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TITLE: Acute Toxicity Determination on Zebrafish Embryos and Octanol/Water Partition Coefficient of Azo Dyes: Experimental and Computational Investigations

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THESIS

ACUTE TOXICITY DETERMINATION ON ZEBRAFISH EMBRYOS AND OCTANOL/WATER PARTITION COEFFICIENT OF AZO DYES: EXPERIMENTAL AND COMPUTATIONAL INVESTIGATIONS

WARAPORN JUNGTANASOMBUT

A Thesis Submitted in Partial Fulfillment of
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Based on EU legislation called REACH, it aims to manage chemicals and increase the protection of human health and environment from chemical used, manufactured and distributed within EU. Synthetic azo dyes that have been extensively used for textile dyeing should be one kind of the most synthetic dyes investigated their toxicity on aquatic organisms. This is because 2-50% of azo dyes can be lost to wastewater during dyeing process and ultimately relased to environment. Zebrafish embryos that is one kind of aquatic life and has been extensively used for toxicity determination because of their various advantages were utilized to investigate the acute toxicity (96 h) of 5 azo dyes including reactive red 239, direct red 80, direct blue 78, direct balck 22, and acid yellow 199 in this study. Endpoints were determined in this work including mortalitiy and embryo abnormalities. Results indicate that almost azo dyes in this study are nontoxic compound to zebrafish embryos due to their median lethal concentration (LC_{50}) is higher than 100 mg/L. However, industry using these dyes should be aware of their adverse effects because they can make developmental abnormalities on zebrafish embryos including yolk sac edema, cardiac edema, bent spine and tail malformation. In this study, acid yellow 199 is the highest toxic compound on zebrafish embryos due to its LC_{50} value is lowest. This results corresponsed to the experimental log *P* value derived from shake-flask method in this research. Acid yellow 199 has the highest log *P* value therefore, it tends to be easily absorbed in tissue of zebrafish embryos more than other compounds which have lower log *P* values. A QSTR model on the small set of 5 azo dyes built up by using linear regression method, it also supported that toxicity of compounds increases with increasing log *P* values. However, the quality of model is not good for toxicity prediction so, more toxicity data are required for developing a new QSTR model.

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Waraporn Jungtanasombut

November, 2012

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LIST OF ABBREVIATIONS

EU	=	European Union
FET	=	Fish Embryos Toxicity Test
LC_{50}	=	Median lethal concentration
log *P*	=	Logarithm of octanol/water partition coefficient
n	=	Number of compounds
$p(1/ LC_{50})$	=	Negative logarithm of median lethal concentration
QSTR	=	Quantitative structure-toxicity relationship
r^2	=	Square of correlation coefficients
REACH	=	Registration, Evaluation, and Authorization of Chemicals
s	=	Standard deviation
SEM	=	Standard error

ACUTE TOXICITY DETERMINATION ON ZEBRAFISH EMBRYOS AND OCTANOL/WATER PARTITION COEFFICIENT OF AZO DYES: EXPERIMENTAL AND COMPUTATIONAL INVESTIGATIONS

INTRODUCTION

Large amounts of dyes have been widely used in various industries including textiles, foods, pharmacuticals, cosmetic, paper, and leather goods (Forgaces *et al.*, 2004; Cirtóvão *et al.*, 2009). The largest and most versatile class of synthetic dyes currently used for coloring are the azo dyes based on azo chromogen (-N=N-) (Benkli *et al.*, 2005; Puvaneswari *et al.*, 2006). Various synthetic dyes such as reactive, direct, and acid azo dyes have been extensively applied in the textile industry for textile processes due to their applications to different materials including synthetic and natural textile fibers (Karadag *et al.*, 2007; Asouhidou *et al.*, 2009; Tavares *et al.*, 2009). 20-50% of these dyes are lost in wastewater during textile dyeing and ultimately released into the environment (Chen, 2002; Ozdemir *et al.*, 2004; Cirstóvão *et al.*, 2008). Reactive, direct, and acid azo dyes are highly soluble in water and cannot be easily removed, causing serious environmental problems, not only aesthetic effects, but more importantly toxic effects (Armaĝan *et al.*, 2003). Many azo dyes and their breakdown products (aromatic amines) have been found to be toxic to aquatic life, and mutagenic to humans (Meriç *et al.*, 2005; Zee and Villaverde, 2005; Tavares *et al.*, 2009).

European Union (EU) legislation called REACH (Registration, Evaluation, and Authorization of Chemicals) aims to manage chemicals and improve the protection of human health and environment from exposure to chemicals by requiring industry to provide toxicity data on the chemical used, manufactured, and distributed (EU, 2001; EU, 2006). Industrial factories in Thailand that export their products to EU will be affected if they do not have sufficient information about the effect, uses,

and safety of their chemicals. Therefore, toxicity information of synthetic azo dyes is an important area of research.

Effluent used during coloring activities in the textile industry is ultimately discharged to the aquatic environment (Umbuzeiro *et al.*, 2005). Thus, aquatic organisms are probably the most appropriate biological indicators to assess the quality of water. Zebrafish (*Danio rerio*) embryos, are aquatic organisms, that have been widely utilized for toxicity testing due to their various advantages. This organism is very sensitive during the life cycle of teleost meaning it is suitable for measuring environmental pollution that occurs at low level of concentration (Bai *et al.*, 2010). Zebrafish eggs are both rapid and synchronous in development. Their optical transparency provides the investigator a means to direct observe the toxic effects of chemicals to their internal organs (Lele and Krone, 1996; Barros *et al.*, 2008; McGrath and Li, 2008). They are also an ethically acceptable vertebrate model based on EU Directives (approximately 5-7 days after fertilization) and therefore can be used as aquatic animals for toxicity test within the framework of REACH (Alshut *et al.*, 2010; Belanger *et al.*, 2010; Ali *et al.*, 2011; Strähle *et al.*, 2011).

Additionally, zebrafish adults are small, easy to maintain, and rapidly and prolifically to breed (Alderton and Berghmans, 2006; Lawrence, 2007). Individual female is capable to lay 200-300 eggs/day every 5-7 days, so this makes the large-scale screens possible (Fako and Furgeson, 2009). Genes of zebrafish are approximately 75% homologous to human on average (Barbazuk *et al.*, 2000; Zhang *et al.*, 2003). Therefore, there are numerous types of toxicity investigations using zebrafish as a surrogate for human, such as reproductive toxicity, acute toxicity, neurotoxicity, cardiotoxicity, and carcinogenicity (Hill *et al.*, 2005)

One of the goals of REACH is to reduce the number of animals for toxicity test by using computational prediction models (EU, 2001). The relationship between the structure of chemical and its activity or toxicity (Lessigiarska *et al*., 2006), called Quantitative Structure-Activity (Toxicity) Relationship or QSA(T)R, is increasingly interested to use for toxicity prediction of chemicals because it can save cost, time and

number of animals used in experimental tests, as required by the European Directive on the protection of Laboratory Animals.

Octanol/water partition coefficient (*P*), commonly quoted on the logarithmic scale as log *P*, is an important parameter in the fields of pharmacology, toxicology, and medical chemistry (Klopman *et al.*, 1994; Golmanhammadi, 2009). Log *P* is defined only for the same (undissociated) species in both phases of octanol and water (James, 1989) due to it is the concentration ratio of the neutral form of a compound in octanol and water phases (Wenlock *et al.*, 2011). Therefore, log *P* can indicate the hydrophobicity of compounds and relates to toxicity (Anderson and Schräder, 1999; Ingram *et al.*, 2011), bioaccumulation (Chapeaux *et al.*, 2007), soil absorption coefficient (Lü *et al.*, 2007), oral absorption, permeability, and solubility (Valkó, 2004). Additionally, log *P* is extensively used to develop QSA(T)R model for predicting the tendency of chemicals to bioaccumulate in the tissues of living organisms (Giri *et al.*, 2009). Compounds with higher log *P* values (lower soluble in aqueous) tend to be absorbed more easily into tissue (Rothwell, 2005). Thus, it is an important property that should investigate in this research work.

Reactive red 239 (RR239), direct red 80 (DR80), direct blue 78 (DB78), direct black 22 (DB22), and acid yellow 199 (AY199), that are synthetic azo dyes, have been widely used in textile industry for coloring purposes however, the toxicity data on them are limited. Thus, the objectives of this research work are to determine the acute toxicity (96 h) of undefined 5 azo dyes on zebrafish (*Danio rerio*) embryos and to investigate the effects of all dyes on embryonic morphology. Logarithm of octanol/water partition coefficient (log *P*) of these 5 compounds is also measure. Additionally, the relationship between toxicity values in term of $p(1/LC_{50})$ and log *P* values of these 5 azo dyes is built up in this work.

OBJECTIVES

1. To determine the acute toxicity of 5 azo dyes including reactive red 239 (RR239), direct red 80 (DR80), direct blue 78 (DB78), direct black 22 (DB22), and acid yellow 199 (AY199) on zebrafish embryos.

2. To investigate the effects of these dyes on morphology of zebrafish embryos.

3. To measure the logarithm of octanol/water partition coefficient (log *P*) of these 5 azo dyes by using shake-flask method.

4. To find the relationship between toxicity values, presented in term of the negative logarithm scale as $p(1/LC_{50})$, with log *P* values of these azo dyes.

LITERATURE REVIEW

In 1867, the synthetic dye industry as we known it was found by William Henry Perkin (Abrahart, 1968). Synthetic dyes, especially azo group, have been extensively used for dyeing processes in textile industry (Carneiro *et al.*, 2010). Additionally, they can also be widely applied to paper printing, photography, pharmaceuticals, food, cosmetics and petroleum products (Chequer *et al.*, 2009). Synthetic dyes can be systematically classified according to their core chemical structure, such as polyene and polymethine, diarylmethine, triarylmethine, nito and nitroso, anthraquinone, and diazo (Doble and Kumar, 2005) as shown in Figure 1. A major class of synthetic dye is the azo type that accounts 65% of the total dyes production in the world. Azo dyes have one or more azo group (-N=N-) and subdivide according to the present of the number of azo groups (Carneiro *et al.*, 2010).

Generally the concentration of dyes contained in wastewater is approximately 10 to 200 mg/L. Characterizations of wastewater released from textile coloration process are highly colored and has low BOD (biological oxygen demand), high COD (chemical oxygen demand) (Doble and Kumar, 2005). Several dyes are toxic to fish and mammalian life, carcinogenic in nature, and can cause intestinal cancer and cerebral abnormalities in fetuses (Doble and Kumar, 2005). Moreover, some dyes such as disperse azo dyes have also a tendency to bioaccumulate (Carneiro *et al.*, 2010). Even though the acute toxicity of azo compounds is generally low, some azo dyes can cause bladder cancer in human and have been shown to cause splenic sarcomas hepatocarcinomas and nuclear anomalies in experimental animal models. Additionally, some of these compounds are able to cause DNA damage (Chequer *et al.*, 2009) and their breakdown products (aromatic amines) are toxic to aquatic life (Zee and Villaverde, 2005).

The mode of activation for azo dyes consists of 3 principal modes containing: (1) after reduction and cleavage of azo dyes to give aromatic amines, (2) azo dyes containing free aromatic amine groups metabolically oxidized without azo reduction, and (3) azo dyes activated via direct oxidation of the azo linkage to diazonium salts,

highly reactive electrophilic compound, (Chung, 2000). The color removal of azo dyes obtaining aromatic amines can be done by microbial species including bacteria, fungi, and algae via biotransformation, degradation or mineralization and can also occur in the body catalyzed by both mammalian cell enzymes and gut microorganisms (Doble and Kumar, 2005). The metabolism of azo dyes by bacteria can be achieved under anaerobic conditions resulting in colorless aromatic amines that are resistant to anaerobic mineralization but are good substrates for aerobic degradation through a hydroxylation pathway involving a ring-opening mechanism. Thus, a combination of anaerobic and aerobic treatment illustrated in Figure 2 could be very effective for azo dyes biodegradation (Zee and Villaverde, 2005). However, Møller and Wallin (2000) reported that the absorbed insoluble azo dyes have been found to be metabolized in the liver. The metabolism of pigments can occurr via various reactions comprising intestinal azo-reduction, hepatic azo-reduction, and hepatic oxidation. As C.I. Solvent Yellow 14 (SY14) that is insoluble in water, it is metabolized by several routes (Figure 3) including ring hydroxylation, azo-reduction, and oxidation by peroxidase enzymes.

The toxicity of azo dyes depends on their metabolites (aromatic amines) that can cause methemoglobinemia resulting in cyanosis of lip and nose, weakness, and dizziness (Gupta, 2006). For example, Acid Orange 7 (Figure 4) can also easily undergo breakdown by enzymes along with reduction and cleavage to give aromatic amines which also tend to oxidize the heme iron of hemoglobin from Fe (II) to Fe (III) and block oxygen binding (Gupta, 2006). Additionally, para-Dimethylaminoazobenzene (DAB) in Figure 5 has been used for coloring polishes and wax products, polystyrene and gasoline. It is carcinogenic in rats when administered orally. Administered daily dosage is major factor to time period of the occurrence of liver tumors in rats. At the higher dose of administration, rats faster produce liver tumors (30 mg DAB per day producing tumors after took up for 34 days while, 1 mg DAB per day producing tumor after took up for 700 days). The major biotransformation reaction of DAB includes stepwise demethylation, acetylation, c-hydroxylation, and reductive splitting of azo linkage shown in Figure 6 (Doble and Kumar, 2005). These studies confirm that excessive exposure of azo dyes might

present a risk to human health and determination of the toxicity of azo dyes is therefore desired.

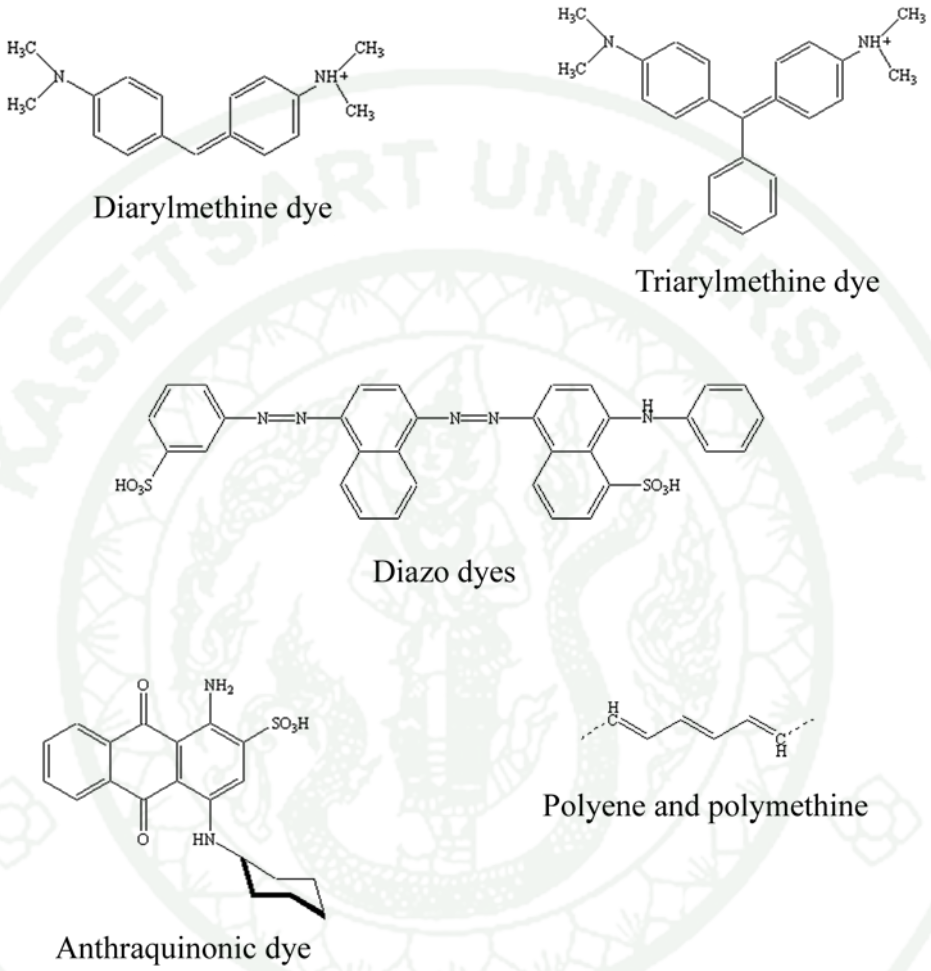


Figure 1 Structure of dyes based on predominant groups

Source: Doble and Kumar (2005)

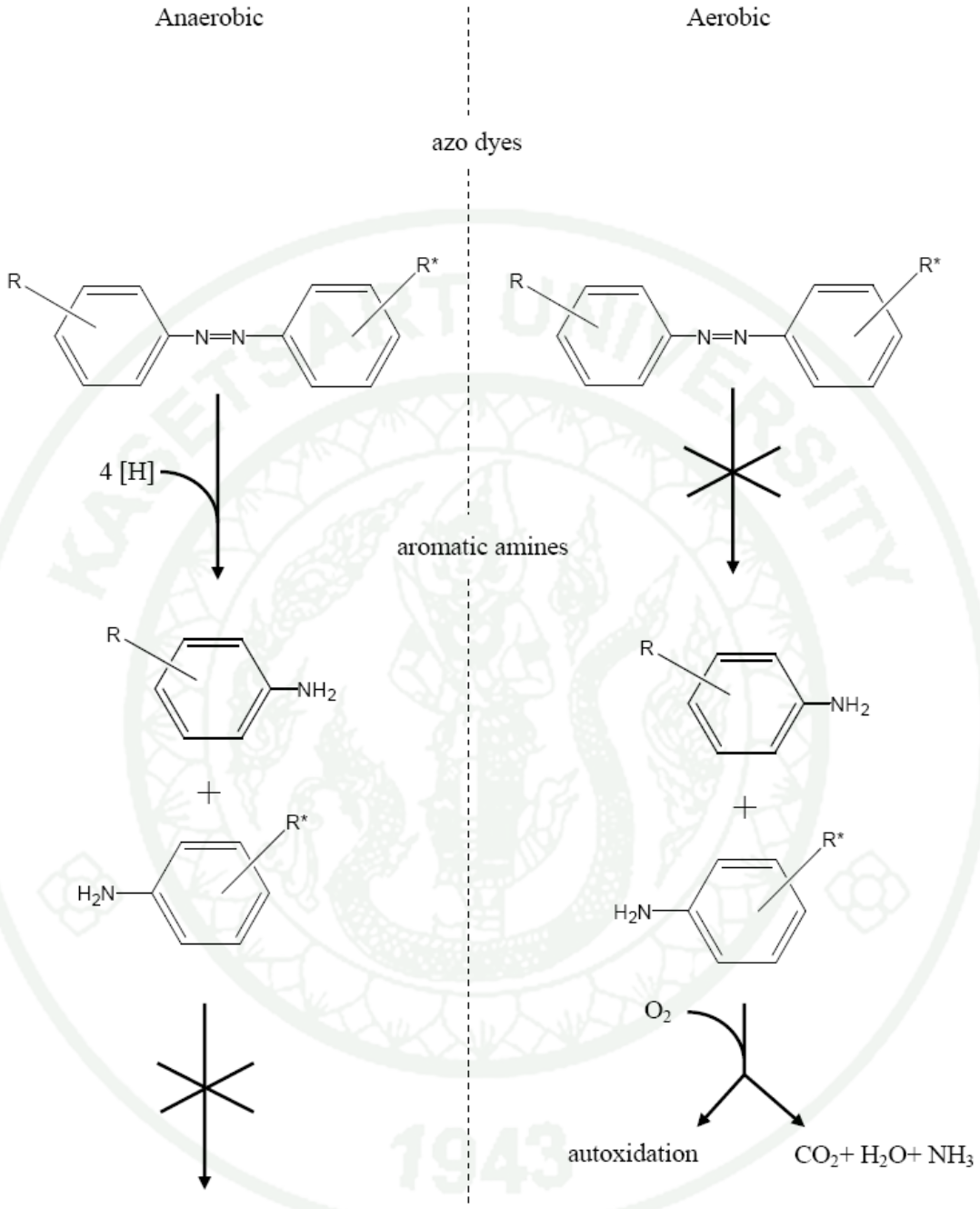


Figure 2 Biodegradation of azo dyes and aromatic amines under anaerobic-aerobic Treatment

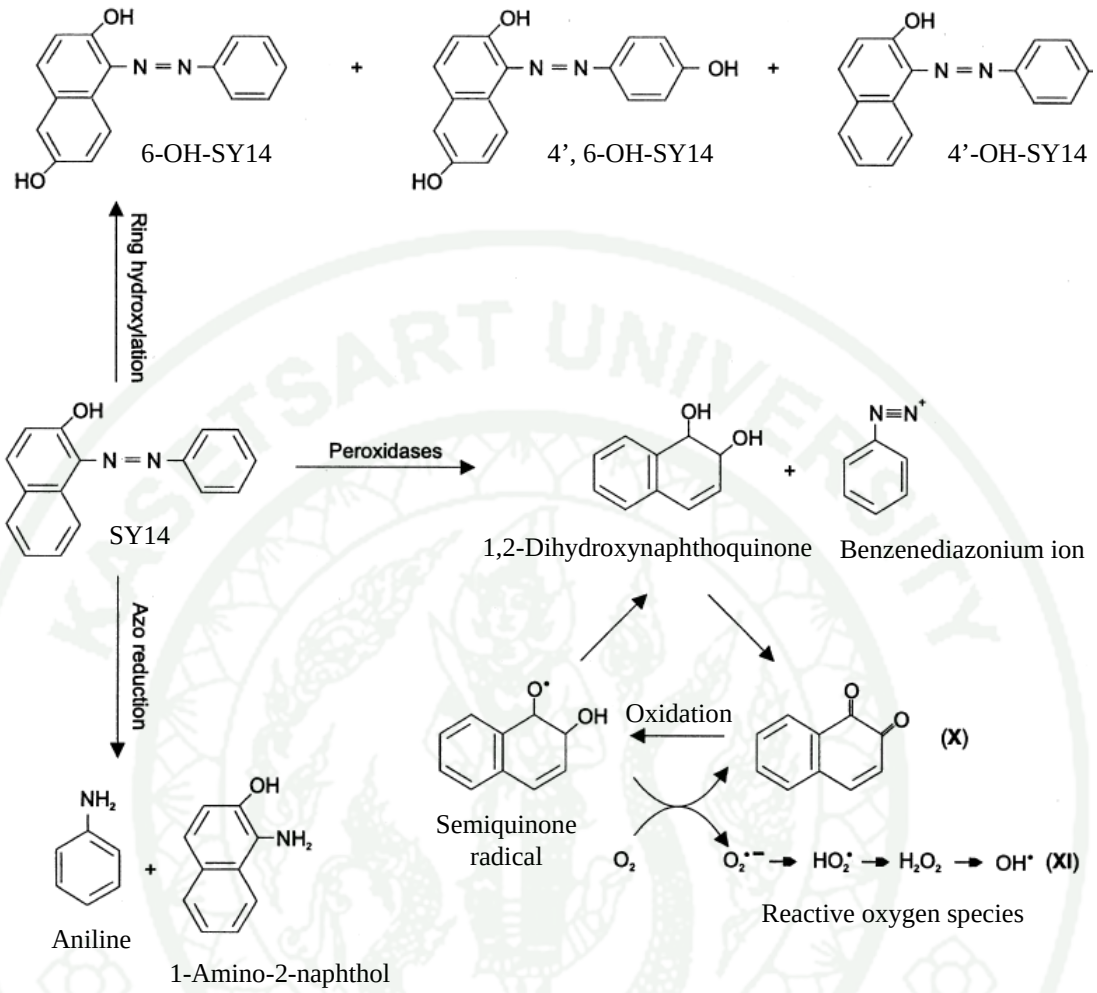


Figure 3 Metabolism of C.I. Solvent Yellow 14 (SY14)

Source: Møller and Wallin (2000)

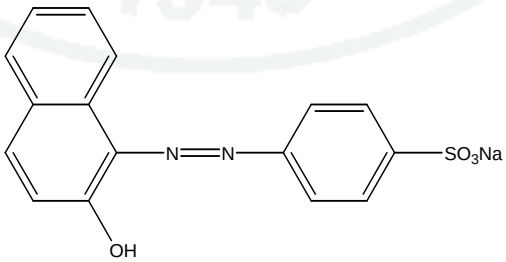


Figure 4 Structure of Acid Orange 7 (p-(2-hydroxy-l-naphthylazo)benzene sulfonic acid, I)

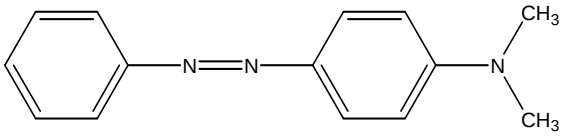


Figure 5 Structures of para-Dimethylaminoazobenzene (DAB)

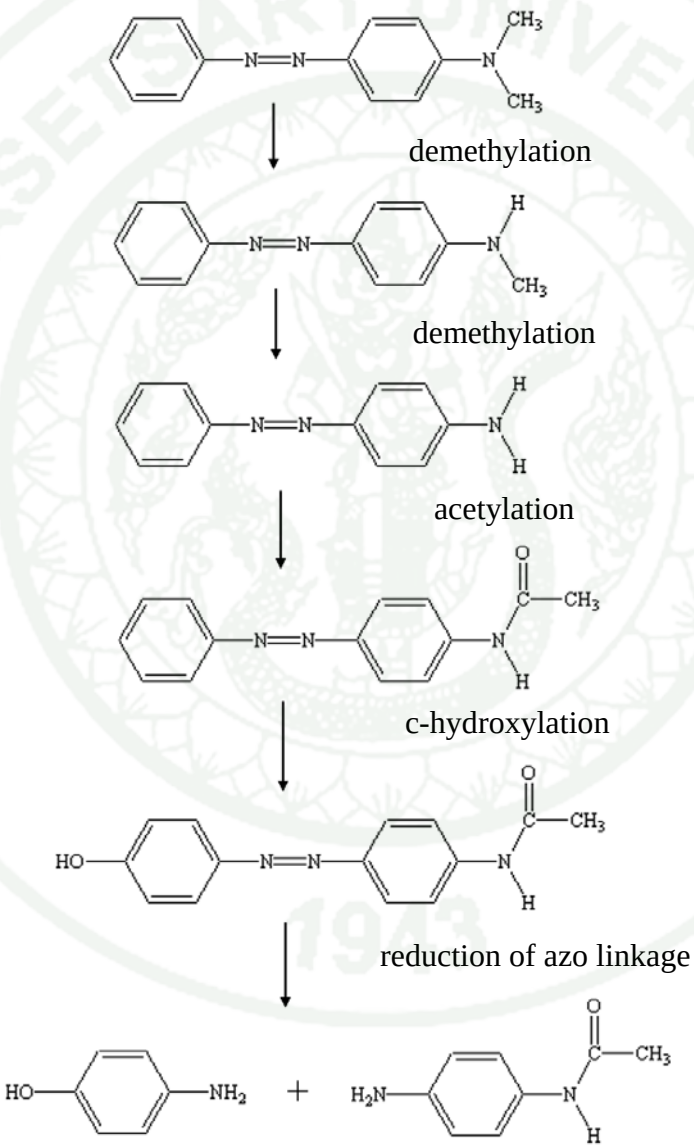


Figure 6 Biotransformation para-Dimethylaminoazobenzene (DAB)

Source: Doble and Kumar (2005)

The presence or absence of aquatic life has been extensively used as a biological indicator (Boateng *et al.*, 2006). One type of aquatic organisms which has been widely used for toxicity test is zebrafish (*Danio rerio*) due to their numerous advantageous as previous mention. Moreover, Hill *et al.* (2005) reported that zebrafish has been probably known the normal of morphological, biochemical, and physiological information at all stages of early development and in juveniles and adults of both sexes more than the other fish species. Therefore, zebrafish has been used to test the toxicity of chemicals in various works as shown in Table 1. The types of toxicity investigation using zebrafish including reproductive toxicity, developmental toxicity, acute toxicity, neurotoxicity cardiotoxicity, ocular toxicity, endocrine disruption, neurobehavioral toxicity, vascular toxicity and carcinogenicity.

Table 1 Toxicity test of chemicals by using zebrafish

Chemical classes	Type of toxicity study	Reference
Metals		
Copper and zinc	Susceptibily to infection by listeria after exposure to metals	Rougier *et al.*, 1996
Copper, nickel, mercury, colbalt, lead	Toxicity: dose response, effects on hatching and survival	Dave and Xiu, 1991
Methylmercury	Fuctional impairment and delayed mortality syndrome	Samson *et al.*, 2001
Aluminium, cadmium, iron	Toxicity: dose response, effects on hatching and survival	Dave, 1985
Lead and uranium	Acute toxicity and toxicokinetics	Labrot *et al.*, 1999
Cadmium	Ectopic apoptosis induction	Chan and Cheng, 2003
Cadmium	Abnormal somite patterning and defects in axonogenesis	Hen Chow and Cheng, 2003
Tributyltin	Reproductive toxicity	McAllister and Kime, 2003
PCBs/PAHs		
PCBs	Transactivation activity of aryl hydrocarbon receptors COS-7	Abnet *et al.*, 1999
PCBs	CYP1A induction in a zebrafish liver cell line	Henry *et al.*, 2001
PCBs	Reproductive	Orn *et al.*, 1998
PCBs	Kinetics of bioconcentration clearance	Fox *et al.*, 1994

Table 1 (Continued)

Chemical classes	Type of toxicity study	Reference
PCBs/PAHs		
PCBs	Bioaccumulation with different routes of exposure	Anderson *et al.*, 2001
PAHs	Morphological abnormalities occuring after cardiac dysfunction	Incardona *et al.*, 2004
Retinoic acid		
Retinoic acid	Abnormal pectoral fin bud morphology and ectopic shh expression	Akimenko and Ekker, 1995
Retinoic acid	Abnormal development of the caudal midbrain and anterior hindbrain	Hill *et al.*,1995
Retinoic acid	RA-mediated gene expression in transgenic reporter zebrafish	Perz-Edwards *et al.*, 2001
Retinoic acid	Pectoral fin duplications	Vandersea *et al.*, 1998
Cyclopamine (inhibitor of hedgehog signaling)		
Cyclopamine	Elimination of primary mononuerons	Chen *et al.*, 2001
Cyclopamine	Role of ssh in the induction and patterning of the pituitary	Sbrogna *et al.*, 2003
Cylcopamine	Inhibition of fin outgrowth	Quit *et al.*, 2002
Cyclopamine	Role of hedgehog signaling in eye Development	Stenkamp and Frey *et al.*, 2003
Fragrances/nitrated benzenes		
Nitro musk-ketones and xylene	Effects on reproduction, mortality, and growth	Carlsson *et al.*, 2000
Nitro musk-ketones, xylene, AHTN, HHCB	Toxicity and mortality	Carlsson and Norrgren, 2004
Nitro musk-ATHN, HHCB	Antiestrogenic effects	Schreurs *et al.*,2004
Pesticides and Herbicides		
Lindane, atrazine, and deltamethrin	Deformations, mortality, growth retardation and hatching rate	Gorge and Negel, 1990
Toxaphene	Toxicity, reproductive success and oviposition	Lee and Payne, 1997

Table 1 (Continued)

Chemical classes	Type of toxicity study	Reference
Pesticides and Herbicides		
Parathion	Acetylcholinesterase inhibition and food consumption rate	Roex *et al.*, 2003
Endosulfan	Primodial germ cell migration and distribution	Willey and Krone, 2001
Sevin	Effects on reproduction and hatching	Todd and Van Leeuwen, 2002
Chlopirifos	Effects on survival, response latency and spatial discrimination	Levin *et al.*, 2003
Atrazine (2-chloro-4-ethylamino-6-isopropylamine-s-triazine)	Morphological and functional abnormalities	Wiegand *et al.*, 2001
3,4-dichloroaniline, lindane	Toxicity and effects on reproduction	Ensenbach and Nagel, 1997
4-chloroaniline	Effects on hatching and ultrastructural changes in liver and kidney	Oulmi and Braunbeck, 1996
Estrogenics		
17-beta estradiol, diethylstilbestrol	Effects on mortality and hatching, consequences for CNS	Kishida *et al.*, 2001
Nonylphenol, ethinylestradiol, bezo[a]pyrene	CYP19 expression induction	Kazeto *et al.*, 2004
Nonylphenol	Primordial germ cell migration and distribution	Willey and Krone, 2001
Nonylphenol, 17alpha-ethinylestradiol	Effects on sex ration and breeding success	Hill and Janz, 2003
Nonylphenol, 17beta-estradiol	Vitellogenin as an estrogenic biomarker	Van de Belt *et al.*, 2003
Phytosters were isolated from wood and soy beans	Reproduction/altered sexual ratio	Nakari and Erkomaa, 2003
Other investigations		
Saxitoxin	Morphological abnormalities and sensorimotor deficits	Lefebvre *et al.*, 2004
1,2,3-trichlorobenze	Reproductive impairment by non-polar narcosis	Roex *et al.*, 2001

Table 1 (Continued)

Chemical classes	Type of toxicity study	Reference
Other investigations		
Ammonium perchlorate	Reproductive performance and thyroid follicle histology	Patino *et al.*, 2003
Flavopiridol, Brefeldin A, Neomycin, and caspase inhibitors	Bioassays for assessing toxicity, angiogenesis, and apoptosis	Parng *et al.*, 2002
7,12dimethylbenz[a] anthracence (DNBA)	Neoplasia	Spitsbergen *et al.*, 2000
Triphynyltin acetate	Effects on survival, hatching success, and liver ultrastructure	Strmac and Braunbeck, 1999

Source: Hill *et al.* (2005)

Various fish embryo toxicity test (FET) procedures have been proposed by various organizations (USEPA, 1996b; OECD, 1992 1998 2006a). The FET test method has been reported by Lammer et al. (2009) as shown in Figure 7. There are many studies which investigate the acute toxicity of chemicals on zebrafish which are now discussed. Gallo and co-worker (1995) determined the acute toxicity of carbaryl and aldicarb (carbamate pesticides) to Guppy (*Poecilia reticulata*) and zebrafish (*Danio rerio*). They found that 96-h LC_{50} values with carbaryl were 46.0 μmol/L for the zebrafish and 12.5 μmol/l for the Guppy. And LC_{50} values of aldicrab were 52.9 μmol/L for the zebrafish and 3.5 μmol/L for the Guppy. The acute toxicity of 1,2,3-trichlorobenzene (123TCB), is nonpolar narcotic with a non-specific mode of action, and parathion that is acetylcholinesterase (AChE) inhibitor was studied by Roex *et al.* (2002). In addition, they also compared these with LC_{50} values obtained from the literature by using the same test organism. In 2004, the histological changes of acute toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in zebrafish were analyzed by Zodrow *et al.* (2004). They found that histophatological changes of adult zebrafish including lipidosis and hypertrophy of liver hepatocytes and hypertrophy of gill lamellae occurred after 5 days injection of TCDD (70 ng/g).

Pretti *et al.* (2006) investigated the acute toxicity of 15 ionic liquids (ILs) to the zebrafish and found that 96 h LC_{50} values of thirteen out 15 of ILs were greater than 100 mg/L, while the LC_{50} values of the remaining ILs (AMMOENG 100 and AMMOENG 130) were 5.2 mg/l for AMMOENG 130 and 5.9 mg/l for AMMOENG 100. In 2007, Liu and co-worker determined the toxicity of HC Orange No. 1 used as color additive in hair dyes to *Daphnia magna*, Zebrafish (*Bracchydanio rerio*) embryos, and goldfish (*Carassius auratus*). The 48 h EC_{50} value for the restrained mobility of *D. magna* was 1.54 mg/L. 96-h LC_{50} values for zebrafish and goldfish were 4.04 and 5.37 mg/L, respectively.

Recently, the *in vivo* biodistribution and acute and long-term effects of functionalized multi-walled carbon nanotubes (MWCNTs) on zebrafish were studied by Cheng *et al.* (2009). They concluded that the biocompatibility of CNTs can be improved by the purification and functionalization. Additionally, the long-term toxicity effects of purified CNTs may occur when they were delivered into the body. Kammann *et al.* (2009) determined and compared the acute toxicity of technical nonylphenol (t-NP), 353-nonylphenol (353-NP), and its metabolites for zebrafish embryo. They found that the toxicity of 353-NP (EC_{50} for lethal endpoints 6.7 mg/L) was higher than the toxicity of its metabolites, for example 4-(3,5-dimethyl-3-heptyl)-2-bromophenol (EC_{50} for lethal endpoints 27.1 mg/L). The acute toxicity of two organophosphates (dichlorvos and phoxim) and four pyrethroids (permethrin, tetramethrin, bifenthrin, and etofenprox) and their binary mixtures on zebrafish was studied by Zhang *et al.* (2010). They reported that the toxicity of all pyrethroids were very high because the 24, 48, 72 and 96 h LC_{50} values were found to be less than 0.1 mg/L. For organophosphate, dichlorvos, it showed low toxicity to zebrafish (24 h and 96 h LC_{50} values were 51.3 and 13.0 mg/L, respectively) while phoxim showed high or intermediate toxicity to zebrafish (24 h and 96 h LC_{50} values were 1.28 and 0.469 mg/L, respectively). The toxicity of their binary mixtures was in the high to very high range.

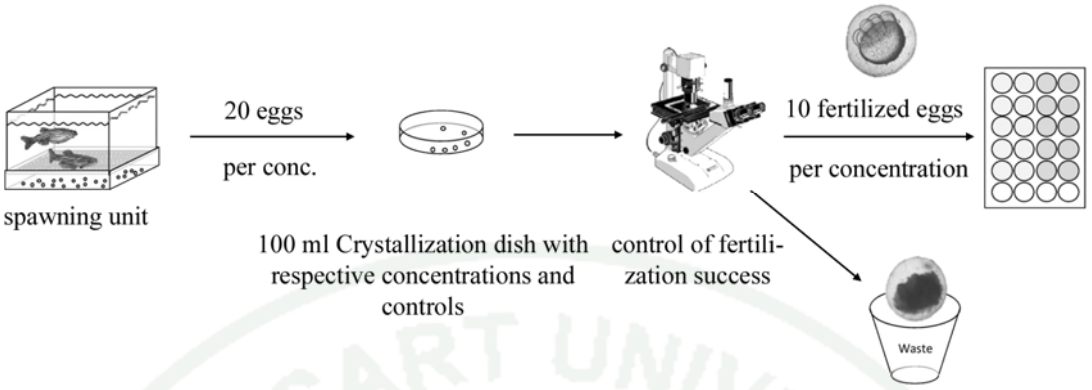


Figure 7 Scheme of the FET test procedure (from left to right): collection of the eggs, pre-exposure of the eggs immediately after fertilization in crystallization dishes, selection of fertilized eggs with an inverted microscope or binocular and distribution of the fertilized eggs into prepared 24-well microtiter plates

Source: Lammer *et al.* (2009)

In the field of toxicokinetic context, octanol/water partition coefficient (*P*) is closely related to absorption, distribution, metabolism, and excretion (ADME) properties of xenobiotics (Jia, 2005; Sabb *et al.,* 2011). It plays a major role for determining the partition and transport of chemicals in the global environment (Zeng *et al.,* 2012). Therefore, it is an important parameter that should be investigated in this research work. The organization for Economic Cooperation and Development (OECD) has proposed various experimental log *P* measurement consisting of shake-flask, high-performance liquid chromatography (HPLC), and slow-stirring method (OEDC, 1995, 2004, 2006). The conventional and most reliable method for log *P* determination in the range of -3 to 6 is shake-flask method (Selassie, 2003). General formular of partition coefficient is defined by the following equation (Short et al., 2010):

$$P = C_{octanol} / C_{water}$$

Where $C_{octanol}$ is the concentration of neutral form of test compound in the octanol phase, and C_{water} is the concentration of un-ionized form of test compound in the aqueous phase.

Therefore, log *P* that is closely related to absorption, distribution, metabolism, and excretion (ADME) of chemicals (Jia, 2005) has been extensively used as molecular descriptor for toxicity prediction. In present, there are various researchs that had used log *P* to develop QSA(T)R models for predicting the toxicity of chemicals on *Tetrahymena pyriformis* (Schultz *et al.*, 1991a), *Daphnia magna* (Marchini *et al.*, 1999; Liu *et al.*, 2003; Davies *et al.*, 2004; Boeije *et al.*, 2006; Hodges *et al.*, 2006; Zvinavash *et al.*, 2206), algae (Van Leeuwen *et al.*, 1992; Lu *et al.*, 2000; Schmitt *et al.*, 2000; Worgan *et al.*, 2003; Yan *et al.*, 2005; Zvinavashe *et al.*, 2006, Chen *et al.*, 2007), and fish (Veith *et al.*, 1983; Hermens *et al.*, 1984; Verhaar *et al.*, 1995; Russom *et al.*, 1997; Roberts and Costello, 2003; Lessigiarska *et al.*, 2004; Papa *et al.*, 2005; Pavan *et al.*, 2005a; Freitas, 2009). Almost models had been reviewed and reported by Netzva *et al.* (2007) and revealed that the toxicity of chemicals increases with increasing log *P* values.

According to the European Union, computation-based quantitative structure-activity relationship (QSA(T)R) model is explicity encouraged and required for toxicology predictions under the framework of REACH legislation (Shi *et al.*, 2012). However, (QSA(T)R) model had developed by using log P and the other molecular descriptos for toxicity prediction of azo dyes has a few. Thus, the relationship between toxicity and log *P* of azo dye is an interesting research topic.

For the studies of QSA(T)R model for azo dyes, They are shown as the following. Garg *et al.* (2002) developed quantitative structure-activity/property-activity relationships (QSAR/QPARs) of 43 aminoazobenzene derivatives, were shown in Figure 8, including 4-aminoazobenzene (AAB), N-methyl-4-aminoazobenzene (MAB) and n,n-dimethyl-4-aminoazobenzene (DAB) derivatives. The models in this study are the correlation between the observed mutagenic activity of aminoazobenzene derivatives in the *S.typhimurium* TA98 bacterial strain with S9

activation (TA98+S9) with various molecular descriptors using multilinear regression techniques and artificial neural networks. From the best multilinear regression (BMLR) models accounting for more than 80% of the variation in TA98+S9, they found that the hydrophobicity descriptor, log P, does not appear in any of these BMLR correlation equations. This is because aminoazobenzene compounds can have rather similar values of log P but quite different mutagen activities. Moreover, all of the BMLR models suggest that the relative mutagenicity of these aminoazobenzene derivatives increase as the average electrophilic reactivity index ($\overline{ERI}$) for all the nitrogen in the compound increase. For artificial neural network (ANN) models, they summarized that only three descriptors were needed to adequately train these ANN models because of the limited data available. However, they found that these several 3-descriptor ANN models can account for over 90% of the variation in mutagenicity of the compounds.

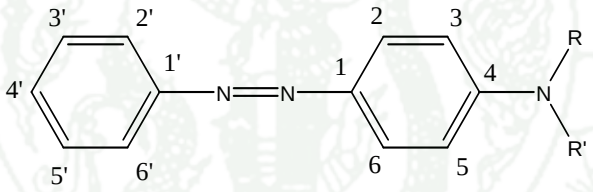


Figure 8 General structure of aminoazobenzene derivatives

The QSAR/QPSRs for the observed mutagenicity in *Salamonella typhimurium* TA98 bacterial tester strain in the present of an induced rat-liver S9 (TA98+S9) mix of an enhanced collection of aminoazo dyes and their reductive-cleavage metabolites were constructed and modified by Sztandera *et al.* (2003). The quantitative mutagenicity data of 74 compounds (aminoazobenzene derivatives, disazo derivatives and reductive cleavage products) illustrated in Figures 9 was obtained from a variety of laboratories which the ranges of the mutagenic activity of these compounds about 10^{-3} to 10^{2} rev/nmol. They showed that multilinear regression techniques using 8 descriptors were shown to account for about 73% of the variation in the relative mutagenic activity of aminoazo dyes (62 aminoazo derivatives and 12 of their reductive cleavage products). For aminoazobenzene derivatives and disazo derivatives,

the main descriptor is either the minimum or maximum values of the electrophilic reactivity index (EIR_N) for an N atom, while the the main descriptor of reductive cleavage compounds is average of this index. The large values of this index occur at azo nitrogen atoms whereas smaller values occur at amino nitrogen atoms. The mutagenicity increases as the maximum values of EIR_N increase for 8 descriptors equation. Additionally, they also used artificial neural networks (ANNs) for the relative mutagenic activity in TA98+S9 of these 74 compounds. They summarized that the 8-descriptors ANN can account for about 95% of the reported variation. The total dipole moment and various polarizabilities play important roles in the neural networks.

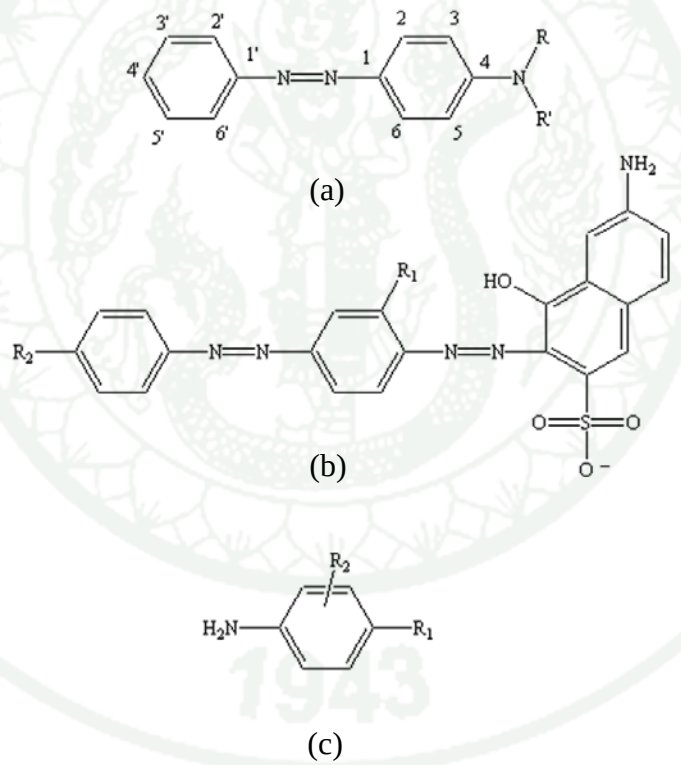


Figure 9 The general structures of (a) aminoazobenzene derivatives
(b) diazo derivatives
(c) reductive cleavage products

MATERIALS AND METHODS

Materials

1. Equipments

1.1 5 Azo dyes including reactive red 239 (RR239), direct red 80 (DR80), direct blue 78 (DB78), direct black 22 (DB22), and acid yellow 199 (AY199)

1.2 Octanol

1.3 Deionized (DI) water

1.4 NaOH

1.5 85% ortho-Phosporic acid

1.6 Parental zebrafish (*Danio rerio*)

1.7 Glass aquarium

1.8 Water system for maintenance of parental zebrafish and breeding

1.9 Oxygen pump

1.10 Breeding chamber

1.11 Fluorescent lamp

1.12 Separatory funnel

1.13 Dropper (diameter at least 2 mm)

1.14 Pipettes (1, 5 and 10 ml)

1.15 Micropipettes (1 - 10 µl, 100 – 1,000 µl, and 1 – 10 ml)

1.16 Volumetric flask (25 and 50 ml)

1.17 Beaker (50, 250 and 600 ml)

1.18 Funnel

1.19 Filter paper

1.20 Test tube

1.21 UV spectrophotometer

1.22 Incubating shaker

1.23 Bio-Vortex

1.24 Stereomicroscope

1.25 Light microscope

1.26 pH meter

1.27 Conductivity meter

1.28 Reversing thermometer

2. Hardware

2.1 LCAC super computer cluster (Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok)

2.2 HP workstations; Intel Pentium 4 CPU 2.60 GHz, RAM 2 GB (Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok)

3. Software

3.1 GraphPad Prism6

3.2 SPSS (Statistical Package for Social Scientists) program

3.3 Gaussian 03 program

3.4 Gaussian View 03 program

3.5 EditPlus

3.6 Web Lab Viewer Lite

3.7 AlogPs 2.1 program (web-based server: www.vcclab.org/lab/alogps/)

3.8 Pallas program (Demo version)

3.9 ACDLab 12.0 program (freeware)

3.10 XLogP3 program (web-based sever: www. Sioc-ccbg.ac.cn/?p=42&software=xlogp3)

3.11 Molinspiration program (web-based server: www.molinspiration/cgi-bin/properties)

3.12 Marvin program (web-based server: www.chemaxon/marvin/sketch/index.php)

3.13 Ecological Structural-Activity Relationship (ECOSAR) program

3.12 Piline Pilot student version 6.1 program

Methods

1. Experimental methods

1.1 Chemicals

Azo dyes used in this work were kindly obtained from DyStar (Thai Co., Ltd). They consist of reactive red 239 (Remazol Brilliant Red 3BS), direct red 80 (Sirius Red F3B), direct blue 78 (Sirius Blue GN), direct black 22 (Sirius Black vsh/c), and acid yellow 199 (Telon Yellow 4R micro 01). Their chemical structures were given in Table 2. Analytical grade of 1-octanol (99% purity) from Panreac and phosphoric acid buffer at pH 1 were used for log *P* determinations. Cadmium chloride ($CdCl_2$) and phenol were used to validate the methods of fish embryos toxicity (FET) test and log *P* experiment, respectively.

1.2 Preparation of test solution for toxicity test

A stock solution of all azo dyes was obtained by dissolving them in distilled water. Nominal concentrations of all dyes were prepared by diluting a stock solution with distilled water mixed with rearing water. Rearing water utilized in the assay came from a water system for maintenance of parental zebrafish, and had undergone passing the process of water treatment. In addition, it was filtered with filter paper before use. Finally, solvent used as a control is 50% distilled water with rearing water due to all zebrafish larval can be survival in this solvent and precipitation of each test solution prepared by using this solvent wasn't found. Quality of solvent used as control (50% distilled water) was displayed in Table 3.

1.3 Preparation of buffer solution for log *P* determination

Phosphoric acid buffer (1 M) was prepared by pipetting 101.1 ml of 85% ortho-phosphoric acid to 750 mL of volumetric flak and then add DI water to volumetric flask until the volume of solution equal to 750 mL. This buffer was

checked the pH value by using pH meter and if the pH of buffer wasn't 1, it was adjusted until equal to 1 by using 50 mM NaOH or 50 mM phosphoric acid.

Table 2 Chemical structures of azo dyes used in this research work

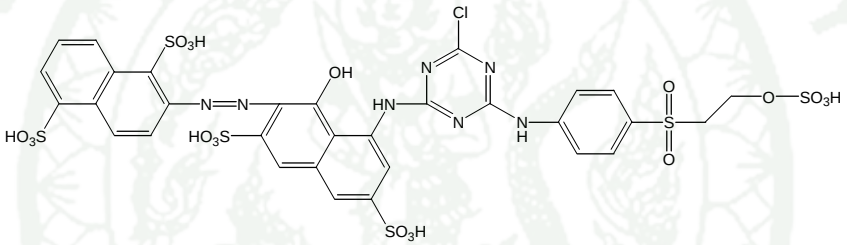
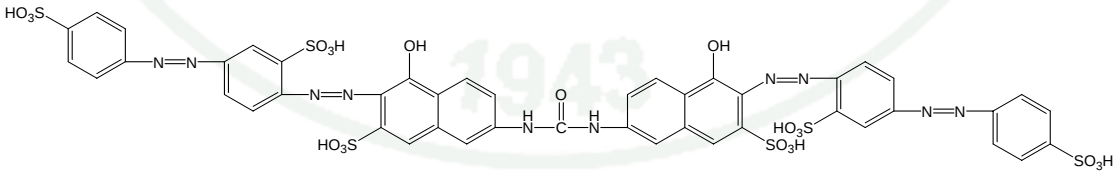
No	Topic	Information
1.	Colour index	Reactive Red 239
	Abrreviation	RR239
	Trade name	Remazol Brilliant Red 3BS
	Structure	
2.	Colour index	Direct Red 80
	Abrreviation	DR80
	Trade name	Sirius Red F3B
	Structure	

Table 2 (Continued)

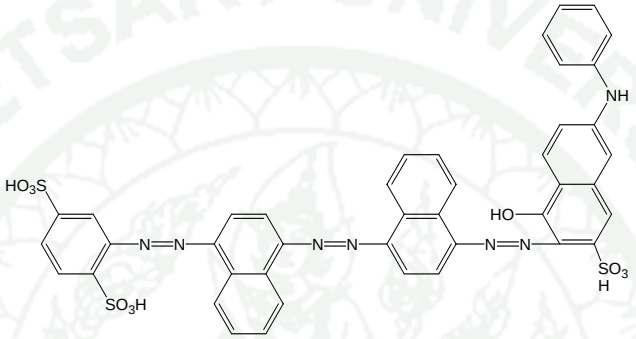
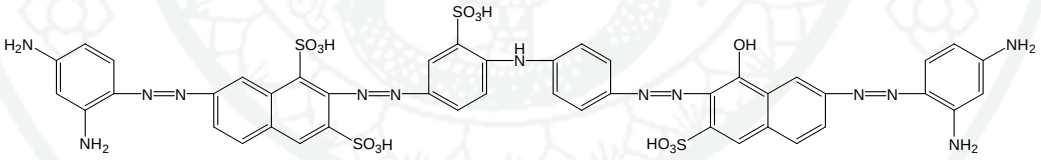
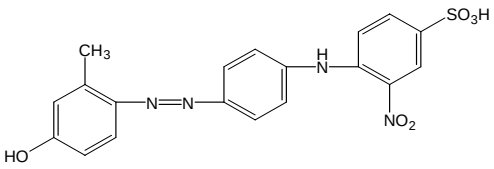
No	Topic	Information
3.	Colour index	Direct Blue 78
	Abrreviation	DB78
	Trade name	Sirius Blue GN
	Structure	
4.	Colour index	Direct Black 22
	Abrreviation	DB22
	Trade name	Sirius Black VSH/C
	Structure	
5.	Colour index	Acid Yellow 199
	Abrreviation	AY199
	Trade name	Telon Yellow 4R Micro 01
	Structure	

Table 3 Quality of solvent (50% distilled water with rearing water) used as control in this study

Parameters	**Rearing water**	**Distilled water**	**Control (50% distilled water)**
Dissloved O_2 (mg/L)	7.3	7.4	7.9
Conductivity (mS)	353.0	2.5	197.5
pH	8.48	7.15	8.36
Result of observation	Precipitation of test solution was occured	Zebrafish larval was dead	Zebrafish larval was survival

1.4 Maintenance of adult zebrafish

Zebrafish between 3 and 6 months old were used for egg production. They must have been free from externally visible diseases and not used in any prior with pharmaceutical treatment studies. Males and females were separately reared in 80 L of glass aquaria (loading ≥ 2.5 L per fish) under natural dark/light cycle. They are fed twice daily with ultrafine powder food containing 35% protein produced by aquaculture research laboratory at department of agriculture, Faculty of Science and Technology, Bansomdejchaopraya Rajaphat University. Moreover, they were also fed once a day with 30 hr hatched *Artemia salina* nauplii of appropriate size. The quality of water used for housing and breeding was maintained under the following condition: water temperature (25.91±0.71 °C), pH (8.11±0.10), conductivity (291.91±14.98 µs/cm), ammonia (0.36±0.03 mg/L NH_3-N), dissolved oxygen (7.13±0.12 mg/L O_2), alkalinity (75.42±1.11 mg/L $CaCO_3$), and hardness (142.82±6.76 mg/L $CaCO_3$). These parameters were measured every week. To avoid an undesirable change in water quality, feces and any remaining food were removed daily and aquarium was cleaned once a week.

1.5 Eggs production

Parental zebrafish in the ratio of 2 males to 1 female were used for egg production. Prior to the test day females which had swollen bellies were selected and transferred to the breeding chamber after the onset of darkness. At the same time, males were chosen and placed outside the breeding chamber. The breeding chamber was made from a plastic net covered with a plastic bag to protect the egg from predation by adults. Eggs that fell through grid of plastic chamber could be easily collected. Before the onset of light in the morning male zebrafish were transferred to the breeding chamber for mating, spawning, and fertilization. Eggs were collected approximately 1 hr later and rinsed several times with rearing water.

1.6 Acute toxicity test using zebrafish embryos

After collection of eggs, they were checked for fertilization under a stereomicroscope. Fertilized eggs were selected to examine the 96 h toxicity of synthetic azo dyes whereas, unfertilized eggs appearing in milky white and darkness under microscope were discarded. Since the fish embryo toxicity test (FET) should be performed as soon as possible, and not later than 3 hour-post fertilization (hpf), 128-cell stage, for starting exposure to test chemical (Lammer *et al.*, 2009). Thus, the FET studies in this work used randomly selecting fertilized eggs at 64-cell stage (~ 2 hpf) which were then placed in petri-dish containing test solution of at least 30 eggs per test concentration and control. Furthermore, each egg was transferred to a test tube filled with 2 ml of test solution and control (20 embryos / each test concentration and control). In order to determine the median lethal concentration (LC_{50}), zebrafish embryos were exposed to range of nominal concentrations of each azo dye. Test tubes were covered with cotton to avoid the evaporation of the test solution. The illustration of the protocol of toxicity test by using zebrafish embryos of this study was displayed in Figure 10. In this test, we had started from the step of the range finding test for finding the range of concentration of compound causing embryos mortality between 0% and 90%. After that we had further performed the definite test for constructing

dose-reponse dose and finding the median lethal concentration (LC_{50}) value of test compound.

The temperature of room was maintained at 26±1 °C. A constant dark/light cycle of 12/12 h was used throughout the experiment. The intensity of fluorescent light was 1,300 Lux. Mortality of the embryo was observed and recorded at 2, 4, 8, 16, 24, 36, 48, 60, 72, 84, and 96 h of exposure. The criterias for mortality determination were (a) coagulation, (b) failure of tail detachment, (c) somite malformation, and (d) lack of heartbeat. Additionally, sublethal endpoints including undeveloped eyes, reduced heart beat, reduced pigmentation, edema formation, and spine malformation were recorded. The measurement of lethal and sublethal effects was given in Table 4. Characteristic of coagulation, tail not detachment and no somite formation were shown in Figure 11.

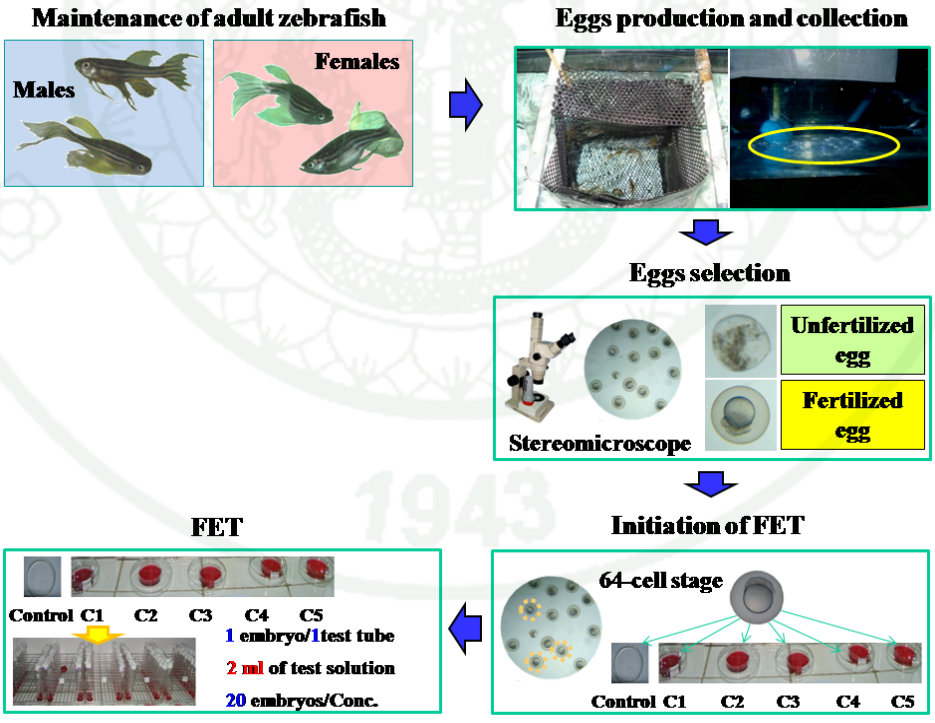


Figure 10 Illustration of protocol of fish embryos toxicity test (FET) of this work including maintaining parental zebrafish, eggs production, eggs collection, eggs selection, initiation of FET, and FET

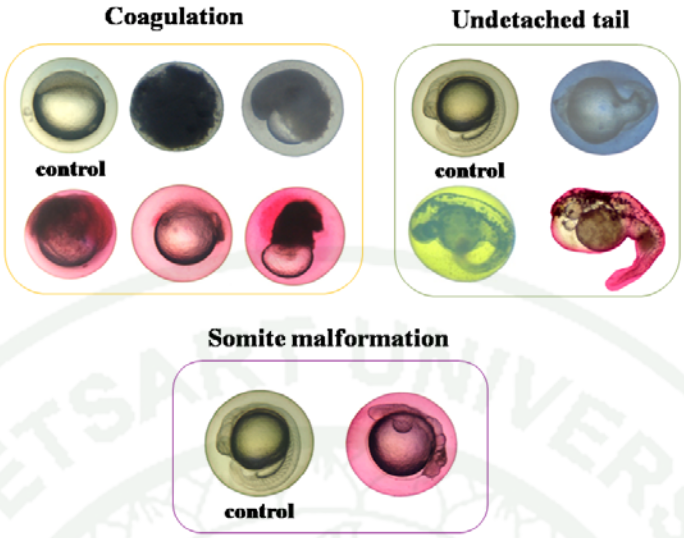


Figure 11 Characteristics of coagulation, tail not detachment, and somite malformation

Table 4 Lethal and sublethal endpoints in the fish embryo toxicity test (FET) with zebrafish (*Danio rerio*) after 96 h of exposure (Lammer *et al.*, 2009; Zielke *et al.*, 2011)

Lethal enpoints	Sublethal endpoints
Coagulation	Undeveloped eyes
Tail not detached	Reduced Heart beat
Somite malformation	Reduced pigmentation
Lack of heartbeat	Edema formation
	Spine malformation

1.7 Vadility criteria of FET test

Each experiment was considered to valid if (1) there was no mortality of embryos in the control group (2) there was no abnormal development of embryos

in the control group, and (3) the hatching rate of fertilized embryos in each breeding replicate was equal or higher than 70%. Normal development of embryonic zebrafish published in literature (Kimmel *et al.*, 1995; Parichy *et al.*, 2009) was used as the model in this study.

1.8 Gross morphology analysis of zebrafish embryos

Gross morphology analysis was performed by placing 10 fertilized eggs at 64-cell stage into the 50 ml of beaker containing 20 ml of individual test concentration and control (10 embryos/individual test concentration and control). The morphology of control and each azo dye-exposed embryos was observed at 4, 8, 24, 36, 48, 72, and 96 h under stereomicroscope. Morphological malformation of zebrafish embryos exposed to each azo dye were transferred to a petri-dish and investigated again using an Olympus model CHS-2 for imaging with a digital camera (Sony DSC-W55). The normal morphology in the control group was also photographed for the purpose of comparision.

1.9 Statistical analysis

The sigmoidal fit for concentration-response curves required to calculate LC_{50} was operated by GraphPad Prism software (version 6.0). Data were presented as mean ± standard error (SEM).

1.10 Shake-flask method for log *P* determination

Partition coefficient (log *P*) of 5 azo dyes was investigated by using shake-flask method according to OECD Guideline Test No. 107 (OECD, 1995). Octanol saturated by buffer (phosphoric acid buffer at pH 1) and buffer (phosphoric acid buffer at pH 1) saturated by octanol were used as organic phase and aqueous phase, respectively. Each azo dye was weighed and transferred to test tube (diameter = 2 cm). Then, 5 mL of 1 M phosphoric acid buffer at pH 1 was added into test tubes for log *P* determination. After that test compound and buffer solution were mixed

together as homogeneous solution by using Bio-Vortex. Next, pH of solutions was measured by using pH meter and if pH of solutions wasn't equal to 1 to adjust pH of solutions by using 50 mM NaoH or 50 mM phosphoric acid. Afterward, 2.5 mL of octanol was added into the test tube. Test tube was covered with aluminium foil and sealed with paraffin film to protect the spill of solution during the shaking process. Next, test tube was shaken for 1 minute by using Bio-Vortex and for 1 hour by incubating shaker. Finally, two phases were allowed to completely separate at least 1 hour. The quantity or concentration, as displayed in term of peak area, of test compound in each phase was analyzed by using UV-spectrophotometer (JASCO V-670, Japan). The maximum wavelength (λ_{max}) used to determine the quantification of individual test compound was shown in Table 5.

Table 5 Maximum wavelength, λ_{max}, (nm) of individual azo dye

Dyes	λ_{max} (nm)
Reactive red 239	541.2
Direct red 80	542.0
Direct blue 78	600.0
Direct black 22	494.2
Acid yellow 199	377.8

2. Computational methods

2.1 Development of relationship between toxicity, $p(1/LC_{50})$, and log *P* values of 5 azo dyes

In this study, the relationship between toxicity and log *P* values for 5 azo dyes including reactive red 239, direct red 80, direct blue 78, direct black 22, and acid yellow 199 was built up by using linear regression method (LR) implemented in SPSS program. This relationship can be called a small QSTR model because of small set of toxicity data. Dependent variable used to develop model was the median lethal concentration (LC_{50}) values of these 5 azo dyes on zebrafish embryos obtained in the part of FET test in this study. The toxicity values were converted into the negative logarithm, $-p(LC_{50})$ or $p(LC_{50})^{-1}$ or $p(1/LC_{50})$. For independent variable used to build up model, it was experimental log *P* values of 5 azo dyes derived from the part of log P determination in this research.

Linear regression (LR) method is a statistical method which can be simply defined as the following equation:

$$Y = \beta_0 + \beta_1 X_1$$

where Y is dependent variable such as toxicity data. X_1 is independent variable or predictor variable such as log *P*. β_0 and β_1 are the regression constant and the coefficients obtained from the fit, respectively.

The quality of QSTR model for prediction has been determined from the square of correlation coefficients (r), standard deviation (s), and F-test (F) values which can be displayed as the following equations:

Correlation coefficients (r)

$$r^2 = 1 - \frac{SSQ}{SS_T}$$

Standard deviation (s)

$$s = \frac{SSQ}{\sqrt{n-k-1}}$$

and F-test (F)

$$F = \frac{r^2 \cdot (n-k-1)}{k(1-r^2)}$$

where SSQ is the prediction error sum of squares and SS_T is the sum of squares of deviation between the affinities of the fitting set. Y_{obs}, Y_{mean}, Y_{calc} and *n* are observed, mean, calculated values of the target property and number of observations, respectively. *k* and *n* are number of variables and observations, respectively. SSQ and SS_T can be defined as below equations:

$$SSQ = \sum (Y_{obs} - Y_{calc})^2$$

and

$$SS_T = \sum (Y_{obs} - Y_{mean})^2$$
$$= \sum y^2 - (\sum y)^2/n$$

Methodology of QSTR model construction for azo dyes in this research work was summarized as shown in Figure 12.

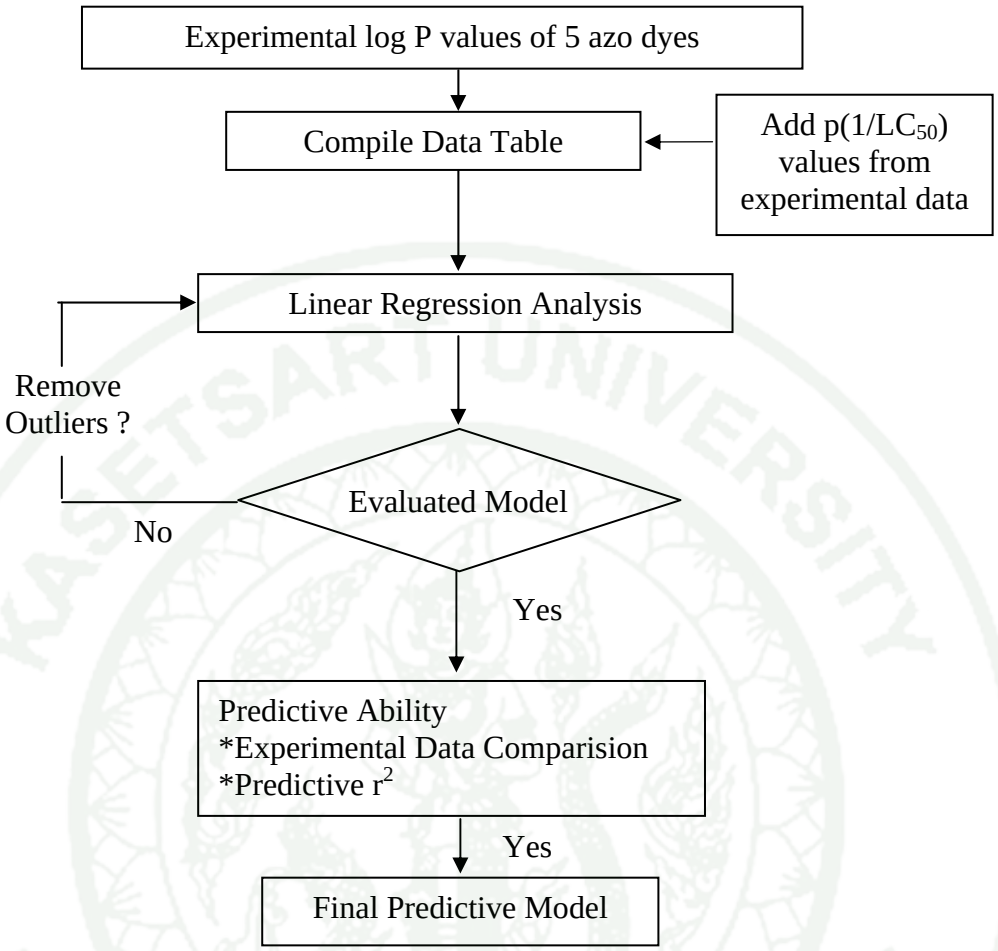


Figure 12 Scheme of the steps of QSTR model construction for azo dyes in this study

RESULTS AND DISCUSSION

Results

1. Fish embryos toxicity test (FET) experiment

In this study, the method of FET test had been validated by using $CdCl_2$ as reference compound which are well known to be a high toxic chemical on zebrafish embryos. The obtained results as shown in Figure 13 revelaed that the protocol of FET test in this work can be reliable and accuracy for testing the toxicity due to the differenc between the results obtained from this study and the result derived from literature review (Cheng *et al.*, 2000) is small.

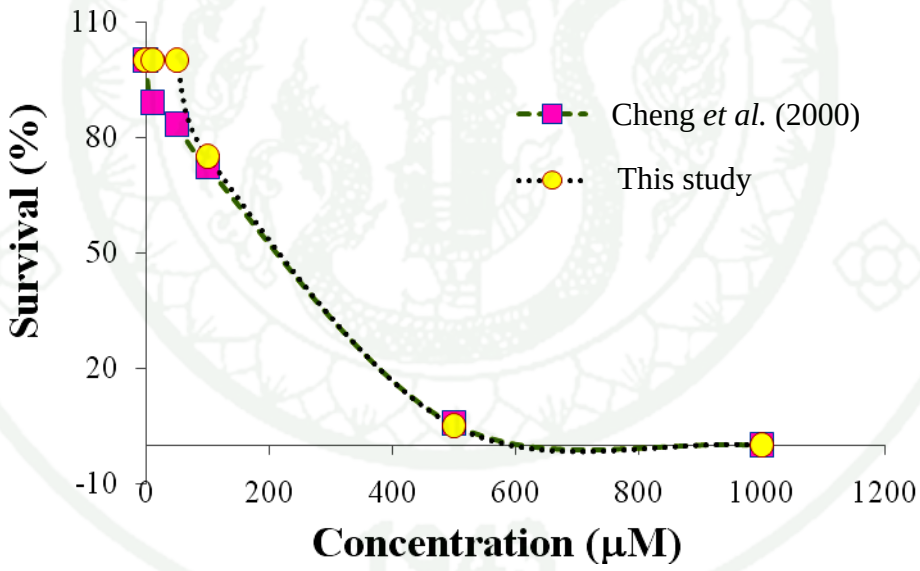
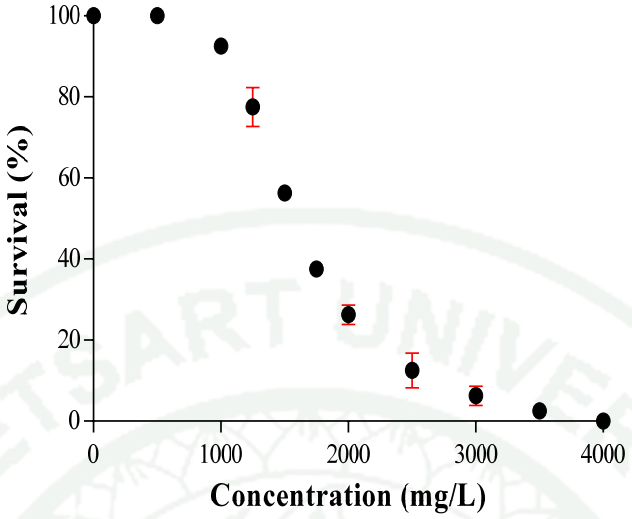


Figure 13 Dose-response curve of Cadmium chloride ($CdCl_2$). Black dot line through yellow circle was the data obtained in this study. Green dash line through pink square was the data derived from Cheng *et al.* (2000)

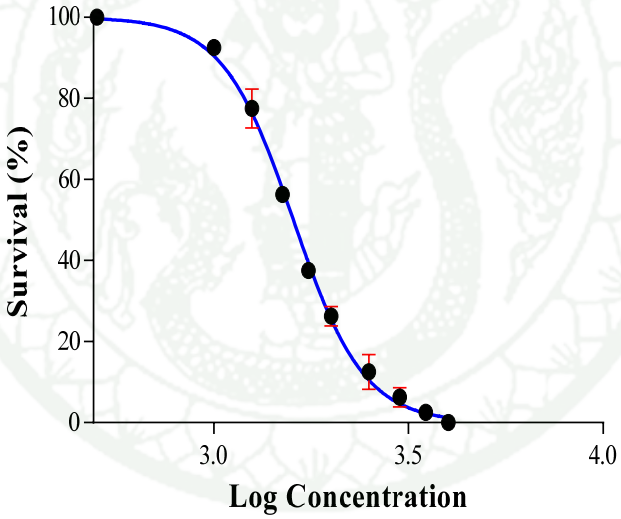
Results of the FET experiments can be divided into 2 parts including acute toxicity of 5 azo dyes (reactive red 239, direct red 80, direct blue 78, direct black 22, and acid yellow 199), and effects of these azo dyes on morphology of zebrafish embryos.

1.1 Acute toxicity of 5 azo dyes on zebrafish embryos

Zebrafish embryos were selected and exposed to various nominal concentrations of individual azo dye solution at ~2 hpf and were scored at 96 h of exposure. Embryo survival decreased with increasing concentration of azo dye as shown in Figures 14-18. In the control group (50% distilled water), there was no mortality and zebrafish embryos normally developed as described by Kimmel *et al.* (1995). One hundred percent survival of embryos occurred for the lowest concentration of individual azo dye solution: 500 mg/L, 1,000 mg/L, 1,000 mg/L, 1,000 mg/L, and 5 mg/L for reactive red 239 (RR239), direct red 80 (DR80), direct blue 78 (DB78), direct black 22 (DB22), and acid yellow 199 (AY199), respectively. Whereas, mortality reached to 100% at concentration higher than 3,500 mg/L for reactive red 239, 4,000 mg/L for direct red 80, 4,000 mg/L for direct blue 78, 5,500 mg/L for direct black 22, and 40 mg/L for acid yellow 199. The 96 h median lethal concentration (LC_{50}) of all azo dyes determined with 95% confidence interval (CI) was displayed in Table 6. Ranking order of toxicity level of these 5 azo dyes as shown in Mean±SEM from high to low is acid yellow 199 (25.82±0.88 mg/L), reactive red 239 (1,600±11.17 mg/L), direct red 80 (2,594±72.50 mg/L), direct blue 78 (3,526±21.50 mg/L), and direct black 22 (3,751 mg/L), respectively.

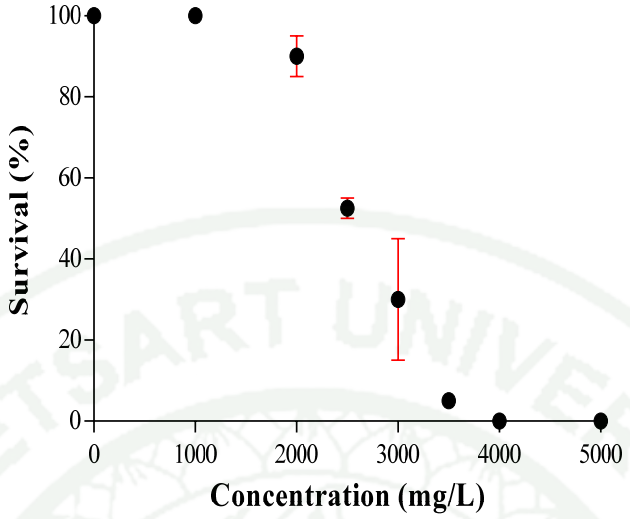


(a)

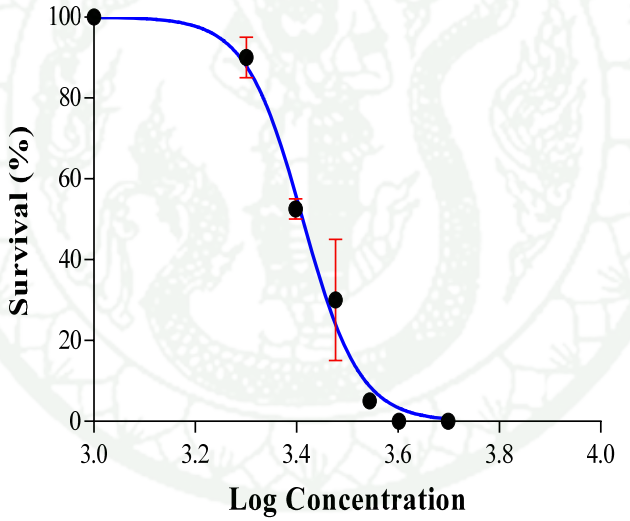


(b)

Figure 14 Survival rate of zebrafish embryos after exposure to control and different concentrations of RR239 for 96 h. Data were averaged from four replicate experiments (n=80 embryos for each treatment) and are shown as the mean±standard error (a) before transforming concentration to log unit (b) after transforming concentration to log unit and fitting curve

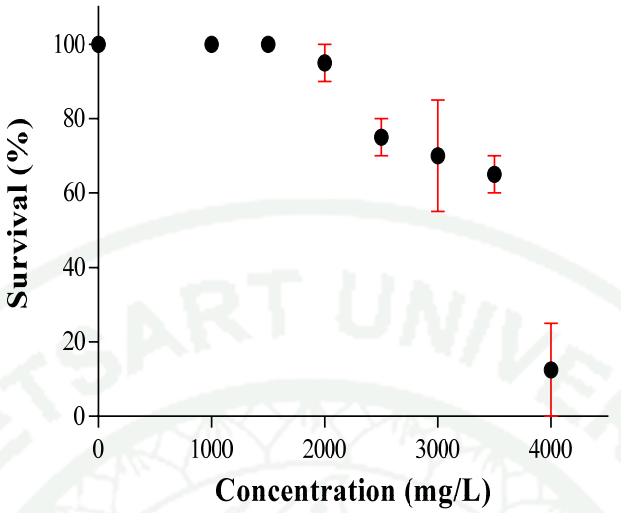


(a)

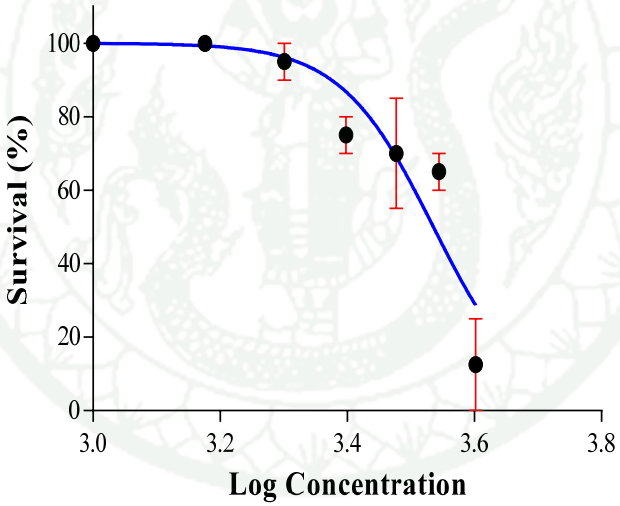


(b)

Figure 15 Survival rate of zebrafish embryos after exposure to control and different concentrations of DR80 for 96 h. Data were averaged from two replicate experiments (n=40 embryos for each treatment) and are shown as the mean±standard error (a) before transforming concentration to log unit (b) after transforming concentration to log unit and fitting curve

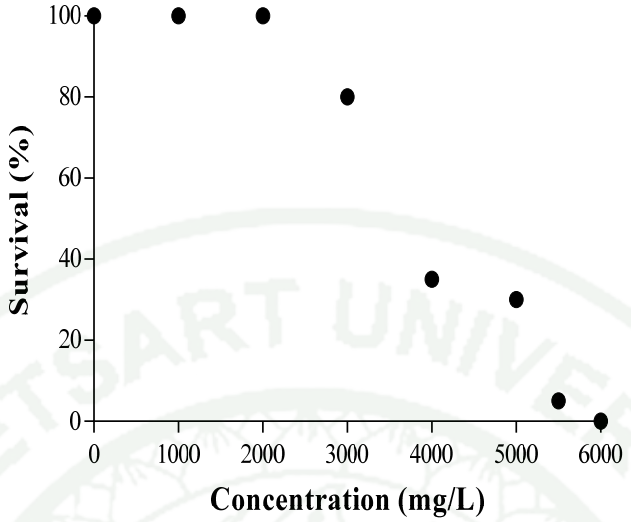


(a)

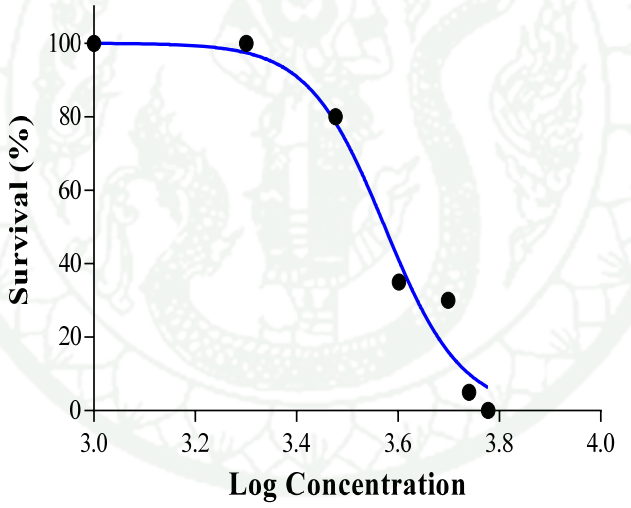


(b)

Figure 16 Survival rate of zebrafish embryos after exposure to control and different concentrations of DB78 for 96 h. Data were averaged from two replicate experiments (n=40 embryos for each treatment) and are shown as the mean±standard error (a) before transforming concentration to log unit (b) after transforming concentration to log unit and fitting curve

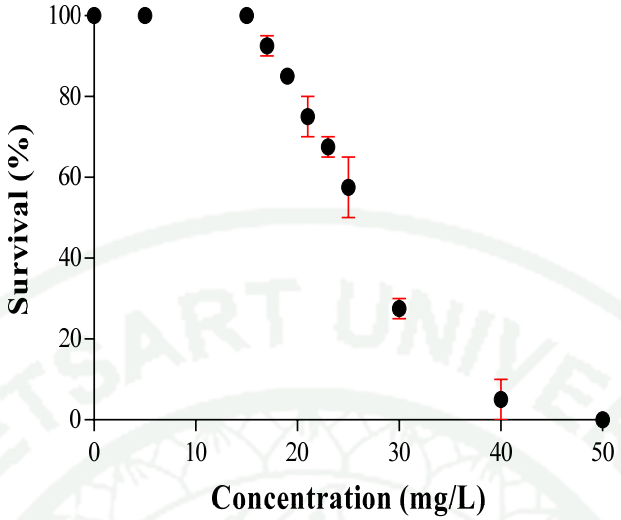


(a)

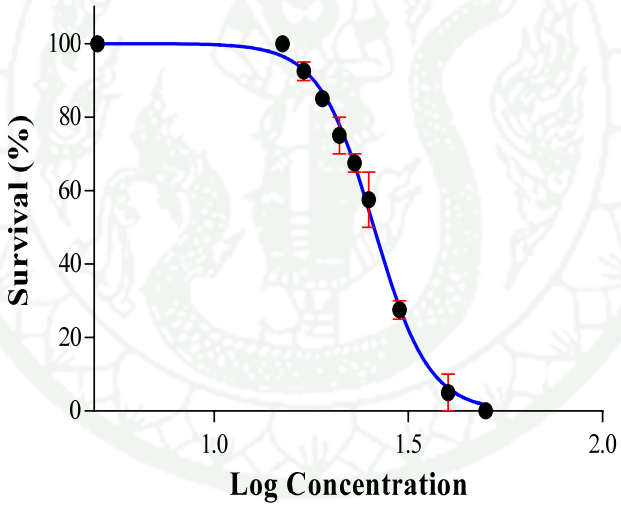


(b)

Figure 17 Survival rate of zebrafish embryos after exposure to control and different concentrations of DB22 for 96 h. Data were averaged from one replicate experiment (n=20 embryos for each treatment) (a) before transforming concentration to log unit (b) after transforming concentration to log unit and fitting curve



(a)



(b)

Figure 18 Survival rate of zebrafish embryos after exposure to control and different concentrations of AY199 for 96 h. Data were averaged from two replicate experiments (n=40 embryos for each treatment) and are shown as the mean±standard error (a) before transforming concentration to log unit (b) after transforming concentration to log unit and fitting curve

Table 6 Median lethal concentration (LC_{50}) of azo dyes at 96 h

Dyes	LC_{50} (mg/L)	Mw	LC_{50} (mM)	1/ LC_{50}	P(1/LC_{50})
Reactive red 239	1,600	1,026.42	1.559	0.642	-0.193
Direct red 80	2,594	1,241.21	2.090	0.478	-0.320
Direct blue 78	3,526	887.937	3.971	0.252	-0.599
Direct black 22	3,751	1,082.11	3.466	0.288	-0.540
Acid yellow 199	25.82	428.428	0.060	16.593	1.220

1.2 Effects of azo dyes on morphology of zebrafish embryos

Embryos at 64 cell-stages that were exposed to several concentrations of each dye solution, were monitored for development abnormalities. The occurance of coagulated eggs was first mortality found for azo-dyes-exposed embryos. Coagulated eggs appeared in milky white and darkness under the microscope (Figure 19) (Liu *et al.*, 2007). Embryos exposed to reactive red 239 at concentration between 3,000 and 4,000 mg/L and acid yellow 199-exposed embryos at 40 and 50 mg/L initiated coagulation at 4 h and 8 h of exposure. After that, coagulation of remaining azo dyes-exposed embryos appeared at 24 h of exposure: direct red 80 in the range of 4,000 to 5,000 mg/L, direct blue 78 at 4,000 mg/L, and direct black 22 in the range of 5,000 to 6,000 mg/L, respectively. Embryos in the control group showed no deformities and presented a well developed tail, head and a normal body structure (Figures 20-21).

At 24 h, 36 h, and 48 h of exposure, zebrafish embryos exposed to reactive red 239 (2,500 and 3,000 mg/L), direct red 80 (4,000mg/L), direct blue 78 (4,000 mg/L), direct black 22 (5,500 mg/L) and acid yellow 199 (40 mg/L) showed embryo malformations including head and eye hypoplasia (HE), tail malformation (TM), and cardiac edema (CD) as illustrated in Figure 20. Afterwards, various developmental

abnormalities of embryos exposed to these 5 azo dyes were found at 72 h and 96 h of exposure. Morphological malformations of azo dyes-exposed embryos consisted of yolk sac edema (YS), pericardial edema (CD), spine bending (BS), and tail malformation (TM) as displayed in Figure 21. Characteristics of developmental abnormalities of the bent spines of embryos include lordosis, kyhosis, and scoliosis (Figure 22).

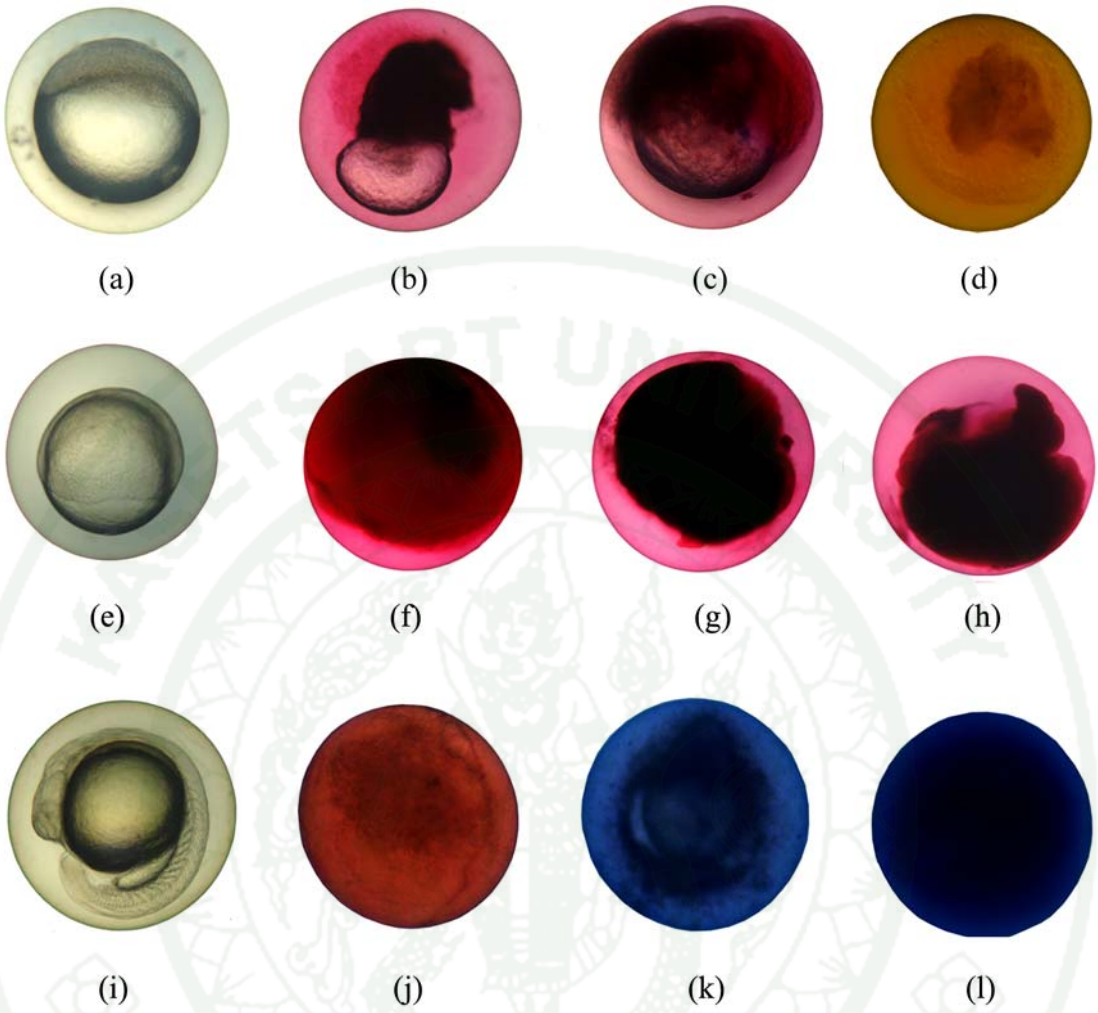


Figure 19 Coagulation of zebrafish embryos after exposure to azo dyes at 4 h ((a) to (d)), 8 h ((e) to (h)), and 24 h ((i) to (l)). (a), (e), and (i) control; (b) and (g) reactive red 239-exposed embryos at 3,500 mg/L; (c) and (h) reactive red 239-exposed embryos at 4,000 mg/L; (d) acid yellow 199-exposed embryo at 50 mg/L; (f) reactive red 239-exposed embryo at 3,000 mg/L; (j) direct red 80-exposed embryo at 5,000 mg/L; (k) and (l) direct blue 78-exposed embryos at 4,000 and 5,000 mg/L, respectively

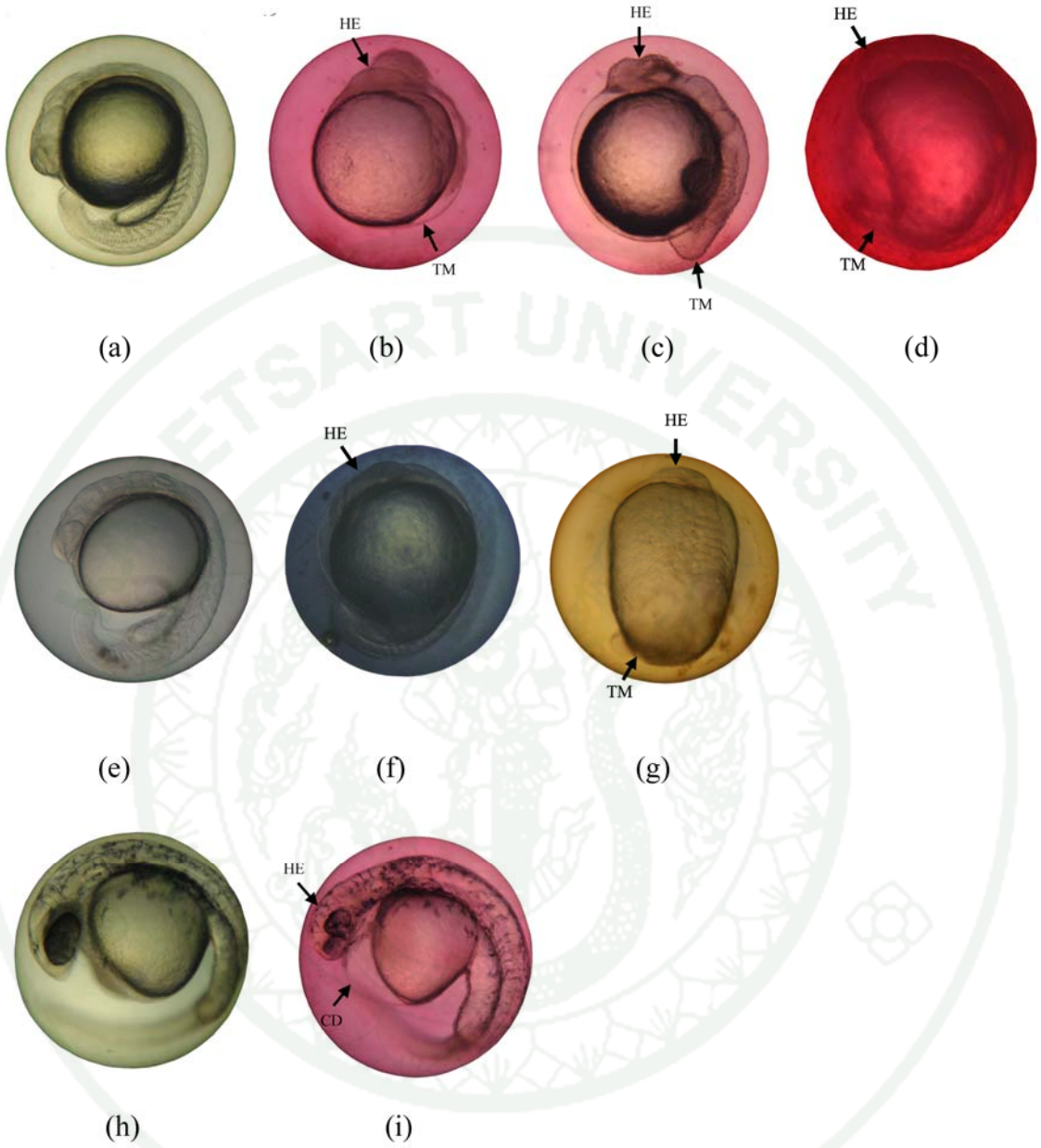


Figure 20 Gross morphology of control-exposed embryos at (a) 24 h, (e) 36 h, and (h) 48 h. (b) and (i) reactive red 239-exposed embryos at 2,500 mg/L; (c) reactive red 239-exposed embryo at 3,000 mg/L; (d) direct red 80-exposed embryo at 4,000 mg/L; (f) direct blue 78-exposed embryo at 4,000 mg/L; (g) acid yellow 199-exposed embryo at 40 mg/L. HE, head and eye hypoplasia; TM, tail malformation; CD, cardiac edema.

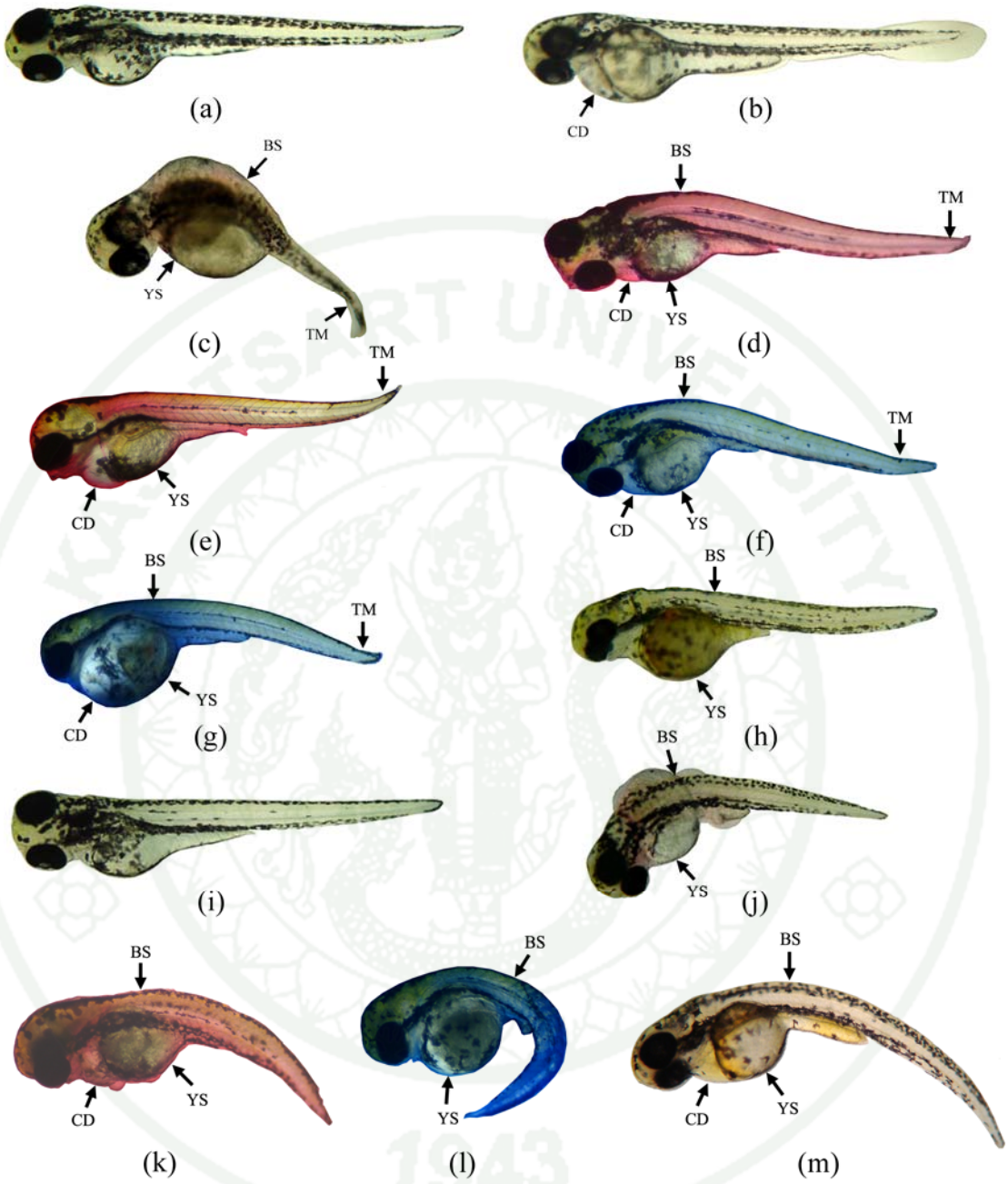


Figure 21 Control and azo dyes-exposed embryos for 72 h ((a)-(h)) and 96 h ((i)-(m)), respectively. Reactive red 239-exposed embryos at (b) 1,000 mg/L, (c) 1,500 mg/L, and (j) 2,000 mg/L. Direct red 80-expose embryos at (d) 2,000 mg/L, (e) and (k) 3,000 mg/L. Direct blue 78-exposed embryos at (f) 1,500 mg/L, (g) 2,000 mg/L, and (l) 3,000 mg/L. Acid yellow199-exposed embryos at (h) 50 mg/L, and (m) 30 mg/L. YS, yolk sac edema; BS, bent spine.

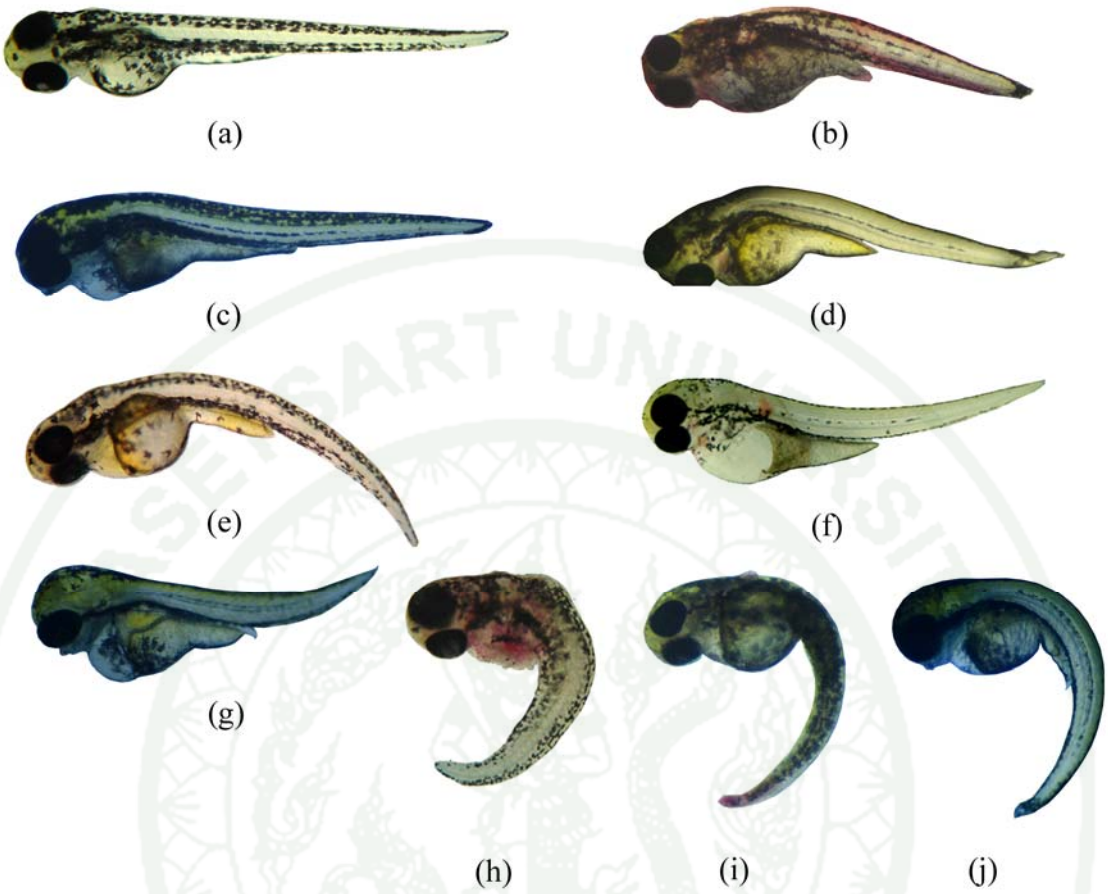


Figure 22 Zebrafish embryos after exposure to control (a) and azo dyes ((b)-(j)). Azo dyes-exposed embryos caused bent spine including kyphosis ((b)-(e)), lordosis ((f)-(g)), and scoliosis ((h)-(j)). (f) and (g) embryos exposed to reactive red 239 at 1,500 and 2,500 mg/L, respectively. (b) and (i) embryos exposed to direct red 80 at 2,000 and 5,000 mg/L, respectively. (c), (g), and (j) embryos exposed to direct blue 78 at 2,000 mg/L. (d) embryo-exposed to direct black 22 at 3,000 mg/L. (e) embryo-exposed to acid yellow 199 at 30 mg/L.

2. Shake-falsk method for log*P* determination

Prior to determining the log *P* of the 5 azo dyes (reactive red 239, direct red 80, direct blue 78, direct black 22, and acid yellow 199), preliminary estimates of the log *P* were performed. Log *P* values were calculated using various programs including ALogPs 2.1, Marvin, KOWWIN implemented in ECOSAR, ACDLABS 12.0, XLogP3, Molinspiration, Pipeline Pilot student version 6.1, and Pallas programs. Theoretical log *P* values of 5 azo dyes in this study were displayed in Table 7. The obtained results showed that calculated log *P* values derived from various programs were in the range of -7 to 8. Therefore, this preliminary information from some programs might not be accuracy and reliable because all of these azo dyes in this study can be highly soluble in water so, their log *P* should be low. However, some programs such as AlogPs, Molinspiration, Pipeline Pilot, and Pallas programs giving log *P* values between -7 and 3 might have the accuracy for calculating log *P* of these 5 azo dyes.

Thus, shake-flask method that is a conventional method for log *P* measurement and has the accuracy for determining log *P* of chemicals in the range of -3 to 6 (Selassie, 2003) was used to investigate log *P* values of 5 azo dyes in this research. In addition, in this study phenol was used as the reference compound for validating method of log *P* measurement. The log *P* values of phenol derived from this work, literature reviews (OECD, 1989 and Sangster, 1989), and Marvin program were displayed in Table 8. The results showed that the protocol of log *P* determination in this research can be reliable and has the accuracy due to the log *P* value obtained from this study was slightly different from literatures and calculation.

Table 7 Experimental and theoretical log *P* values for 5 azo dyes

Compounds	Experimental log*P*	AlogPs	logP	KOWWIN	ACD/LogP	XlogP3	miLogP	AlogP	PrologP
Reactive red 239	-4.86	0.10	4.16	-1.31	-3.15	2.32	-5.44	-7.50	0.10
Direct red 80	-3.75	0.51	9.2	6.34	-1.16	3.87	-4.52	-4.14	1.72
Direct blue 78	-1.68	2.28	10.84	8.34	5.33	8.43	4.43	4.24	3.49
Direct black 22	-0.52	2.63	8.12	4.37	2.91	5.53	-0.62	-0.20	2.36
Acid yellow 199	1.97	2.85	6.45	2.71	4.92	4.38	2.90	2.98	3.74

AlogPs obtained from ALogPs program

logP obtained from Marvin program

KOWWIN obtained from Ecological Structure-Activity Relationship (ECOSAR) program

ACD/LogP obtained from ACDLABS 12.0 program

XlogP3 obrained from XLogP3 program

miLogP obtained from Molinspiration program

AlogP obtained from Pipeline Pilot program

PrologP obtained from Pallas program

Table 8 Log *P* values of phenol derived from this research, literature reviews, and calculation

Compound	log *P* value	Reference
Phenol	1.63±0.01	This research
Phenol	1.5	Hansch and Leo (1979)
Phenol	1.55	Sangster (1989)
Phenol	1.67	Marvin program

Characteristics of solutions between octanol and aqueous phases after adding test compounds and shaking for 1 hour with shaking incubator at 25±1°C was shown in Figure 23. This revealed that almost compounds should have low log *P* values, except acid yellow 199, because the colors of solution in octanol phase more brighter than in aqueous phase.

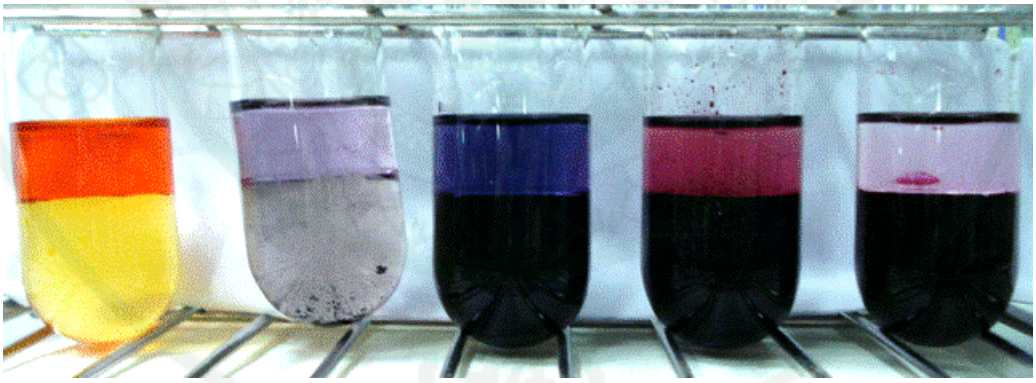


Figure 23 Characteristics of solutions between octanol and aqueos phases of 5 azo dyes after shaking for 1 hour with shaking incubator at 25±1°C. Above and below phases are octanol and aqueous phases, respectively.

The obtained log *P* values of 5 azo dyes determined by using shake-flask method were shown in Table 9. Log *P* experiment of 5 azo dyes was repeated for 3 replicats for each test compound. Data were reported as mean±SEM values.

Table 9 Log *P* values of 5 azo dyes obtained from shake-flask method

Compound	log P value
Reactive red 239	-4.86±0.11
Direct red 80	-3.75±0.02
Direct blue 78	-1.68±0.02
Direct black 22	-0.51±0.06
Acid yellow 199	1.97±0.02

From experimental log *P* values of 5 azo dyes, it can imply that acid yellow 199 tends to be absorbed more easily into tissue compared to the other compounds due to the ranking order of log *P* values for 5 azo dyes from high to low is acid yellow 199 > direct black 22 > direct blue 78 > direct red 80 > reactive red 239. Therefore, acid yellow may be the highest toxic compound on zebrafish embryos compared to the remaining 4 azo dyes in this study.

Additionally, the correlation between experimental log *P* and theoretical log *P* values of 5 azo dyes, reported in Table 8, were ploted in this study. The obtained results showed that calculated log *P* values from ALogPs 2.1 program had the highest correlation with experimental log *P* values obtained from this research comparing to the other calculated log *P* derived from the other programs as shown in Figures 24-25. Thus, these results can indicat that it exists the possibility of the prediction of log *P* on the basis of ALogPs program for 5 azo dyes in this study.

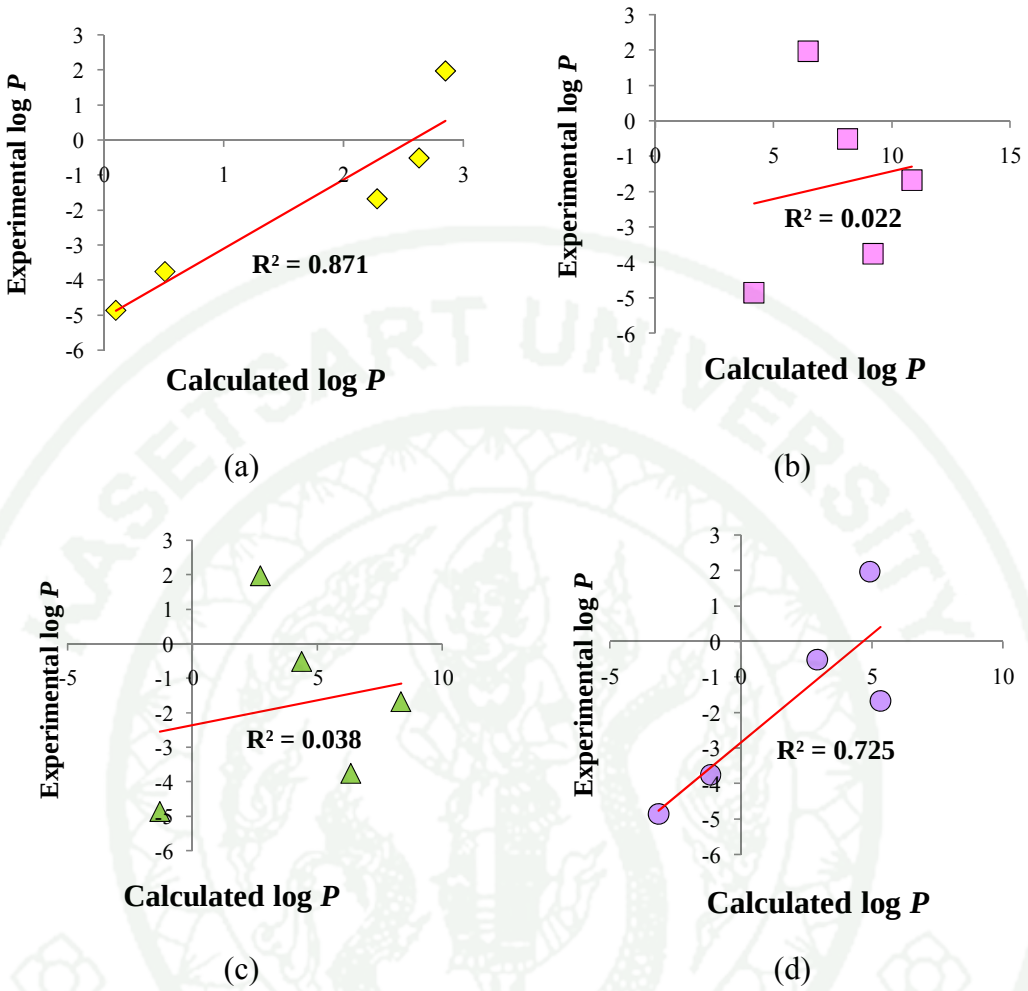


Figure 24 Relationships between the experimental log *P* and theoretical log *P* values
(a) ALogPs
(b) logP
(c) KOWWIN
and (d) ACD/LogP

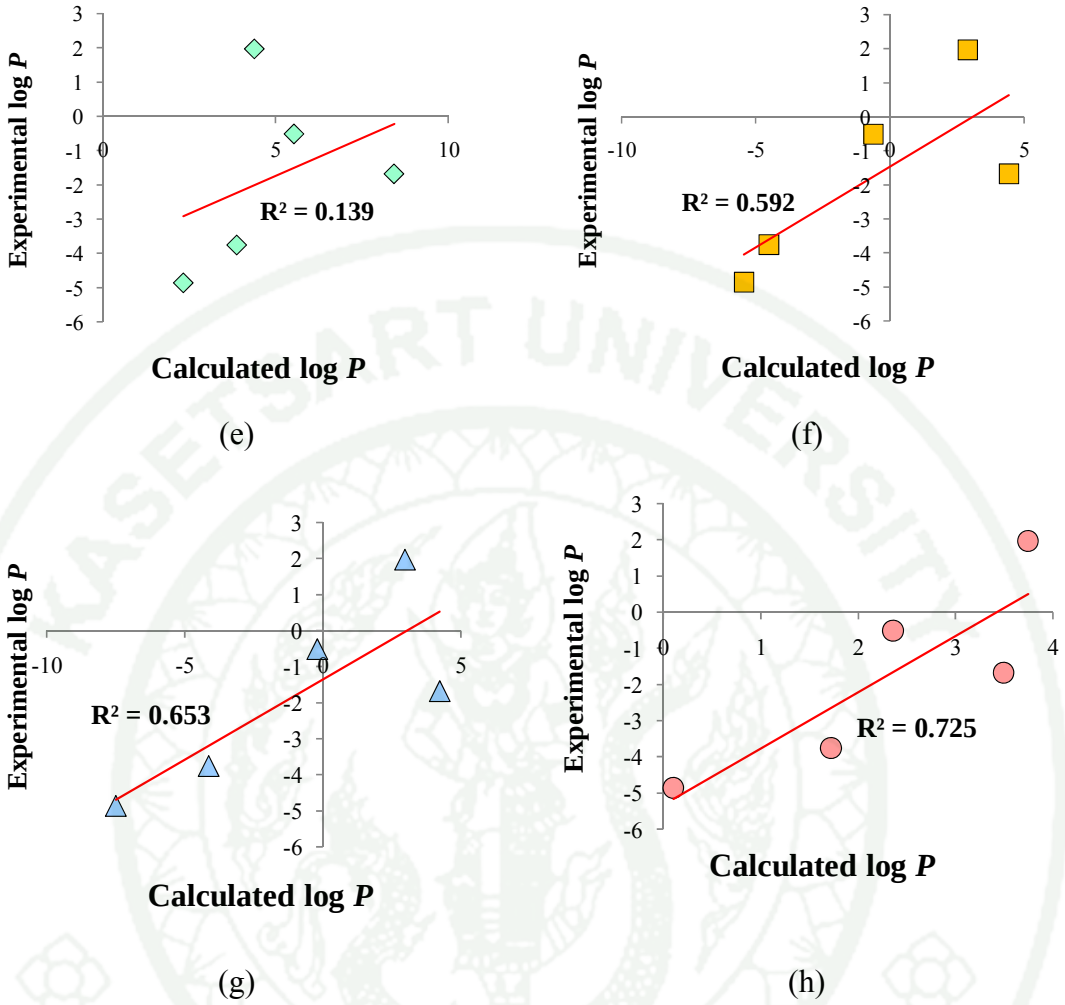


Figure 25 Relationships between the experimental log *P* and theoretical log *P* values
(e) XlogP3
(f) miLogP
(g) AlogP
and (h) PrologP

3. QSTR model construction

QSTR model of 5 azo dyes (reactive red 239, direct red 80, direct blue 78, direct black 22, and acid yellow 199), that displayed the relationships between the negative logarithm of toxicity ($p(1/LC_{50})$) values and experimental log *P* values, was built up in this work by using linear regression method. The toxicity values as reported in term of $p(1/LC_{50})$ and experimental log *P* values of 5 azo dyes used to develope QSTR model were shown in Table 10. The obtained QSTR model was shown as equation (1). The observed $p(1/LC_{50})$ values versus experimental log *P* values of these 5 azo dyes was illustrated in Figure 26.

Table 10 Toxicity values, $p(1/LC_{50})$, and experimental log *P* values of 5 azo dyes obtained in this study

Chemical	$p(1/LC_{50})$	log *P*
Reactive red 239	-0.193	-4.857
Direct red 80	-0.320	-3.752
Direct blue 78	-0.599	-1.676
Direct black 22	-0.540	-0.512
Acid yellow 199	1.220	1.969

$$p(1/LC_{50}) = 0.22(\pm 1.190) + 0.174(\pm 0.398)\ \log P \quad (1)$$

$(n = 5, r^2 = 0.392, s = 0.674, F = 1.393, \text{sig}(F) = 0.259$

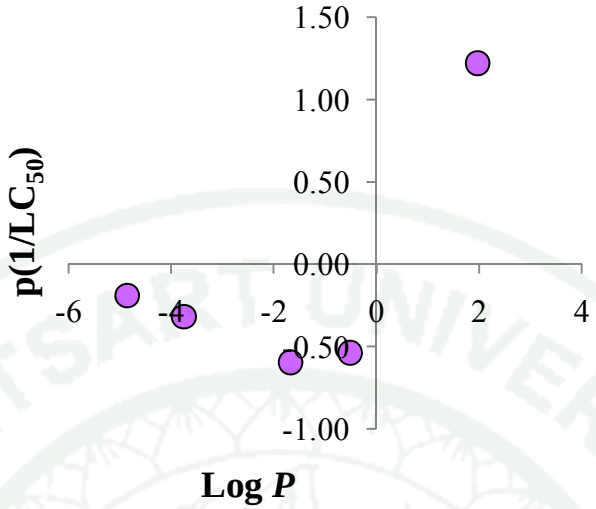


Figure 26 Observed p($1/LC_{50}$) values versus experimental log *P* values of 5 azo dyes

From the obtained results, it revealed that QSTR model as shown in equation (1) wasn't significant due to its statistical value in term of significance of F-test value, sig (*F*), showed non-significance. Actually, the square of correlation coefficients (r^2) of this model is low. Thus, this model isn't optimal used for toxicity prediction. This is because the distribution of toxicity data isn't good. However, the relationships between p($1/LC_{50}$) and experimental log *P* values can imply that the toxicity of azo dyes increases with increasing log *P* values corresponding to the literature reviews (Brust, 2001; Sachidanandan *et al.*, 2008; Ni *et al.*, 2011).

Discussion

Synthetic dyes have been extensively used for dyeing process in textile industry (Carneiro *et al.*, 2010). As much as 2-50% of them may be lost to wastewater and are ultimately released into the environment (Novotný *et al.*, 2006). They can cause harmful effects to fish and mammalian life (Doble and Kumar, 2005). Especially, azo dyes and their metabolites (aromatic amines) are well known to be toxic to aquatic life (Zee and Villaverde, 2005). Therefore, zebrafish embryos that are aquatic organisms have been widely used to assess the toxic effects of chemicals due to they are the most sensitive stage in the life cycle of the teleost (Hallare *et al.*, 2006; Wu *et al.*, 2010). Additionally, chemicals will exert their adverse effects to embryonic development at lower concentration than adults. This toxicity threshold can be used to determine the quality of water for protection of aquatic life (Zhu *et al.*, 2007).

According to the objectives of REACH, synthetic azo dyes including reactive red 239, direct red 80, direct blue 78, direct black 22, and acid yellow 199 which have been extensively used for dyeing textile and are highly soluble in water were determined their acute toxicity on zebrafish embryos in this study. 96h-LC_{50} values of these 5 azo dyes were 1,600, 2,594, 3,526, 3,751, and 25.82 mg/L for reactive red 239, direct red 80, direct blue 78, direct black 22, and acid yellow 199, respectively. Criteria used for classification of chemicals with regard to the aquatic organisms are shown in Table 11. These demonstrated that almost azo dyes in this study are nontoxic compounds on zebrafish embryos, except acid yellow 199. Acid yellow 199 is slightly toxic and can be classified to be harmful chemical on aquatic organisms due to its LC_{50} value is between 10 and 100 mg/L (Tišler and Končan, 2003). However, the concentration of some azo dyes such as reactive azo dye occurring in the textile dyehouse wastewater is in the range of 5-1,500 mg/L (Gottlieb *et al.*, 2003). Therefore, industries use these azo dyes should be aware their harmful effects. This is because they can induce various adverse effects. Several morphological malformations on zebrafish embryos that found in this study as shown in Figures 20-22 can be

considered as an effect of azo dyes due to observed developmental abnormalities were not found at control group.

Based on log *P* values of 5 azo dyes determined by using shake-flask method in this research, they can demonstrated that acid yellow 199 is the highest toxic compound compared to the other compounds. This is because it has the highest log *P* value therefore it tends to be easily absorbed in the tissue of zebrafish embryos leading to cause adverse effects on embryos. For QSTR model of the small set of azo dyes, it showed toxicity of compounds increases with increasing log *P* values. However, its predictive power isn't good due to the distribution of toxicity data is low. Toxicity values of 5 azo dyes obtained in this study can be classified in two two group including slightly toxic compound (acid yellow) and nontoxic compounds (reactive red 239, direct red 80, direct blue 78, and direct balck 22). Therefore, more toxicity information of azo dyes is required to develop a new model which may have a better quality for predicting the toxicity of azo dyes.

Table 11 Classification of chemicals with regard to the aquatic organisms (USEPA, 1012)

Toxicity Category	Acute concentration (mg/L)
Very highly toxic	< 0.1
High toxic	0.1 – 1
Moderately toxic	> 1 – 100
Slightly toxic	> 10 – 100
Practically nontoxic	> 100

CONCLUSION

In this study, acute toxicity on zebrafish embryo (*Danio rerio*) and log *P* of 5 azo dyes including reactive red 239, direct red 80, direct blue 78, direct black 22, and acid yellow 199 were investigated in this research. The study could be summarized as the following:

(1) Almost azo dyes in this work are nontoxic on zebrafish embryos due to their median lethal concentration (LC_{50}) values at 96 h are higher than 100 mg/L, except acid yellow 199. It can be classified to be slightly toxic compound on zebrafish embryos because its LC_{50} value is between 10 – 100 mg/L. 96h – LC_{50} values of azo dyes are 1,600 mg/L for reactive red 239, 2,594 mg/L for direct red 80, 3,526 mg/L for direct blue 78, 3,751 mg/L for direct black 22, and 25.82 mg/L for acid yellow 199.

(2) However, industry using these dyes should be aware their harmful effects because they can cause various developmental abnormalities including yolk sac edema, cardiac edema, bent spine, and tail malformation.

(3) Acid yellow is the highest toxic compound compared to the other compounds because it has the lowest 96h- LC_{50} value. This result also corresponded to the experimental log *P* value obtained by shake-flask method in this study. This is because log *P* value of this dye is highest so it tends to be easily absorbed in tissue of zebrafish embryos leading to adverst effects on embryos.

(4) The QSTR model of small set of azo dyes indicated that the toxicity of compounds increases with increasing log *P*. However, the obtained model is not appropriate to use for toxicity prediction due to its square of correlation coefficients (r^2) is low. This may be low distribution of toxicity data. More toxicity information is required for developing model.

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APPENDICES

Appendix A

Raw data of fish embryos toxicity (FET) test

Appendix Table A1 Percentage of survival of zebrafish embryos after exposure to each concentration of reactive red 239 for 96 h used to construct dose-response curve for finding LC_{50} value

Concentration (mg/L)	Survival (%) at 96 h			
	Rep. 1	Rep. 2	Rep. 3	Rep. 4
0	100	100	100	100
500	100	100	100	100
1,000	90	90	95	95
1,250	75	85	85	65
1,500	60	55	55	55
1,750	40	40	35	35
2,000	25	20	30	30
2,500	0	15	15	20
3,000	0	5	10	10
3,500	0	0	5	5
4,000	0	0	0	0

Appendix Table A2 Percentage of survival of zebrafish embryos after exposure to each concentration of direct red 80 for 96 h used to construct dose-response curve for finding LC_{50} value

Concentration (mg/L)	Survival (%) at 96 h	
	Rep. 1	Rep. 2
0	100	100
1,000	100	100
2,000	95	85
2,500	50	55
3,000	45	15
3,500	5	5
4,000	0	0

Appendix Table A2 (Continued)

Concentration (mg/L)	Survival (%) at 96 h	
	Rep. 1	**Rep. 2**
5000	0	0

Appendix Table A3 Percentage of survival of zebrafish embryos after exposure to each concentration of direct blue 78 for 96 h used to construct dose-response curve for finding LC_{50} value

Concentration (mg/L)	Survival (%) at 96 h	
	Rep. 1	**Rep. 2**
0	100	100
1,000	100	100
1,500	100	100
2,000	90	100
2,500	70	80
3,000	85	55
3,500	60	70
4,000	0	25

Appendix Table A4 Percentage of survival of zebrafish embryos after exposure to each concentration of direct black 22 for 96 h used to construct dose-response curve for finding LC_{50} value

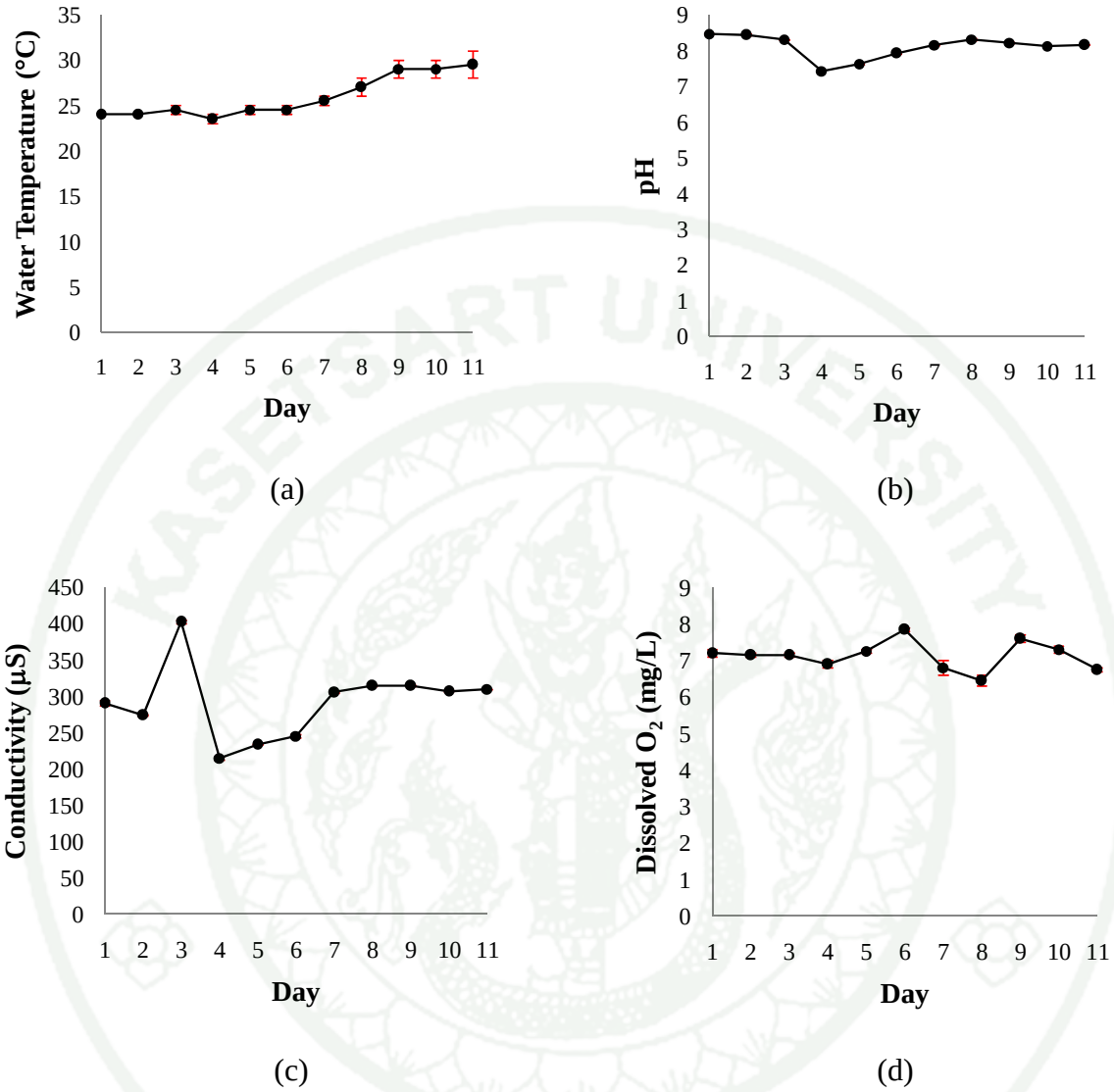
Concentration (mg/L)	Survival (%) at 96 h
	Rep. 1
0	100
1,000	100
2,000	100

Appendix Table A4 (Continued)

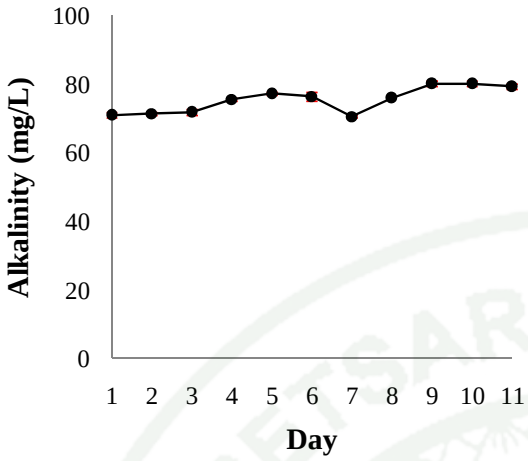
Concentration (mg/L)	Survival (%) at 96 h
	Rep. 1
3,000	80
4,000	35
5,000	30
5,500	5
6,000	0

Appendix Table A5 Percentage of survival of zebrafish embryos after exposure to each concentration of acid yellow 199 for 96 h used to construct dose-response curve for finding LC_{50} value

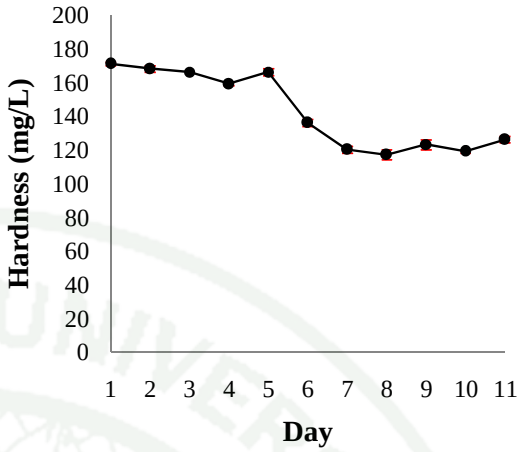
Concentration (mg/L)	Survival (%) at 96 h	
	Rep. 1	Rep. 2
0	100	100
5	100	100
15	100	100
17	95	90
19	85	85
21	70	80
23	65	70
25	50	65
30	25	30
40	0	10
50	0	0



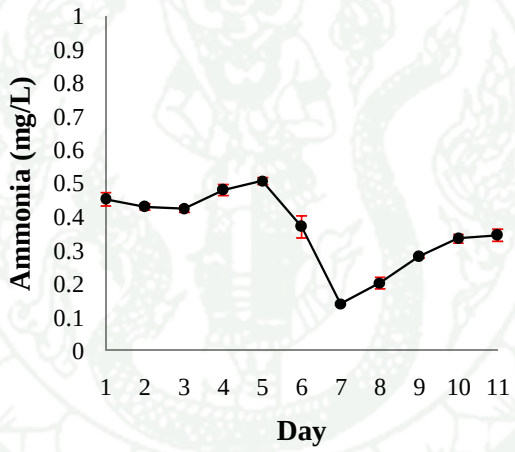
Appendix Figure A1 Quality of water used for maintaning parental zebrafish and breeding zebrafish embryos throughout the experiment
(a) water temperature
(b) pH
(c) conductivity
and (d) dissolved O_2



(e)



(f)



(g)

Appendix Figure A2 Quality of water used for maintaning parental zebrafish and breeding zebrafish embryos throughout the experiment
(e) alkalinity
(f) hardness
and (g) Ammonia

Appendix B

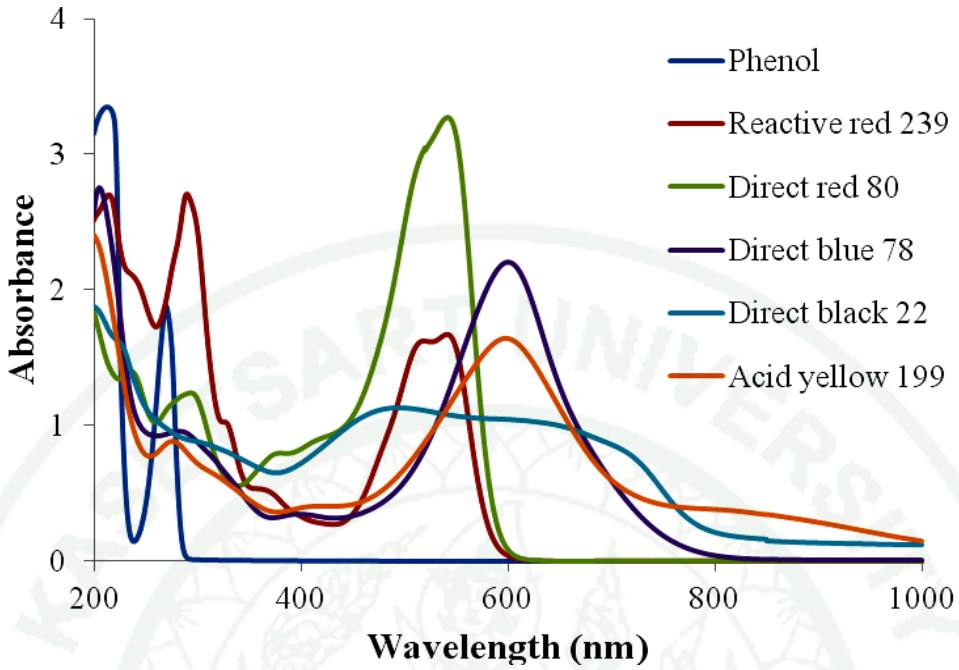
Raw data of log *P* experiment

Appendix Table B1 Peak area of 5 azo dyes in octanol and aqueous phases used to calculated log *P* values

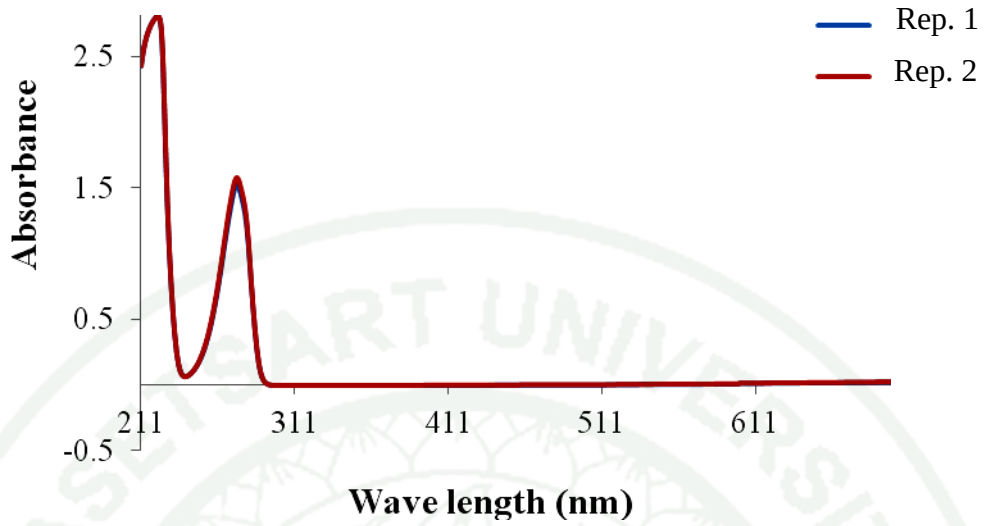
Compound	Octanol phase			Aqueous phase		
	1	**2**	**3**	**1**	**2**	**3**
Phenol	33.2019	34.3891	–	0.7988	0.8009	–
Reactive red 239	1.39822	3.00874	1.37317	121301.8	132579.8	133522.2
Direct red 80	15.6411	16.01	16.7395	86311.6	98901.2	88316.8
Direct blue 78	70.4544	67.0344	73.4341	3532.94	3352.19	3116.97
Direct black 22	2.49691	2.53061	4.19136	8.74482	10.046	10.33778
Acid yellow 199	5480.1	5086.7	4719.965	58.246	51.3742	54.3364

Appendix Table B2 Ratio of peak areas between octanol and aqueous phases, called *P*, and the log *P* values for 5 azo dyes

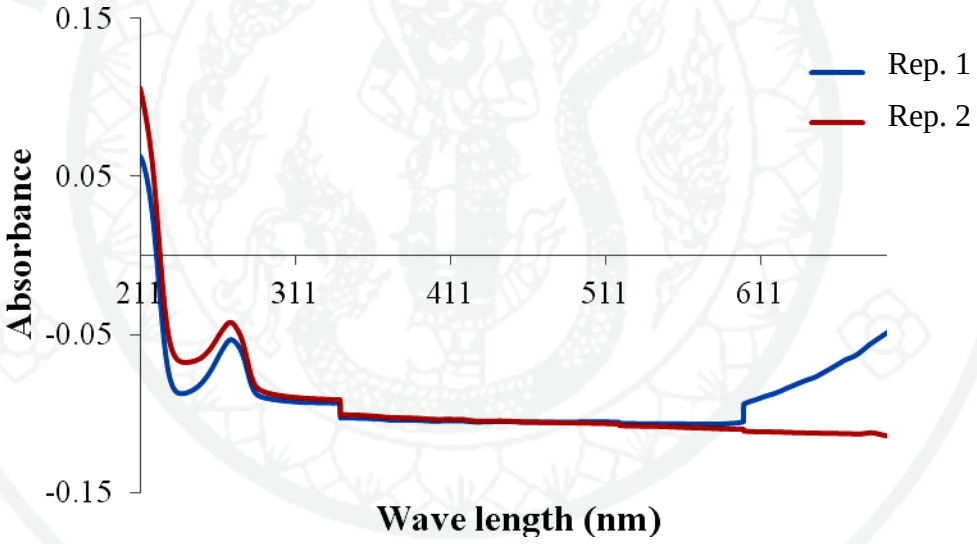
Compound	*P* (octanol/aqueous)			log *P*			Mean	SD
	1	2	3	1	2	3		
Phenol	4.156E+01	4.294E+01	–	1.619	1.633	–	1.626	0.010
Reactive red 239	1.153E−05	2.269E−05	1.028E−05	−4.938	−4.644	−4.988	−4.857	0.186
Direct red 80	1.812E−04	1.619E−04	1.895E−04	−3.742	−3.791	−3.722	−3.752	0.035
Direct blue 78	1.994E−02	2.000E−02	2.356E−02	−1.700	−1.699	−1.628	−1.676	0.041
Direct black 22	2.855E−01	2.519E−01	4.054E−01	−0.544	−0.599	−0.392	−0.512	0.107
Acid yellow 199	9.409E+01	9.901E+01	8.687E+01	1.974	1.996	1.939	1.969	0.029



Appendix Figure B1 UV spectral of phenol, reactive red 239, direct red 80, directblue 78, direct black 22, and acid yellow 199

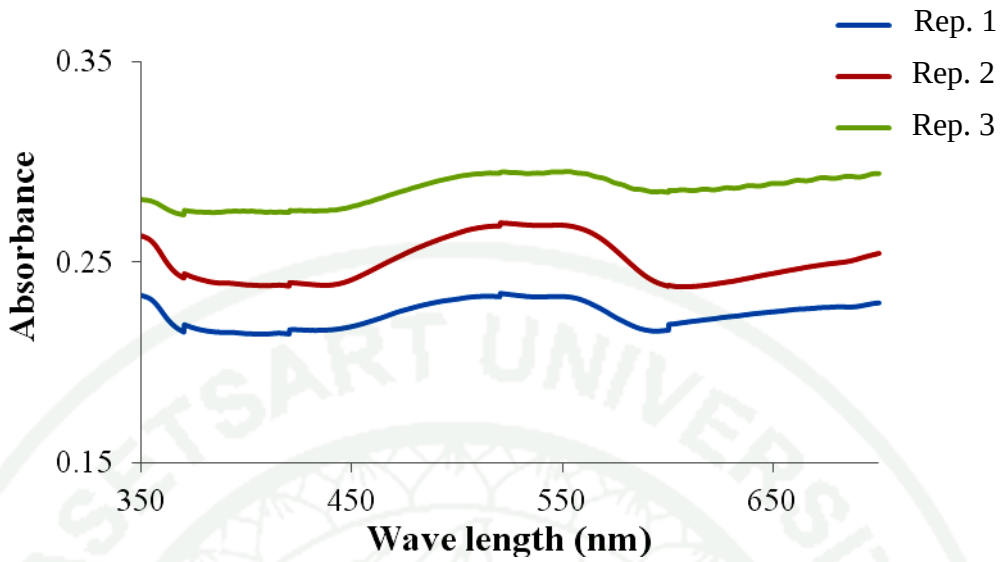


(a)

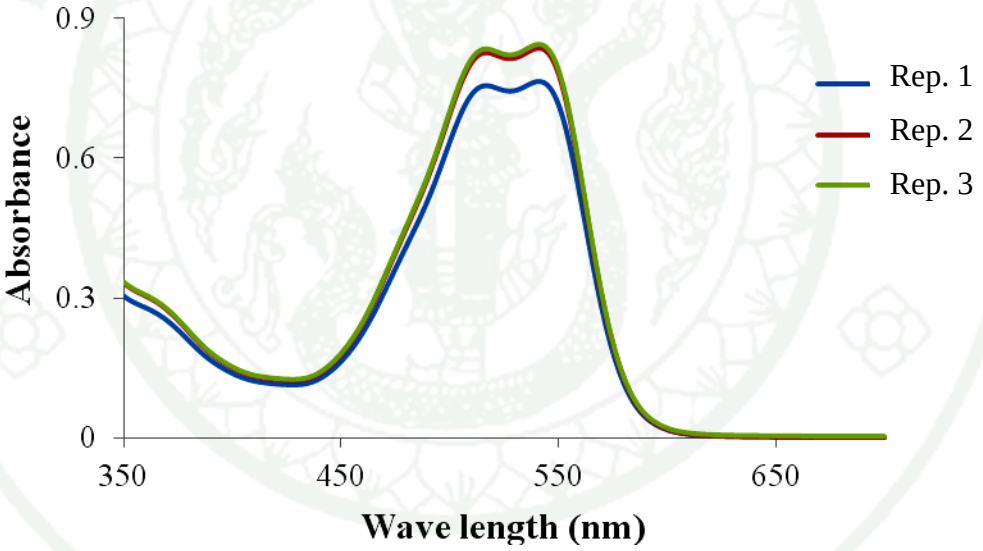


(b)

Appendix Figure B2 UV spectral of phenol in
(a) octanol phase
and (b) aqueous phase

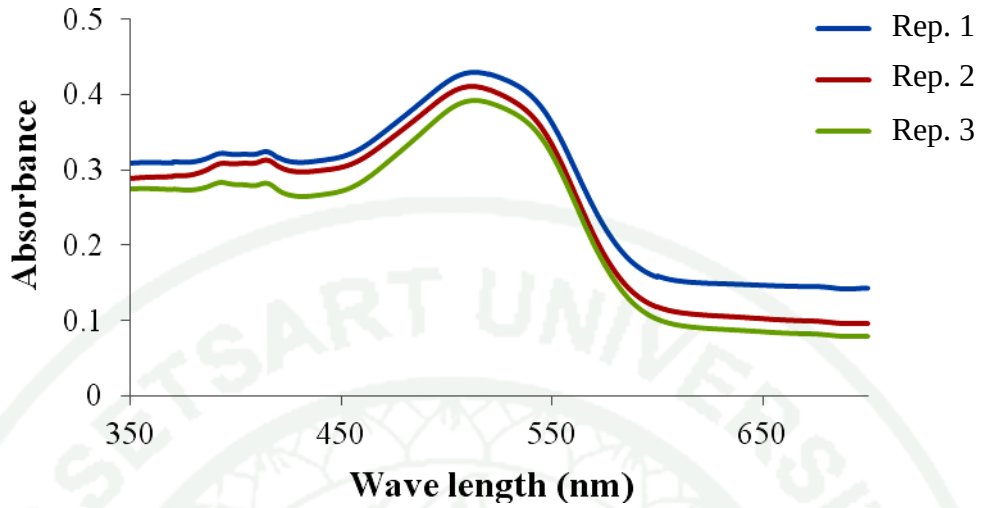


(a)

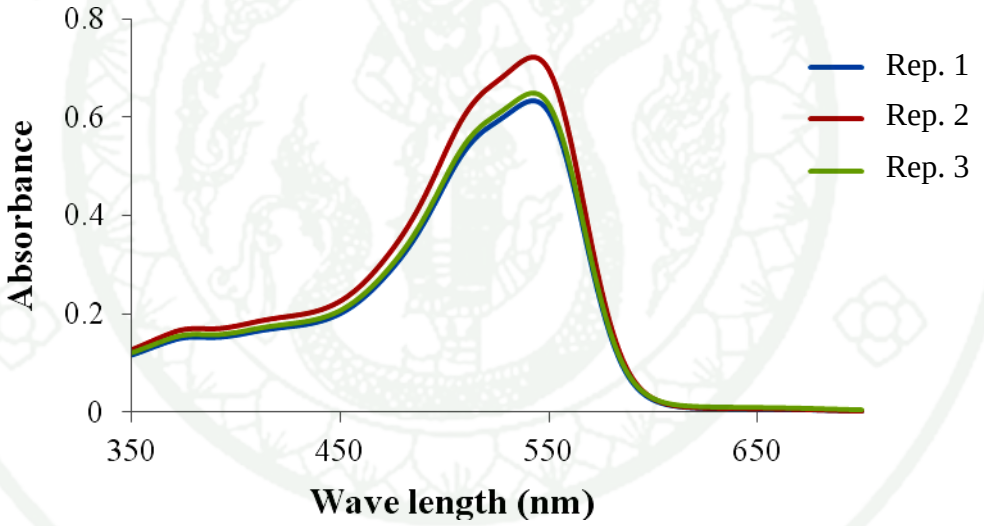


(b)

Appendix Figure B3 UV spectral of reactive red 239 in
(a) octanol phase
and (b) aqueous phase

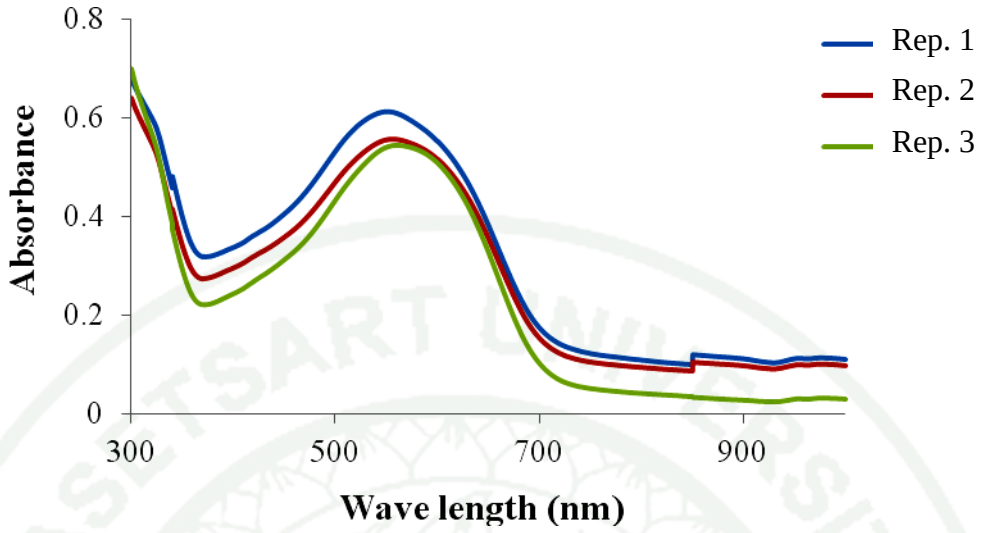


(a)

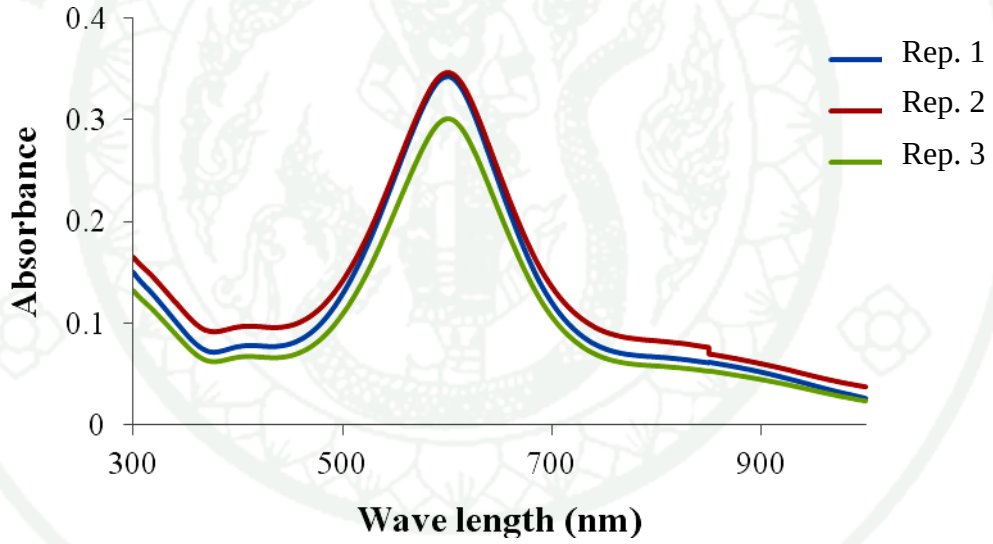


(b)

Appendix Figure B4 UV spectral of direct red 80 in
(a) octanol phase
and (b) aqueous phase

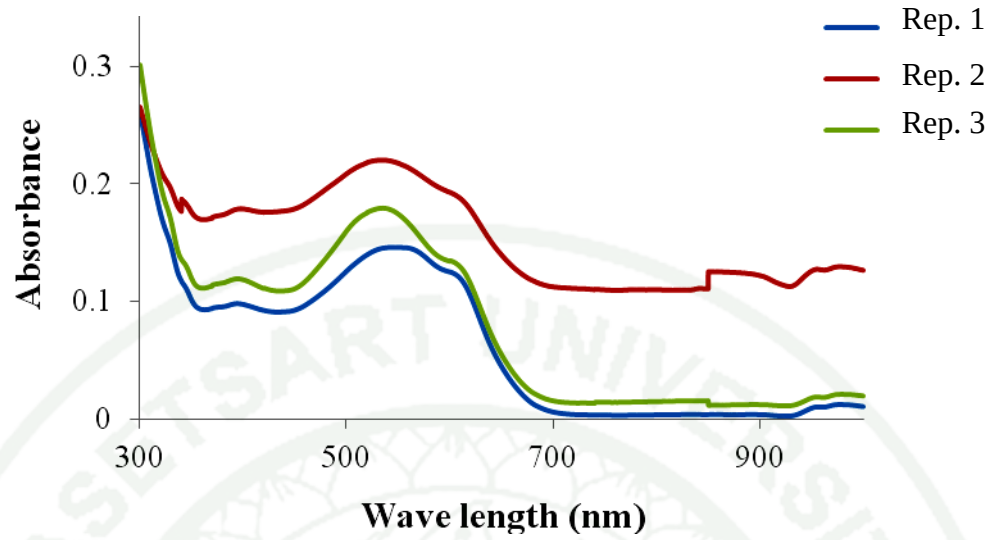


(a)

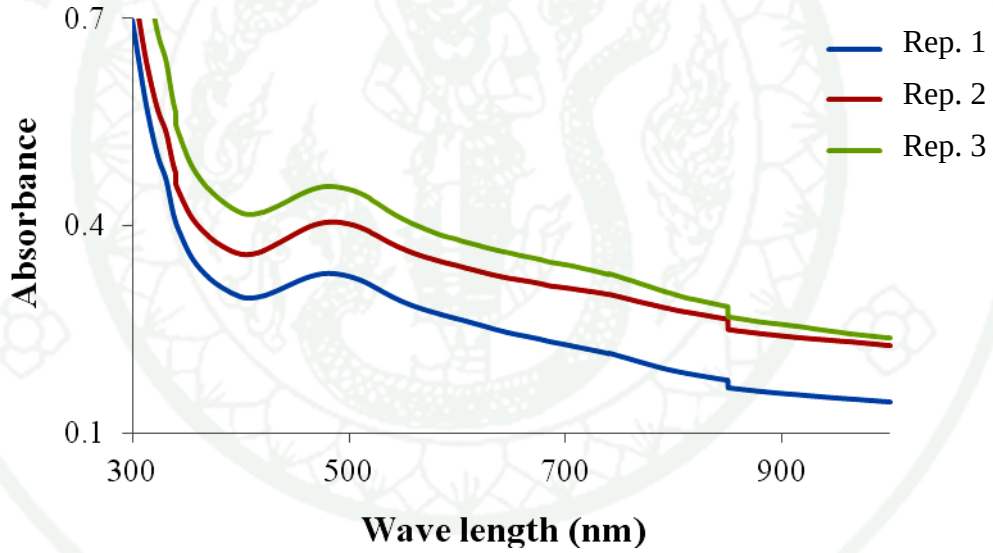


(b)

Appendix Figure B5 UV spectral of direct blue 78 in
(a) octanol phase
and (b) aqueous phase

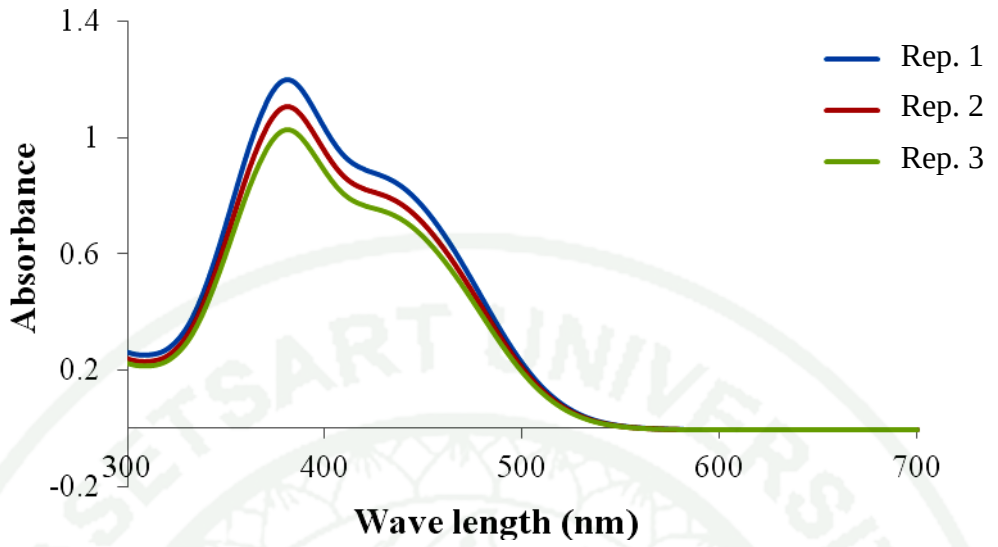


(a)

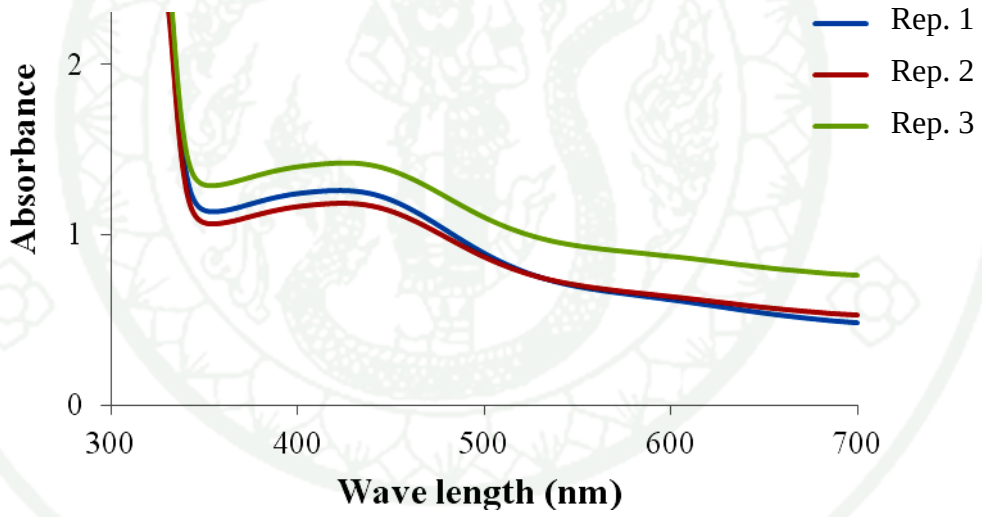


(b)

Appendix Figure B6 UV spectral of direct black 22 in
(a) octanol phase
and (b) aqueous phase



(a)



(b)

Appendix Figure B7 UV spectral of acid yellow 199 in
(a) octanol phase
and (b) aqueous phase

Appendix C

Publication, proceeding, and presentations

Publication

1. Waraporn Jungtanasombut, Krittikar Noytanom, Thitinun Karpkird Waraporn Parasuk and Supa Hannoungbua*, **Quantitative Structure-Activity Relationships and Log P_{ow} Investigation of Newly Synthesized UV Protector: Cinnamate and Cinnamic Acid Derivatives.**

2. Waraporn Jungtanasombut, Pichapop Preeprem, Uthaiwan Kovitvadhi, Supa Hannongbua* and Satit Kovitvadhi*, **Acute Toxicity of Reactive Red 239 (RR239) on Zebrafish (*Danio rerio*) Embryos**.

Proceeding

1. Supawan Sukiattichaisakul, Krittikar Noytanom, Waraporn Jungtanasombut , Waraporn Parasuk, Thitinun Karpkird and Supa Hannongbua. **Calculation of Partition Coefficient (Log *P*) of Synthesized Cinnamic Acid and Cinnamate Derivatives.** Pure & Applied Chemistry Conference (PACCON 2010), Faculty of Science, Ubon Rajathanee University, Thailand, 22-24 January 2010.

2. Krittikar Noytanom, Supawan Sukiattichaisakul, Waraporn Jungtanasombut, Thitinun Karpkird, Waraporn Parasuk and Supa Hannongbua. **Experimental on *n*-Octanol/Water Partition Coefficient (Log P_{ow}) of Synthetic Cinnamic Acid Derivatives.** Nano Thailand 2010: Nanotechnology for a Sustainable World. Thailand Science Park Convention Center (NSTDA) Pathumthani, Thailand, 18-20 November 2010.

Presentations

1. Oral presentation

1.1.1 Waraporn Jungtanasombut, Pichapop Preeprem, Uthaiwan Kovitvadhi, Supa Hannongbua and Satit Kovitvadhi. **Toxicity Investigation of Reactive Read 239 (RR239) on Zebrafish (*Danio rerio*) Embryos**. The 5th Annual Meeting "The Thailand Research Fund Senior Research Scholar, Innovative Research on Anti-AIDS Drug Discovery (Phase II), Rajamangala University of Technology Suvarnabhumi Phra Nakhon Si Ayutthaya Wasukri Campus, Phra Nakon Sri Ayutthaya Thailand, July 28, 2012.

1.1.2 Waraporn Jungtanasombut, Pichapop Preeprem, Uthaiwan Kovitvadhi, Supa Hannongbua and Satit Kovitvadhi. **Investigation of the effects of Reactive Read 239 (RR239) on Zebrafish (*Danio rerio*) Embryos**. The 1st Chemistry Postgraduates Symposium ($^{1}/_{2012}$ ChPGS), Department of chemistry, Faculty of Science, Kasetsart University, Thailand, August 8, 2012.

2. Poster presentation

1.2.1 Waraporn Jungtanasombut, Thanapop Yiamsawad, Pichapop Preeprem, Satit Kovitvadhi and Supa Hannongbua. **Toxicity of C.I. Direct Red 80 on Zebrafish Embryo**. Commission on Higher Education Congress IV University Staff Development Consortium (CHE-USDC Congress IV), The Zigh Hotel Pataya, Chonburi, Thailand, 14-16 September, 2011.

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