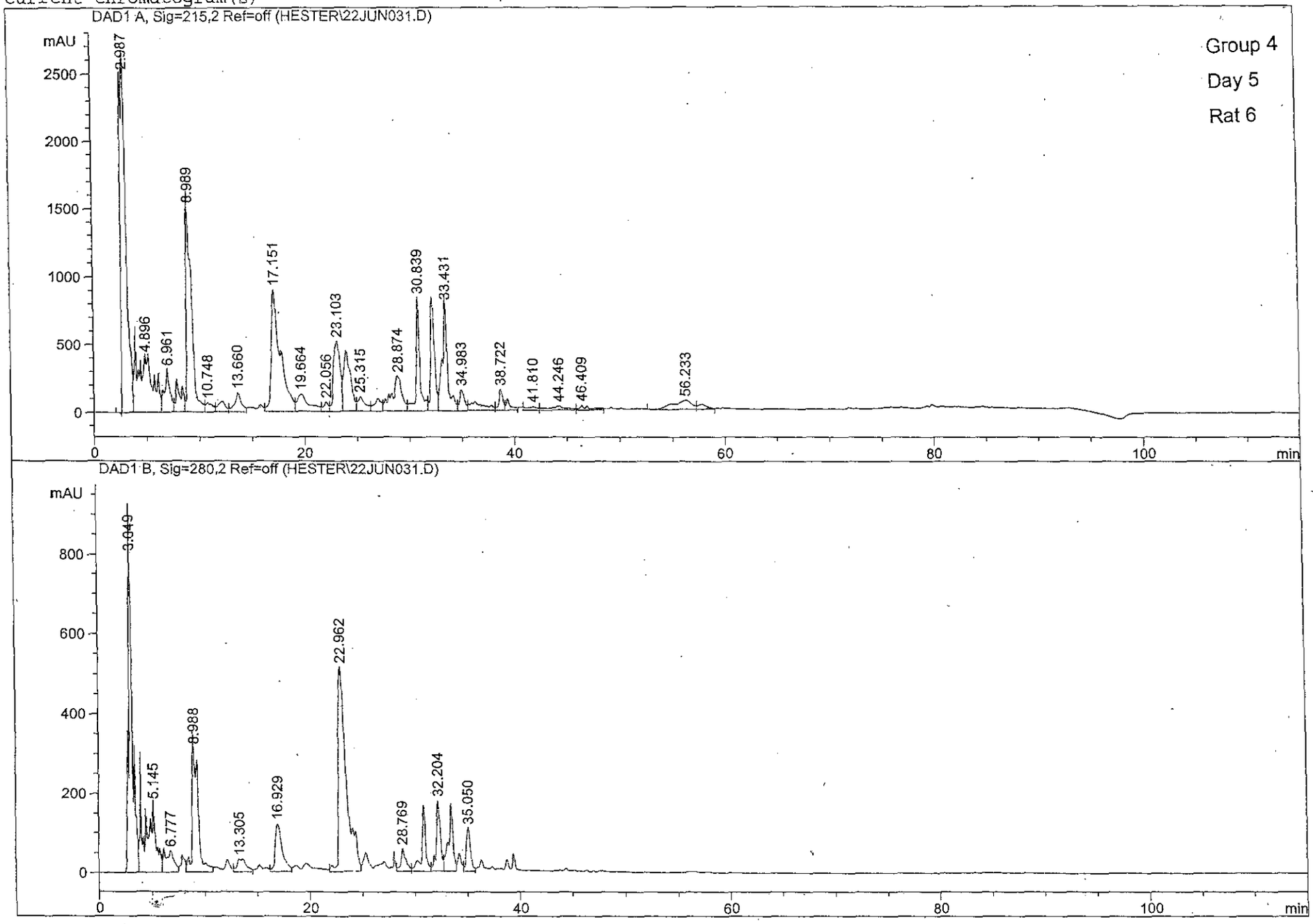

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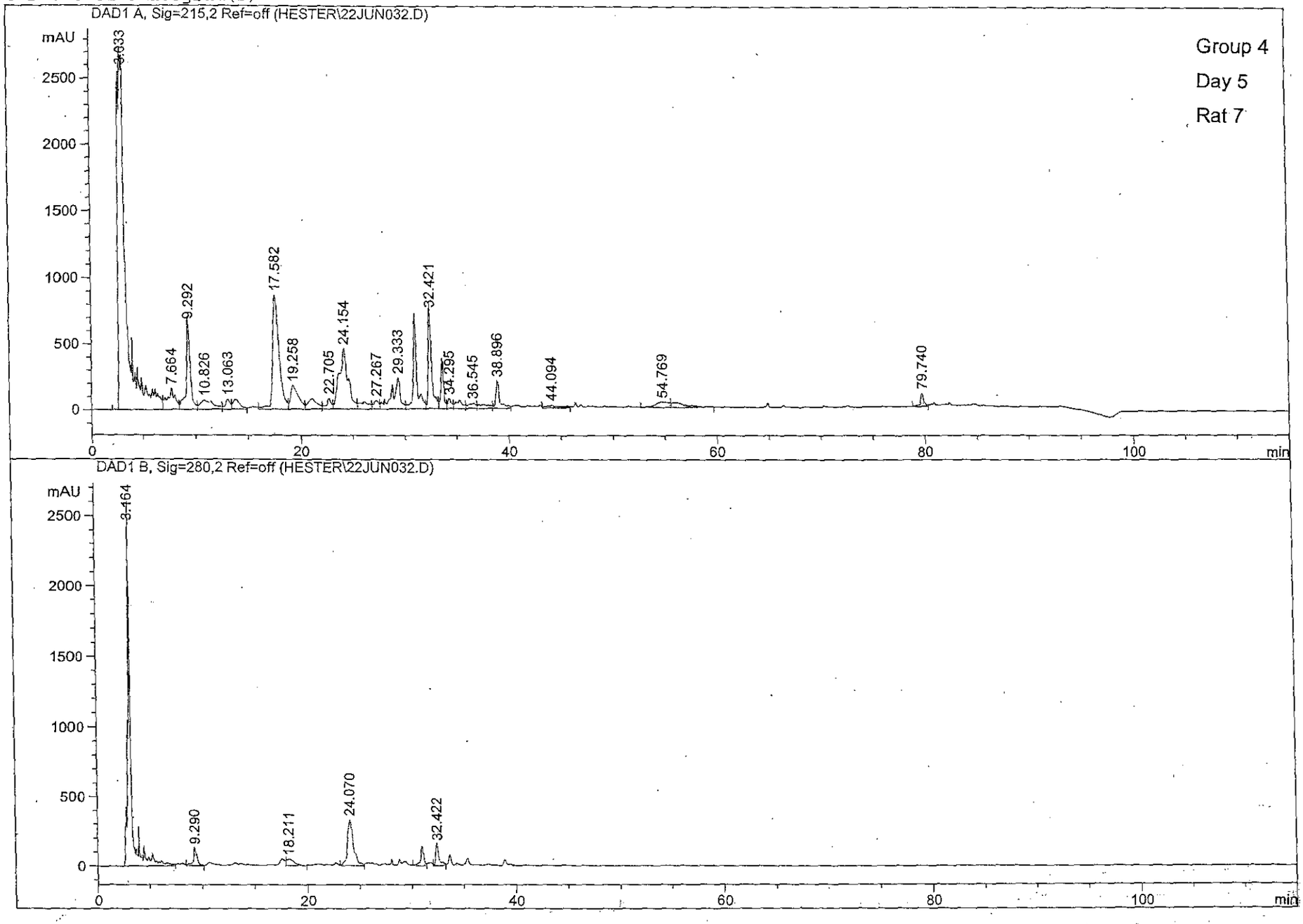

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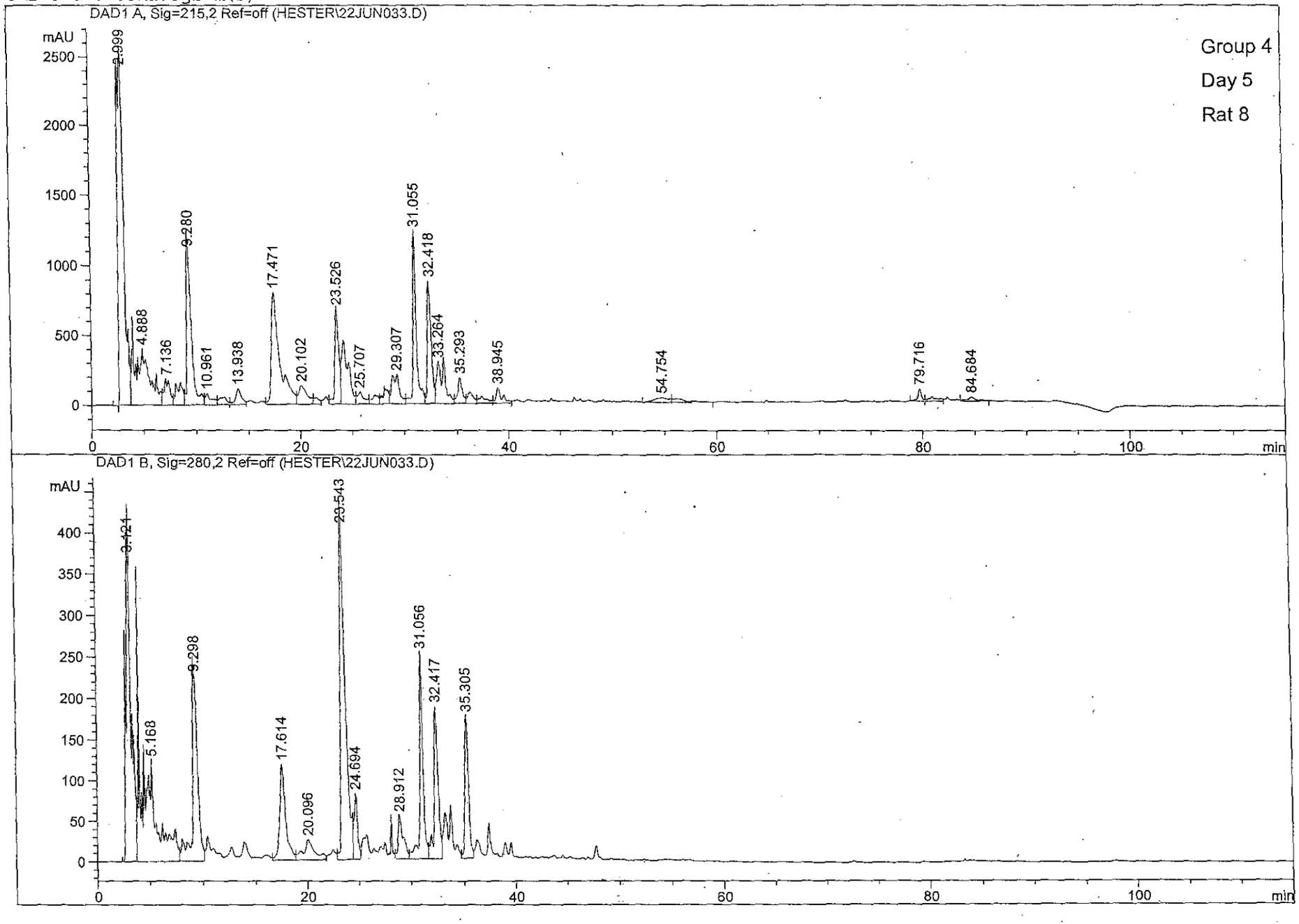


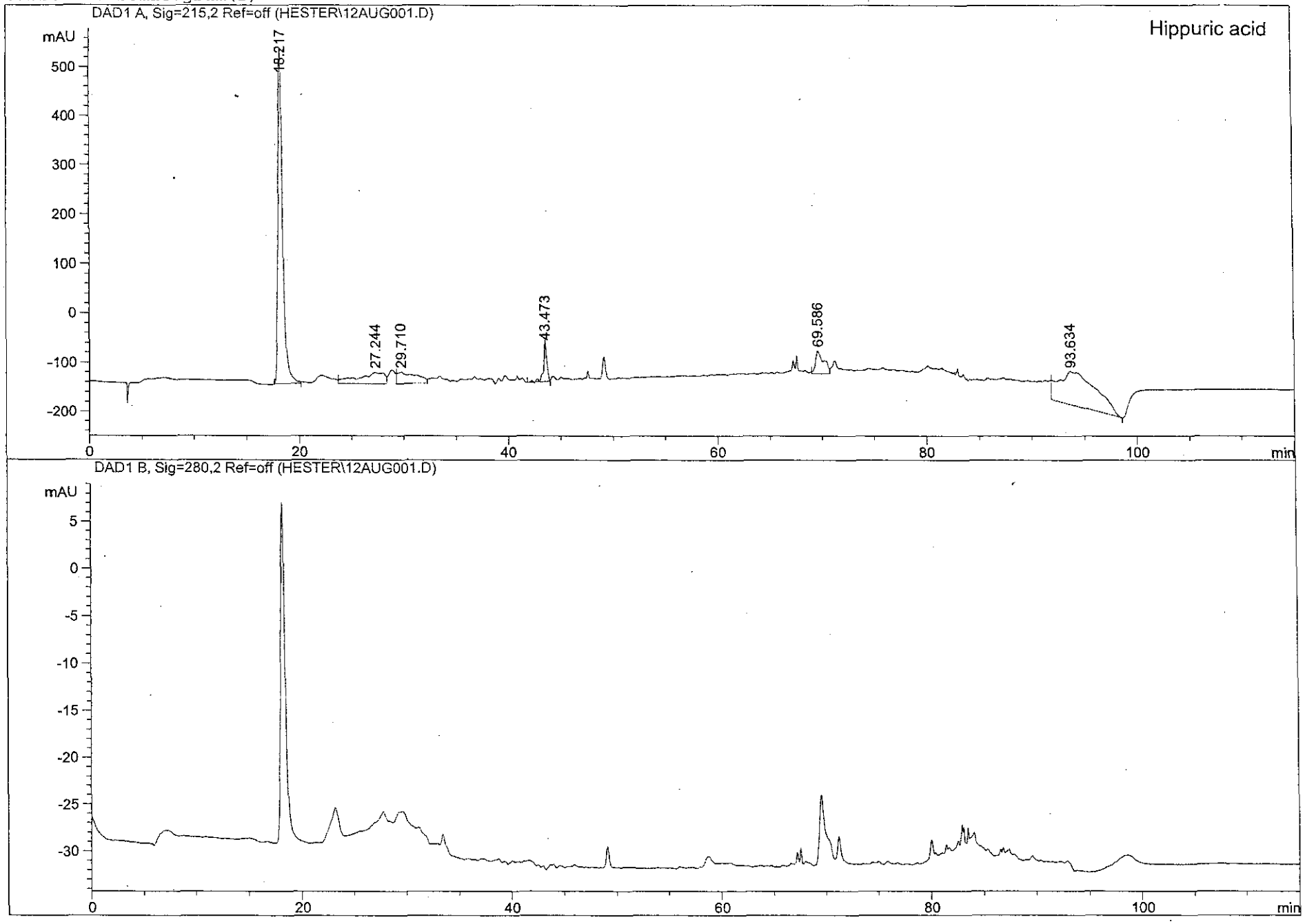
# Appenix C: Standards

Hippuric acid

β-casomorphin

β-casomorphin, fragment 1-5

Current Chromatogram(s)



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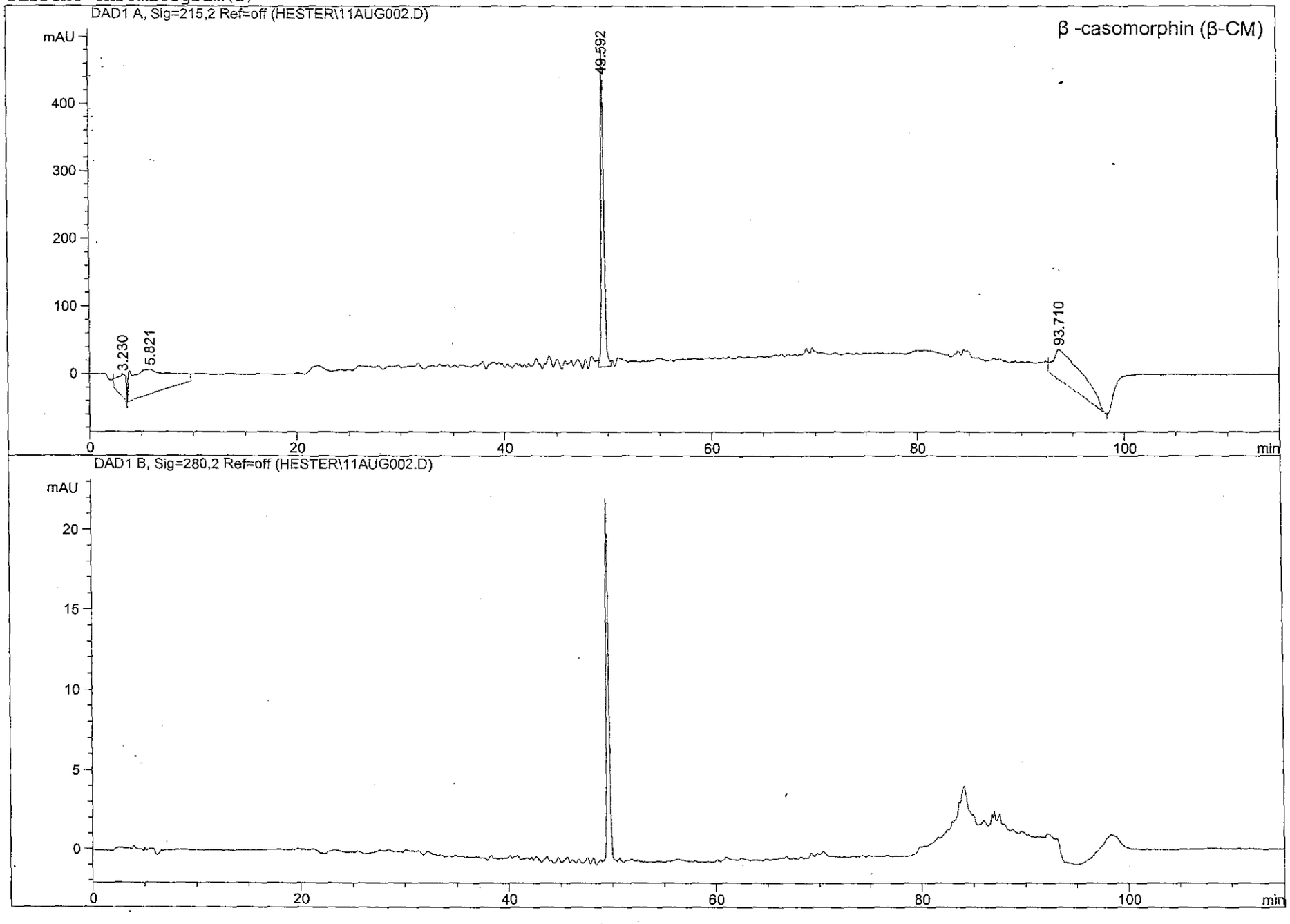

Current Chromatogram(s)