Development of simplified colorimetric assay method of liquid aiming for on-site discrimination test of unknown samples

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Takao Sakurai

Graduate School of Bio-Applications and Systems Engineering,

Tokyo University of Agriculture and Technology

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Chapter 1

General introduction

1.1 Importance of rapid testing method for unknown chemical substances

In our daily life, we receive much benefit from a lot of chemical substances. It is not too much to say that today's convenient lives depend on the developments of chemistry. On the other hand, chemical substances can cause social or domestic damages. Some of accidents, such as fires, explosions, or poisonings are caused when chemicals are handled in the wrong way [1-7]. In the crime scenes, for example, chemical warfare agents [8-11] or explosives [12-13] have been used for terrorism, ignitable liquids for arson [14-15], hazardous material (such as poisons, acids, and bases) for murder or injury [7], drugs or organic solvents for abuse [4, 16-17].

Such chemical substances require careful handling to protect people from the risks, such as poisoning, explosion, and chemical burning [9-10]. The ways to safety handling of chemicals vary widely depending on their chemical properties. When no information about the unknown chemical substance is given, people who is handling are exposed to the large risks. Moreover, when chemicals are mishandled, it is possible that the risk is expended to people around.

Analyses of chemical substances have an important part in the forensic science to investigate the causes of accidents or crimes, and to establish the scientific evidences of crimes. Suitable analytical methods for chemical substances are individually dependent on each chemical properties. Especially, analyses of unknown samples have great difficulties because a suitable analytical method for the sample cannot be predicted when we have no information about the sample. The analyses are carried out through trial and error process. Thus, the procedure tends to be time-consuming and requires

multiple instrumental analyses in the laboratory.

To protect people from risks of chemical substances and to shorten the time for the analyses, as shown in Fig. 1-1, on-site testing methods for the identification or accurate prediction of chemical substances are important in the forensic science field.

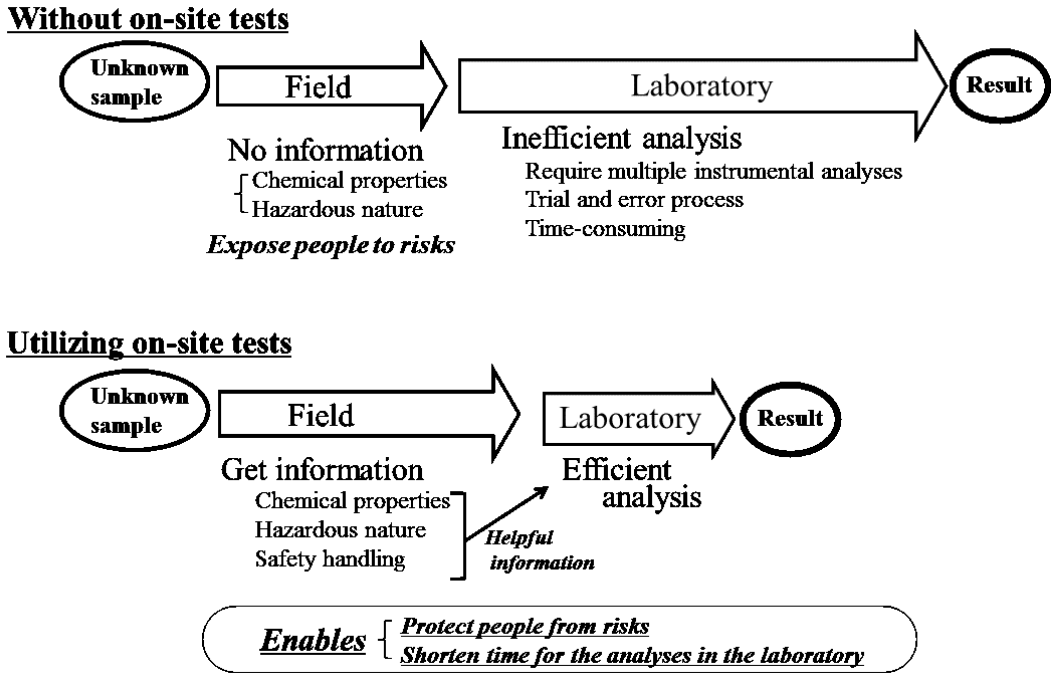

Fig. 1-1 Conceptual diagram for analysis of unknown samples in forensic field comparing the case when on-site tests are utilized or not utilized.

1.2 Detection methods for chemical substances

A lot of on-site detection methods useful in the forensic science field have already been developed. They are used in the specialized tasks and can be classified according to the target reagents and intended use. Examples of target reagents are listed as follows.

1.2.1 Chemical warfare agents

Chemical warfare agents (CWAs) have been used not only in the war and conflict but

also in the terrorisms [8-11]. Moreover, after the war period, CWAs, which are left on battlefields, have sometimes been found out, and have caused health issues [9]. Because of their fast-acting properties, the rapid identification of the kind of the reagent is required to take an appropriate medical treatment and to minimize the damage. Thus, on-site detection of CWAs has a great importance both on the battlefield and in urban security [8-11].

CWAs are categorized into some classes; choking agents, blister agents, blood agents, nerve agents, and riot control agents. Nerve agents are further divided into two categories, G series agent and V series agent. The types of CWAs are summarized in detail by Organisation for the Prohibition of Chemical Weapons (OPCW) as shown in Table 1-1 [18].

Table 1-1 List of CWAs summarized by OPCW [18].

category	agents	dispersal
Choking agents	Chlorine Phosgene Diphosgene Chloropicrin	Gas
Blister agents	Sulfur mustard Nitrogen mustard Phosgene oxime Lewisite	Liquid, aerosol, vapour and dust
Blood agents	Hydrogen cyanide Cyanogen chloride Arsine	Gas
Nerve agents	Tabun Sarin Soman Cyclosarin VX	Liquid, aerosol, vapour and dust
Riot control agents	Tear Gas Pepper Spray	Liquid, aerosol, vapour and dust

1.2.2 Explosives

Explosives have been occasionally used to harm civilians around the world by terrorists. Once bombing occurs, over hundreds of victims can be killed [12-13]. Detection of explosives before the enforcement by terrorists is most important task. However, there is difficulty on the detection of explosives because of the variety of explosive materials, cleverness of packaging, variability of venue, and the low vapor pressures of explosives [12].

Explosives are classified into many types according to their chemical structures and performance and some examples of explosives are listed in Table 1-2 [12, 19]. Detections of explosives have been performed widely with a variety of methods, utilizing dogs [19], standing instruments on the airport using X-ray [20], and so forth. Recently, advanced portable on-site detection systems have been developed [21].

Table 1-2 Examples of commonly used explosives [19].

Compound class	Example	Symbol	Commonly found in the following
Aliphatic nitro	Nitromethane		
	Hydrazine		Rocket fuel and liquid component of two-part explosive
Aromatic nitro	Nitrobenzene	NB	
	2,4,6-trinitrotoluene	TNT	Composition B with equal part RDX, Pentolite with equal part PETN
Nitrate ester	Nitroglycerin	NG	Certain dynamites, pharmaceutical
	Ethylene glycol dinitrate	EGDN	Some dynamites
	Pentaerythitol tetranitrate	PETN	Detonating cord, Detasheet (Flex-X military name), Semtex with RDX
	Nitrocellulose		'Guncotton', main component of single-based smokeless powder
	Nitrocellulose and NG		Double-based smokeless powder
	Nitrocellulose, NG and nitroguanidine		Triple-based smokeless powder
Nitramines	Trinitro-triazacylohexane (cyclonite)	RDX	C-4,tetrytol-military dynamite with TNT
	Tetranitro-tetrazacylooctane (octogen)	HMX	Her Majesty's Explosive
Acid salts	Ammonium nitrate		ANFO with fuel oil, nitro-carbo-nitrates (NCN) w oil[-1]
	Potassium nitrate		Black powder with charcoal and sulfur
Primary explosives	Lead azide		
	Lead styphnate		Blasting caps
	Hexamethylene triperoxide diamine	HMTD	
	Triacetone triperoxide	TATP	

1.2.3 Ignitable fuels and organic solvents

Ignitable fuels such as gasoline and kerosene are used in the fire scene to arson [22-28]. They can kill victims in a short time at fire scene, too [1]. Field detection of

ignitable fuels can help the investigation of the cause of fire and the evidence of crime [15]. Inhalation of organic solvents became social problems in Japan [16, 17]. Inhalation of thinner containing toluene, methanol or ethyl acetate is regulated by law in Japan.

1.2.4 Abused drugs

Abuse of illegal drugs like methamphetamine has been widely spread, causing social problems [29]. On-site detection and policing of abused drugs are getting more important [30].

1.3 Examples of commercially available on-site detector

Many of portable on-site detectors are commercially available and they have been used over the world. No detector can identify all the target reagents comprehensively [10]. Therefore, appropriate detector must be chosen according to the chemical structures or properties of the target reagents. Kinds of detection methods for CWAs [8, 10] or explosives [13] are reviewed. Examples of commercially available detectors which have been recognized as effective ways are listed as follows.

1.3.1 Ion mobility spectrometer

Ion mobility spectrometers (IMSs) allow analytes to be distinguished on the basis of their mass, charge and collision cross-section. Nowadays portable IMS apparatuses are commercially available and they are frequently used to detect CWAs, explosives, and drugs because they can provide confidence in measurement from characteristic spectra of the target substances [31]. Moreover, their instrumental simplicity, fast response, low operation cost, portability, make IMS popular detection instrument [32].

Basically, IMSs are composed of ionization region, drift region, and detector [8].

Sample vapor, which is introduced by aspiration with carrier flow, is first ionized in the ionization region.

For ionization sources, β-emitter radioactive sources such as ^{63}Ni and ^{241}Am are mainly used [33]. Laser desorption ionization, corona discharge ionization, and electrospray ionization, which are free from the regulations of radioactive source usage, have recently been popular [33-35].

For drift region, there are three approaches to separate ions [31]; time of flight type, aspirator type, and the type which is based on the differences of ion mobilities in high and low electric fields, called high-field asymmetric waveform ion mobility spectrometers (FAIMS) or differential mobility spectrometers (DMS). In time of flight type, ions are injected at a given time interval via an electronic shutter into the drift region and then ions drift towards the detector in the voltage gradient and through a gas atmosphere. Ions are separated based on their ionic mobilities which can be associated with the ion structure, mass, charge, and so on, and reach the detector in the drift time. At the detector, ions collide and release their charge, which is registered as a current and the plot of the current generated for a series of the peaks over drift time, given an ion mobility spectrum (schematic diagram is shown in Fig. 1-2a) [8, 31, 36]. In aspirator type, ions are introduced continuously into a parallel plate structure by carrier gas. In the parallel plate structure, an electric field is applied in a direction perpendicular to the introduced ion direction and ions move by each ion mobility towards the plate. The position where an ion strikes the plate is detected by collectors fixed in line on the plate. The mobilities of ions are registered according to the distance from the inlet to the point where an ion strike on the plates (schematic diagram is shown in Fig. 1-2b) [31, 37-40]. In the FAIMS or DMS, asymmetric electric field is established in the gap between the conducting surfaces where ions introduced by carrier gas. When no other voltage is

applied, ions collide with the plate and lost. If a constant DC voltage (called compensation voltage, CV) of the correct magnitude and polarity is applied, ions will continue to travel between the two plates and reach the detector. By scanning the CV, ion mobility can be determined and the ion mobilities are measured (schematic diagram is shown in Fig. 1-2c) [31, 41].

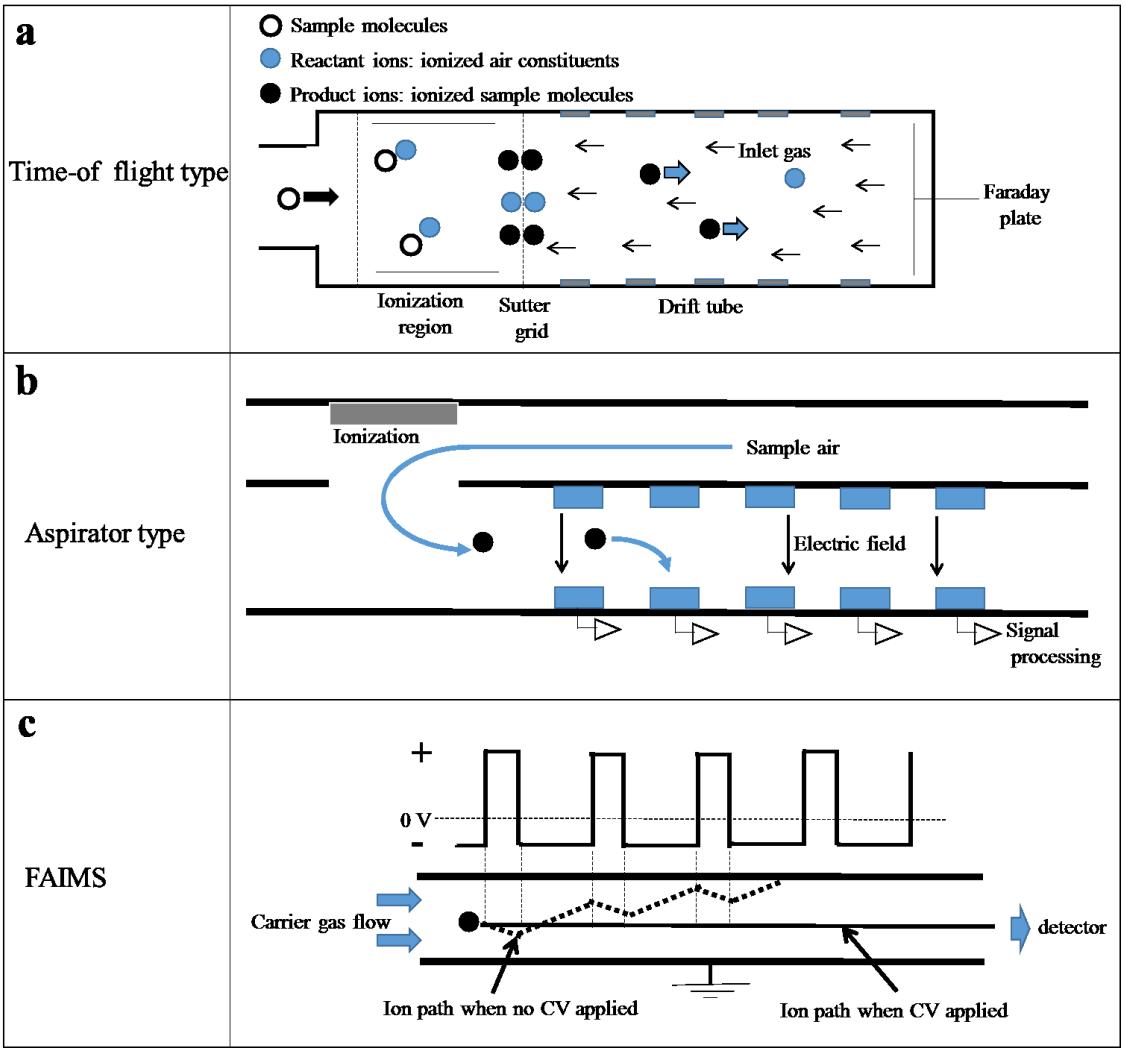

Fig. 1-2 Schematic diagrams of three types of IMS [31, 41].

Portable detector measures the ion mobility of the sample and alarms if mobility of sample is the same as target chemicals. For CWAs detection, detectors can discriminate

the vapor samples into some groups, such as Nerve gas, Blister gas and so on [9, 34, 38]. Recently, solid phase micro-extraction is coupled with IMS to be applied not only for vapor samples but also for liquid samples [32]. IMS is one of the most popular instruments for the detection of CWAs and explosives [42], because of its portability and rapidity. Moreover, they are simple, rugged, handled with easy maintenance. On the other hand, because of its poor separation capability, identification cannot be achieved occasionally, and some gas vapors such as acetone, ethanol, and cigarette smoke can bring about a false alarm [9, 10, 34, 38].

1.3.2 Surface acoustic wave sensor array

Surface acoustic wave (SAW) sensor array has been mainly used to detect volatile organic compounds [8, 43-45], and vaporized some chemical agents [8-10, 46-52]. The detection principle of SAW sensor is briefly described as follows; SAW sensor device consists of an input transducer, a chemical adsorbant film, and an output transducer on a piezoelectric substrate. The piezoelectric substrate is typically made of quartz, and the chemical adsorbant polymeric film is coated between interdigital transducers. The input transducer launches an acoustic wave that travels through the film, which is detected by the output transducer as shown Fig.1-3. When analyte hits the surface, the analyte with high affinity to the polymer can be adsorbed on the polymer surface. Adsorption of analyte on polymer film, gives rise to changes in the characteristics of the propagation path affecting the velocity and amplitude of the wave. The resulting shift, caused by the changes in mass, viscoelastic constants, and electrical conductivity of the polymer, is monitored. SAW sensor array includes multiple SAW sensor devices with different polymer coatings, which exhibit different adsorption behavior of the organic compounds. The wave shift pattern of each array for each analyte is recognized by the

recognition system that uses a clustering technique, and various chemicals are discriminated [8-10, 43, 47].

Commercially available SAW sensor array detectors have good portability, high sensitivity and higher discrimination capability for CWAs than IMSs [51]. However, the false alarm occurs more frequently for some chemicals other than CWAs [9, 10, 51]. Additionally, the long recovery time is necessary for some CWAs in order to release the chemicals from the polymer [9, 51].

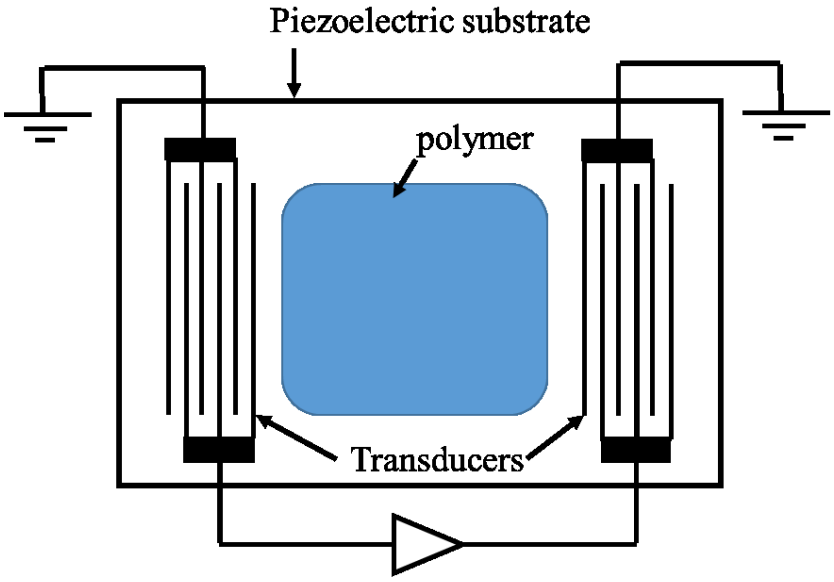

Fig. 1-3 Schematic diagram of construction of a SAW sensor [44].

1.3.3 Gas chromatograph-mass spectrometer

Gas chromatograph-mass spectrometer (GC-MS) is one of the most powerful tools for identification of the substance in the laboratory. Portable GC-MS is commercially available to analyze the volatile organic compounds (VOCs) [9]. HAPSITE ER and HAPSITE smart plus have been developed by the U. S. company, Inficon [53], and detection performance of CWAs by HAPSITE system have been evaluated [54]. These instruments are utilized not only for the hazardous compounds of operational

environments but also for the volatile metabolites of agricultural products [55-57]. The vapor sample is separated by GC with a capillary column under the elevated temperature control, followed by the analysis of each resolved component via electron-ionized mass spectrometry with quadrupole mass spectrometer. Solid phase micro extraction (SPME) and Thermal Desorption (TD) are applicable as a sampling probe in order to enhance sensitivity [58]. Some degradation products of VX gas offer poorly diagnostic mass spectra with electron-ionized mass spectrometry with quadrupole mass spectrometer. To overcome this problem, a portable gas chromatograph-toroidal ion trap mass spectrometer, which can produce pseudo-molecular ions, have been invented [59].

GC-MS can separate chemicals, and can afford information related to the retention time and the mass spectrum. Comparing the given mass spectrum with that of a mass spectral library, on-site identification of the CWAs can be achieved [9]. Due to this advantage, GC-MS is regarded as a powerful method for the identification of volatile CWAs. However, some disadvantages exist; carryover phenomenon[54], requirement of training because of the complicated operation, relatively long analyzing time for detection and identification (longer than other methods such as IMSs), heavier and larger apparatus than other portable sensors. Comparison of size, weight, and response time for GC-MS and other methods is listed in Table 1-3.

Table 1-3 Comparison of some example of available portable detectors [34, 40, 52, 53].

Type	IMS (TOF type) [34]	IMS (Aspirator type) [40]	SAW sensor [52]	GC-MS [53]
Product name	LCD-3.2E[a)]	ChemPro100[b)]	HAZMATCAD[c)]	HAPSITE ER[d)]
Size H × W × D (cm)	18 × 11.5 × 4.5	23 × 10 × 5.5	20 × 7 × 6.5	46 × 43 × 18
Wight (kg)	0.545	0.8	0.63	19
Response time	1-30 s	5-175 s	10-120 s	several minutes

a): Smith detection, Inc., United Kingdom

b): Environics Oy, Finland

c): Microsensor Systems, Inc., United States

d): INFICON, United States

1.3.4 Raman spectrometer

Raman spectrometer detects the Raman scattering which is radiated from the sample when it is illuminated by monochromic laser light. The Raman scattering is correlated to the molecular structure, and the identification of an unknown sample is conducted by searching a given spectrum through spectra libraries [8].

Raman spectrometer with confocal optics can be utilized for the sample which is enclosed in a container, or trapped between clothing fibers [60]. Measurements can be carried out remotely, without contact to the sample and destruction of the sample [8]. A previous report shows that some instruments can be applied to the explosives as far as 55 m distance [61]. Raman spectrometer is applicable to the various forms of sample, such as liquids, solids, and aerosols, and the presence of water does not affect on the measurement [62]. Because of these advantages, the Raman spectrometer has been widely used as the on-site detector of CWAs and explosives [8, 13, 63-65] On the other hand, limitation exists for Raman spectrometer; if the sample is mixture of more than two chemical components, identification can be false resulting in difficult detection of

the hazardous substance [8].

1.3.5 Infrared ray spectrometer

Infrared ray (IR) spectrometer is applicable to detect and identify the hazardous chemicals by referencing the IR spectrum of sample with the spectra in libraries [8]. Characteristic absorption band at specific wavenumber corresponds to the existence of functional group, affording useful information for the detection and identification of target chemicals. Samples with various forms can be measured with portable gas sensor [66] or with attachments such as gas cell or attenuated total reflection (ATR) discs for solids [8].

For the field operation, use of portable IR spectrometer is currently limited. Major disadvantage of IR spectrometer is subject to environmental affection, which is resulted from strong IR absorption of water and carbon dioxide [8, 10].

1.3.6 Flame photometric detector [8]

Handy portable flame photometric detectors (FPD) are mainly used in a battlefield to detect CWAs. FPD detects the specific emission line from phosphorus and sulfur atoms on burning in a hydrogen-rich flame. It gives an alarm immediately when the emission lines for these atoms are detected, making the rapid detection of vapor CWAs possible. Liquid samples can also be measured together with a sampling probe. However, chemical compounds without phosphorus and sulfur atoms cannot be detected even if they are hazardous. Furthermore, FPD alarms against compounds with phosphorus or sulfur even if they are not hazardous [10].

1.3.7 Detector papers

Detector papers are one of the least expensive ways, and they are useful for testing liquids or aerosols of CWAs. It can detect CWAs and indicate which type the sample is [8-10]. There are three types; G, H, and V agents. Detector paper usually contains two dyes and one pH indicator, which are impregnated into cellulose paper. Some agent dissolves one dye, and another dye is dissolved by other type of agent. When pH is changed by some agent, pH indicator changes its color. Some types of detector paper are commercially available, and they have been used by military and police [8-10].

M8 detector paper, one type of detector paper used in American army, shows three color patterns against CWAs depending on their species [8, 10]. When a G agent, such as sarin, soman, is absorbed on the paper, yellow color appears. When a H agent like mustard gas, is absorbed, the color changes to red. If a V agent like VX, is absorbed, the pH changes turning to green. In Japan, a similar detector paper is provided by Toyo Boseki [9]. Although this detector exhibits a false positive sign for some organic solvents, there are some advantages such as the immediate color change and easy operation.

1.3.8 Detector tubes

A detector tube is made of glass and is filled with granular carrier materials which are impregnated with chemical reagents [67]. Gas detector tube technique is simple, inexpensive, and easy to handle, and it is well-known technique for gas measurements [68]. The chemical reagents react with a specific analyte and show particular color change. A measurement is performed by aspirating the air sample through the tube with a pump. The pump is used to draw a specific volume of air sample as shown in Fig. 1-4 [67].

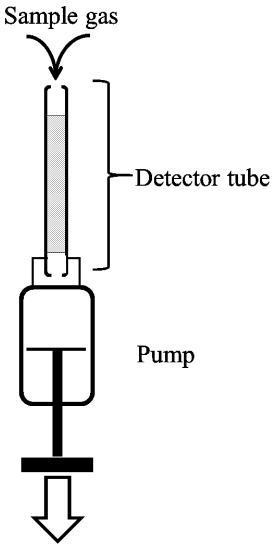

Fig. 1-4 Schematic diagram of operation of a detector tube.

Detector tubes have been used in various fields such as agriculture, oil drilling, construction industry [67], quantification of breath alcohol [69], workplace or environmental monitoring [68, 70-74], light fuel oil [15], and to detect hazardous chemicals in the crime scene, after the accident, or battlefield [8-10, 67, 75].

There are many different types of tubes using variety of chemical reagents, which are specific to analytes, respectively. Thus, particular detector tubes are used taking target substances into consideration. In one of the simplest reagent systems, only one layer with pH reagent is used to detect acidic or basic substance; for example, a tube for the determination of ammonia consists of the granular carrier material coated with an acid and bromophenol blue as a pH indicator [67]. The mixture of iodine pentoxide, sulfuric acid and silica gel is used to detect light mineral oils for a field test at fire-scenes [15]. For detection of CWAs, six types of tubes, phosphoric acid tube, thioether tube, organic arsenic compounds and arsine tube, hydrogen cyanide tube, and cyanogen chloride tube, are available from Dräger (Lübeck, Germany) [76], and are commonly used [8-10, 75]. Results of detection tests using these tubes have been reported by Takayama *et al.* [75].

Detector tube is used as the specific detection method for selected chemicals, and it provides few false positive reaction [8]. This method is easy to use, inexpensive [68] affording a reliable way as an on-site detector [8, 75]. However, due to its selectivity, many kinds of tubes would be required [8], especially when the target compounds are mixtures or unknown.

1.3.10 Other on-site detection methods

Photo ionization detector (PID) is a non-selective detector which can detect chemical substances with lower ionization potential than irradiated UV lamp energy [8]. It can detect vapor of VOCs or CWAs, however, it has no capability to identify or discriminate the chemicals [9, 10].

Flame ionization detector (FID) is also non-selective detector which can detect the vaporous organic substances. It also cannot identify or discriminate the chemicals as PID.

Immunoassay based method is utilized for the detection of biological warfares [10], or for on-site screening test of abused drugs [77]. Many of screening test kits for abused drugs in urine, oral fluid, or hair samples are commercially available [78-82]. Its high selectivity enables to detect low quantities of a target chemical despite the existence of complex matrix, such as proteins. However, some false positive reactions sometimes happen [83].

On-site detection methods described above are summarized in Table 1-4. These methods have characters and they should be selected and used in appropriate scenes.

Table 1-4 Commercially available on-site detectors.

Method	Applicable sample	Specificity (identification potential)	Portavility	Easiness of handling
IMS	volatile	high	high	middle
SAW	volatile	high	high	middle
GC-MS	volatile	very high	low	complecated
Raman spectrometer	solid, liquid, gas	high	middle	middle
IR	solid, liquid, gas	high	middle	middle
FPD	volatile	middle	high	simple
Detector paper	liquid	middle	high	simple
Detector tubes	gas	high	high	differs dipending on target
PID	volatile	low	high	simple
FID	volatile	low	high	simple
Immunoassay	liquid, gas	very high	high	differs dipending on target

1.4 On-site detection method in advance

In this section, some examples of recent researches for on-site detection of chemical substances, other than described above, are briefly described.

1.4.1 Biosensor based detector

Biosensor based detectors based on enzyme inhibition is recently in growing progress [84]. The principle of this type of biosensors is detecting the enzymatic activity of the enzyme, such as butyrylcholinesterase immobilized on the transducer, in the absence or presence of the inhibitor [84].

Since such biosensors based on enzyme inhibition are reliable tools for the detection of a lot of toxic compounds [84], they are applied to the detection of sarin in portable size [85].

1.4.2 Quantum dot based detection method

For a specific detection, quantum dot (QD) based methods have been developed. Luminescent QD conjugated to antibody fragments, based on fluorescence resonance energy transfer (FRET), was applied for the specific detection of the explosive (TNT) [86]. This sensor consists of anti-TNT specific antibody fragments attached to a QD via metal-affinity coordination, which quenches the QD photoluminescence via FRET. Addition of TNT displaces the dye-labeled analogue, eliminating FRET and resulting in a concentration-dependent recovery of QD photoluminescence [86]. Another QD based approach is based on a dual-emissive fluorescent hybrid nanoparticle, where the red-emitting QD is embedded in silica nanoparticles, and the green-emitting QD is covalently linked to the silica surface [87]. The green-emitting QD is functionalized with polyamine which can selectively bind TNT. Bound TNT leads to the green fluorescence quenching, and the fluorescent color changes as illustrated in Fig.1-5 [87].

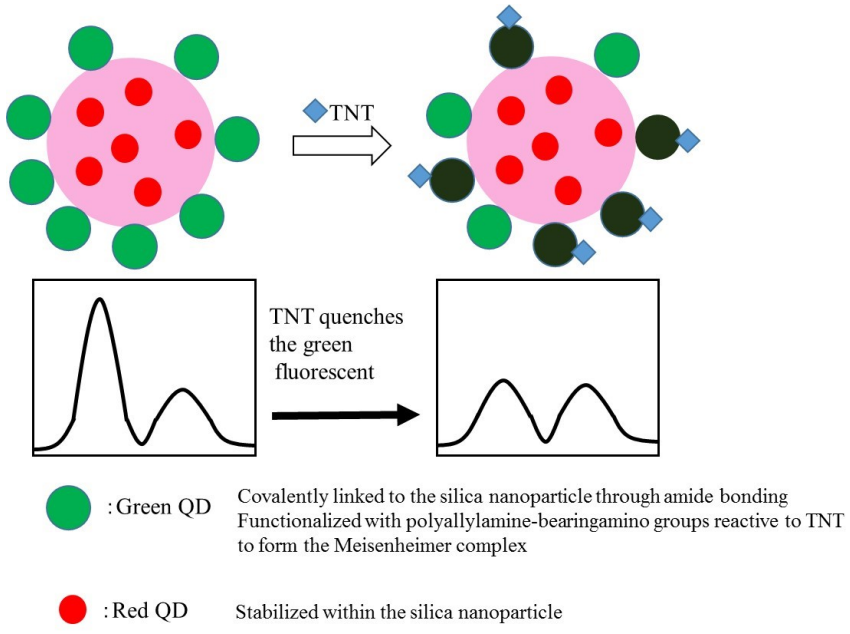

Fig. 1-5 Schematic illustration of a dual-emissive fluorescent hybrid nanoparticles with red and green QDs [87].

1.4.3 Polydiacetylenes based methods

Some colorimetric discrimination methods based on the color change of polydiacetylenes (PDAs) have been developed. PDAs can be easily prepared by the UV irradiation of self-assembled diacetylene (DA) [88]. Four kinds of VOCs, such as chloroform, tetrahydrofuran, ethyl acetate, and hexane, were discriminated by silica-enforced electrospun microfibers embedded with four kinds of PDAs [89]. A litmus-type chemosensor, where polydiacetylene (PDA) and graphene stacked within a composite film, was applicable to sensing and discriminating four kinds of VOCs (tetrahydrofuran, chloroform, methanol, and dimethylformamide) [88].

1.4.4 Colorimetric sensor arrays

As simple and reliable colorimetric sensors with high recognition potential, colorimetric sensor arrays have been developed [90]. The sensor arrays are composed of various kinds of indicators, such as acid-base indicator, vapochromic dyes, metalloporphyrins, which are immobilized independently on one small film as shown in Fig. 1-6. These indicators change their color by exposure to vaporous samples. Color change patterns are scanned, and the digital imaging is evaluated with a hierarchical cluster analysis. The arrays can detect and identify the VOCs [91] or toxic industrial chemicals [92], and the sensitivity can be enhanced by pre-oxidation of sample vapor [93].

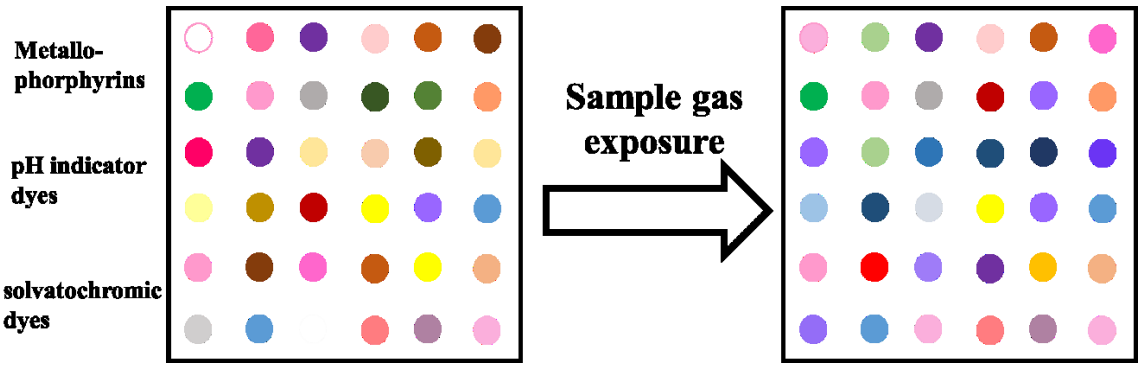

Fig. 1-6 Schematic diagram of colorimetric sensor arrays [91].

1.5 Motivation of this thesis

1.5.1 Problems

As described above, many of on-site detection methods for chemical reagents have been developed and in use. Especially, detecting methods for reagents which give large social and domestic damages such as chemical or biological agents [94], explosives [95], and drugs, are mainly developed. These are very powerful and useful detectors for particular reagents and effective when the types of target reagents are predictable. Selection of a suitable detector is necessary taking into consideration the situation of the field, and this is a key for the protection of people from crimes. On the other hand, if an unexpected chemical reagent is used, these detectors cannot provide any meaningful information.

Sophisticated detectors based on instrumental analysis technique, such as mass spectrometry or photo spectrometry, are expensive, and operators have to receive training before use. These need power supply like battery, and periodic maintenance. Due to these reasons, deployable quantities of these detectors are not so much, and the cases to use these detectors are limited.

Colorimetric detectors such as detector papers and detector tubes are inexpensive and

easy to use. Therefore, these can be used more frequently than instrumental detectors. The advantage of colorimetric methods is simplicity; easy operation, and needlessness of maintenance and power supply. Detector papers possess less discrimination ability, and can give false positive signs, or no information if the reagent other than the target is tested [9, 10]. Detector tubes have high selectivity, however, many different tubes would be required to use in field applications [8].

Crimes such as CWA attack and explosion are caused at low frequency. On the other hand, crimes or accidents related with chemical substances, such as organic solvents or acids, are caused more frequently. In initial phase of these scenes, the related chemical substances tend to be completely unknown. Prediction or identification of unknown chemical substances in early phase is important to keep safe and to make laboratory analyses rapidly. However, on-site testing method, except for partial reagents (such as CWAs, explosives, abused drugs), have paid little attention in forensic science field. Hence, on-site testing methods for unknown samples, which can be used in forensic science field, have not be sufficiently developed today. Much more kinds of on-site testing methods are needed to be developed for better response to various cases.

1.5.2 Aim of this thesis

This research aims the development of novel on-site testing methods of unknown chemical substances that are complementary to existing methods. Presented methods are designed to be utilized in the case that the other on-site tests do not provide any useful information. The methods aim to be simple, inexpensive, easy, rapid, and useful in various scenes. Liquid samples are chosen as target samples since most of existing on-site detectors are designed for vapor samples, and few methods are applicable to liquid samples. As shown in Fig.1-7, simple and widely applicable methods for liquid

samples are focused on in this research.

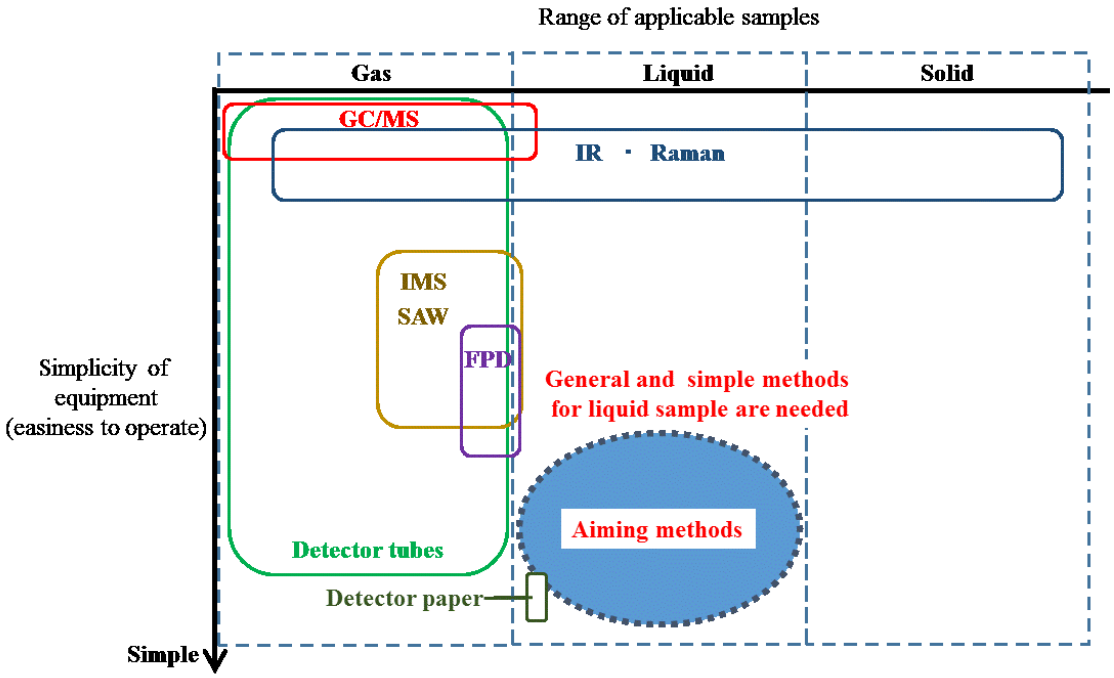

Fig. 1-7 Conceptual diagram of positioning of on-site methods which this research is aiming to.

To satisfy these requirements, colorimetric method is chosen because of its easiness and simplicity in operation, and its portability. Dyes are widely used for many purposes, not only as coloring material. Dyes are used as indicator of nature of liquids. For example, many of dyes such as bromothymol blue, methyl red, have been used as pH indicators. Reichardt's dye [96, 97], nile red [98], 4-nitropyridine *N*-oxide [99], have been used as solvatochromic proves. Dyes such as bromothymol blue and methyl red, are used as indicators of solid surface acidity, too [100]. Cationic dyes, such as methylene blue or brilliant green, are used as ion-association reagents for many analytical applications [101, 102]. In biological studies, oil red O and nile red are used for staining lipids of cell or tissues [103-105]. Some dyes, such is C. I. solvent red 164 and C. I. solvent yellow 124, are used as a marker of fuels to discriminate and to curb

tax avoidance [106, 107].

Various kinds of dyes are available, and dyes have many interesting chemical properties. Dyes are expected to have great potential to be utilized as indicator reagents for aiming methods. For this reason, the color changing nature of dyes is regarded as a key phenomenon and a basic principle for development of novel on-site testing method.

1.6 Outline of thesis

In Chapter 2, development of a rapid and safe method for the field discrimination of various liquid samples is described. Novel pipette was developed for discrimination of liquid samples. The pipette is composed of two dye layers (using two dyes, brilliant green and methyl red) and three colored layers (silica gel, florisil, and polypropylene). When liquid samples were applied to the pipette, various color patterns of the colored layers were observed. The pipette discriminated 8 types of liquid samples, such as water, methanol, ethanol, 2-propanol, acetone, acetonitrile, ethyl acetate and toluene each other.

In Chapter 3, development of novel field sensing method for concentrated acid solutions is described. The sensor is composed of a dye, Oil Red O, and florisil as a support for the dye. When the dye is supported on the florisil surface, its color change properties against the acid solution drastically changes compared to in solution, and the sensor is applicable to sensing for acids with relatively low concentration. The significant phenomenon could not be observed when silica gel was used as a support, suggesting that the florisil plays an important role in the color changing phenomenon. The amount of dye absorbed on the florisil surface is related to the color change properties.

In Chapter 4, investigation of color-changing phenomenon of Sudan III, similar to Oil Red O, in an acetonitrile solution against the addition of concentrated sulfuric acid and

its application for quantification of sulfuric acid is described. Sudan III changes its color from orange to blue against a small volume of sulfuric acid, and the acetonitrile solution of Sudan III is the most suitable for observing the color-change phenomenon. ^{1}H-NMR and UV-Vis spectroscopic studies showed that the color-change mechanism of Sudan III against sulfuric acid is due to the protonation of the dye by sulfuric acid. This phenomenon is applicable to the quantification of concentrated sulfuric acid by introducing the Hammett acidity function. The proposed method requires only a small amount of the sample, 0.04 mL, and enables rapid quantification.

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Chapter 2

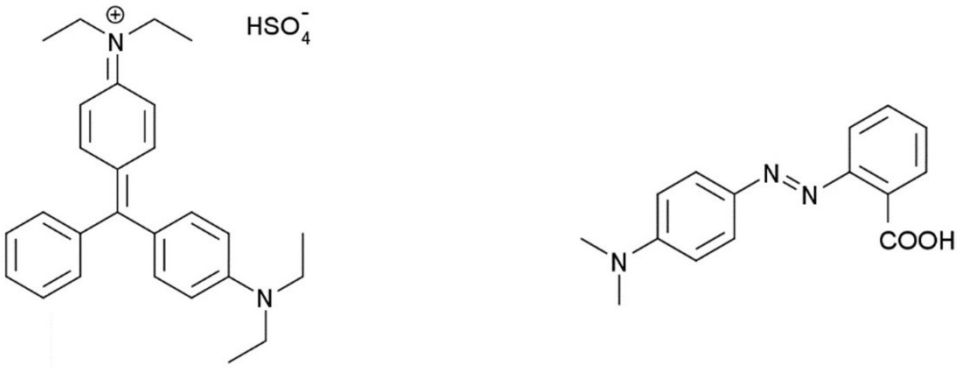
Development of a method for rapid field discrimination of liquid sample using a pipette composed of Brilliant Green and Methyl Red

2.1 Introduction

In the forensic science fields, analyses are conducted for liquid samples with unknown components left on the crime scene or on the public place. Nowadays, unknown samples are first tested using the pH test paper and are collected in the field followed by the instrumental analyses, such as gas chromatograph/mass spectrometer and liquid chromatograph/mass spectrometer, in the laboratory. If some information about the components of unknown liquid samples could be given in the crime fields before the analyses, the safety of the police officer and people around could be ensured, and the most suitable way to collect the samples could be determined. Moreover, the prompt analyses in the laboratory are expected. However, on-site testing method of liquid sample, except for some particular cases where specific chemical warfare agents are used in terrorism for the wrong purpose, can afford only limited information like pH of the liquid. A rapid and easy optical method for the discrimination of various liquid samples is expected to be helpful in the crime scene. However, any method which satisfies these demands has not been developed, and a useful method is strongly required.

In this chapter, the development of a rapid and safe method for the field discrimination of various liquid samples with easy operation is described. In the presented method, two kinds of dyes, Methyl Red (MR) and Brilliant Green (BG), are used as a marker (structures of these dyes are shown in scheme 2-1), and the discrimination mechanism is based on the three principles; 1) the differences of the solvation properties of two dyes against the various liquid samples, 2) the differences of the interaction between

dyes and solid surface with the presence of various liquid samples, 3) the color change properties of the dye when the dye is adsorbed on the solid surface. Utilizing these principles, a novel discrimination tool for unknown liquid samples, called “Pipette for discrimination” have been developed. The pipette shows the color change depending on the samples, and it enables to discriminate the liquid samples by only suctioning the sample into the pipette. It does not require any difficult operation technique, and gives optical information clearly. In this study, eight kinds of liquid samples, including water, methanol, ethanol, 2-propanol, acetone, acetonitrile, ethyl acetate, and toluene are examined as model samples, and it is shown that these eight samples can be discriminated each other by the “Pipette for discrimination”.



Brilliant Green (BG) **Methyl Red (MR)**

Scheme 2-1 Structures of Brilliant Green and Methyl Red.

2.2 Experimental

2.2.1 Reagents and chemicals

The dyes, Brilliant Green (BG) and Methyl Red (MR) were purchased from Wako (Japan) and Tokyo Chemical Industry (Japan), respectively. Silica gel (silica gel 60N,

sphere, neutral) was purchased from Kanto Chemical (Japan), and was used as a support of BG and as a material of colored layer. Molecular sieve (13X) was purchased from GL science (Japan), and was used as a support of MR. For the materials of colored layer, Florisil (purchased from Wako, Japan) and polypropylene fabric were used. Methanol, ethanol, 2-propanol, acetone, acetonitrile, ethyl acetate, and toluene (purchased from Wako, Japan), and Milli-Q water were used as test liquid samples.

2.2.2 Instrumental

Microspectroscopic experiments were performed using the Leica DM2500M microscope equipped with the MSP 800 microspectroscope (J&M Analytik AG, Germany). The reflection of the light from halogen lamp was measured for the samples. Spectra were recorded for the area of 50 mm square using the white florisil without dye as a background.

2.2.3 Procedure

2.2.3.1 Fabrication of "pipette for discrimination"

The powders for dye layers of BG and MR were prepared as follows; 3.0 mg of BG and 2.0 mg of MR were dissolved in about 50 mL of methanol. Silica gel (1.0 g) was added to the BG solution, and 1.0 g of molecular sieve was added to the MR solution. Each suspension was heated with agitating on a water bath (85℃) in order to evaporate methanol.

The overview and schematic diagram of the "pipette for discrimination" are shown in Fig. 2-1. In the tip of the borosilicate glass Pasteur pipette with 146 mm length (purchased from Thermo Fisher Scientific), polypropylene (PP) fabric was filled

followed by filling the 4 mg of BG layer and 7 mg of MR layer. After filling the dye layers, PP was filled again, then 0.015 g of silica gel as the colored layer 1 and 0.05 g of florisil as the colored layer 2 were filled in sequence. Finally, PP fabric as the colored layer 3 was filled.

A rubber ball was attached to the "pipette for discrimination", fabricated as described above, to suction the liquid samples. About 0.5 mL of liquid samples was suctioned during ten seconds.

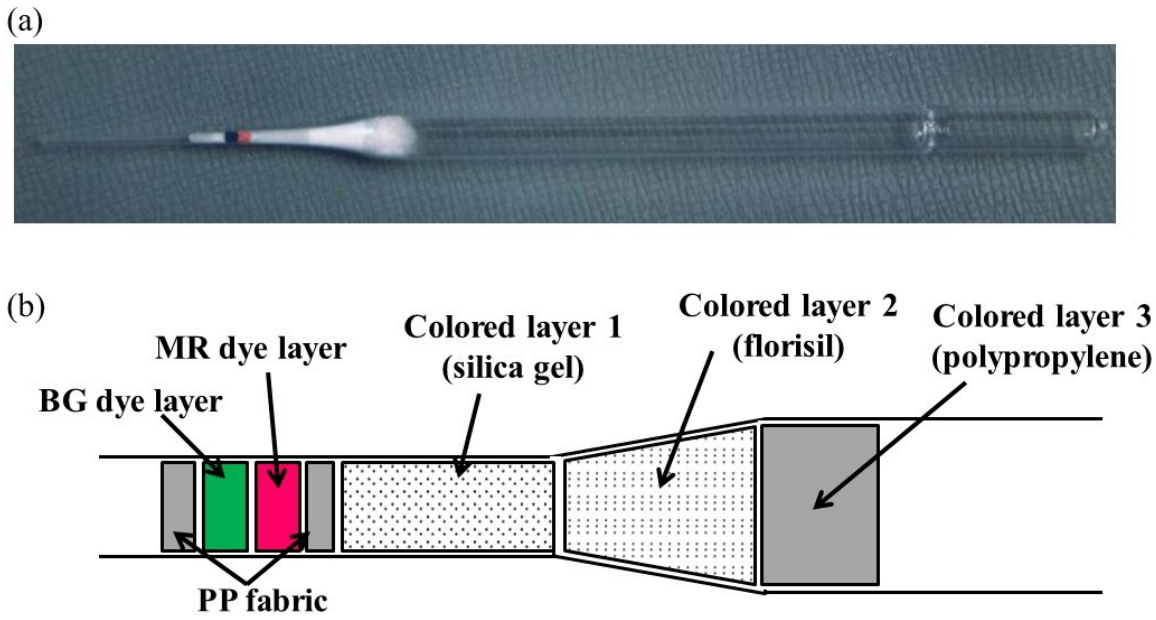


Fig. 2-1 Developed pipette for discrimination
(a) Overview of the pipette.
(b) Schematic diagram of the pipette .BG: brilliant green; MR: methyl red; PP: polypropylene

2.2.3.2 Thin layer chromatography (TLC)

The TLC plate, silica gel 60 plastic plate, was purchased from Merck (Germany). Eight kinds of liquid samples were used as developing solvents. Methanol solution of BG or MR with concentrations of 1.0×10^{-4} M (5 μL) was spotted and developed. The developing length was 8 cm, and the R_f value was determined.

2.3 Results and discussion

By the preliminary examination, the material and layout of each layer in the pipette were optimized to achieve high discrimination performance. The optimized layout, silica gel in front of florisil, as colored layers, showed the best discrimination results. Hence, a pipette for discrimination, filled with silica gel and florisil as the colored layer 1 and the colored layer 2, respectively, was prepared for the following experiments. Experimental results when sample liquids were suctioned into this pipette is shown in Fig. 2-2, and the resulting colors of each colored layer are listed in Table 2-1.

As shown in Fig. 2-2 and Table 2-1, colored layers 1-3 provided different color pattern against each sample, and it is found that obtained patterns made visual discrimination of 8 kind of samples possible.

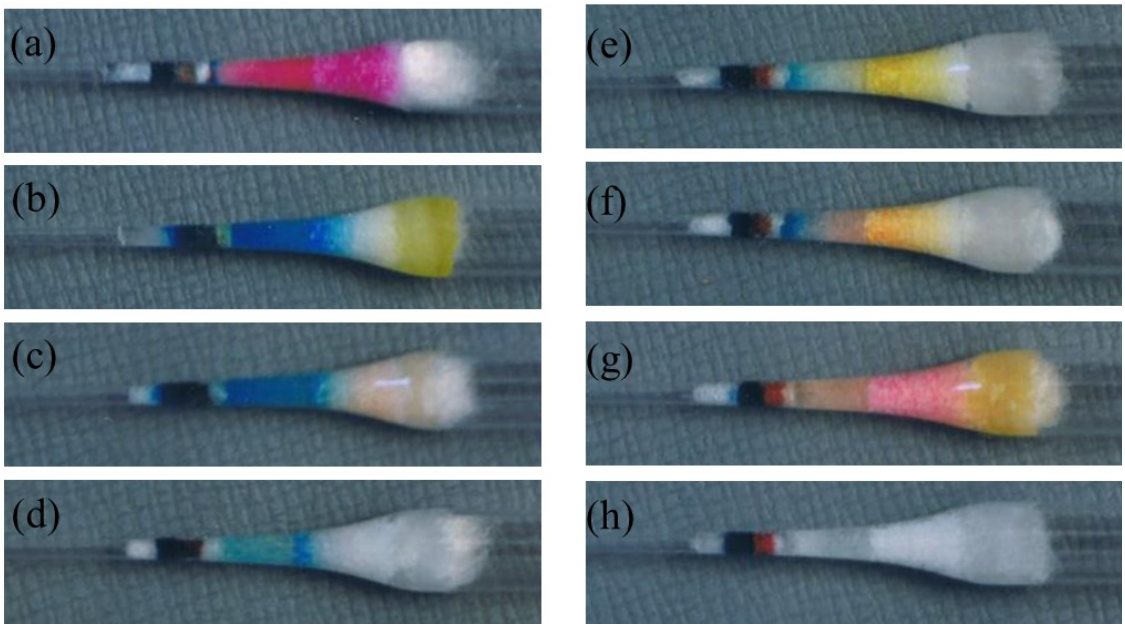


Fig.2-2 Color change of the pipette for discrimination for 8 liquid samples.
(a): Water; (b): Methanol; (c): Ethanol; (d): 2-propanol; (e): Acetone; (f): Acetonitrile; (g): Ethyl acetate; (h) : Toluene

Table 2-1 Results of color change of colored layer 1[a)], colored layer 2[b)], and colored layer 3[c)] in the pipette for discrimination for 8 liquid samples

Liquid sample	Colored layer 1 [a)]	Colored layer 2 [b)]	Colored layer 3 [c)]
Water	Pink (Blue at the front portion)	Pink	White (Colorless)
methanol	Blue	White (Colorless)	Yellow
ethanol	Blue	Light Pink	White (Colorless)
2-propanol	Light Blue	White (Colorless)	White (Colorless)
acetone	Blue at the front portion	Yellow	White (Colorless)
acetonitorile	Blue at the front portion (Light pink at the end portion)	Yellowish Orange	White (Colorless)
ethyl acetate	Pink	Pink	Orange
toluene	White (Colorless)	White (Colorless)	White (Colorless)

a) silica gel; b) florisil; c) polypropylene

2.3.1 Discrimination by difference of solubility of dyes

Colored layers of the proposed pipette are colorized as the result of the dissolution of dyes into sample liquid followed by the transfer to the colored layers. BG possesses tertiary amine and MR includes carboxyl, diazo, tertiary amine moieties. Therefore, solvents with low polarity like toluene show negligible solubility for these dyes. For this reason, no color change of the colored layers was observed against toluene. Consequently toluene could be discriminated from other samples.

2.3.2 Discrimination by interaction between dyes and solid surface

BG is water soluble basic dye [1], and it is strongly adsorbed to silica gel surface. As shown in Fig. 2-2, BG colorized the colored layer 1 blue, and the coloring patterns were different between liquid samples. This result could be explained by the different

interaction between dyes and silica gel surface in the presence of tested liquids, as shown in Fig.2-3.

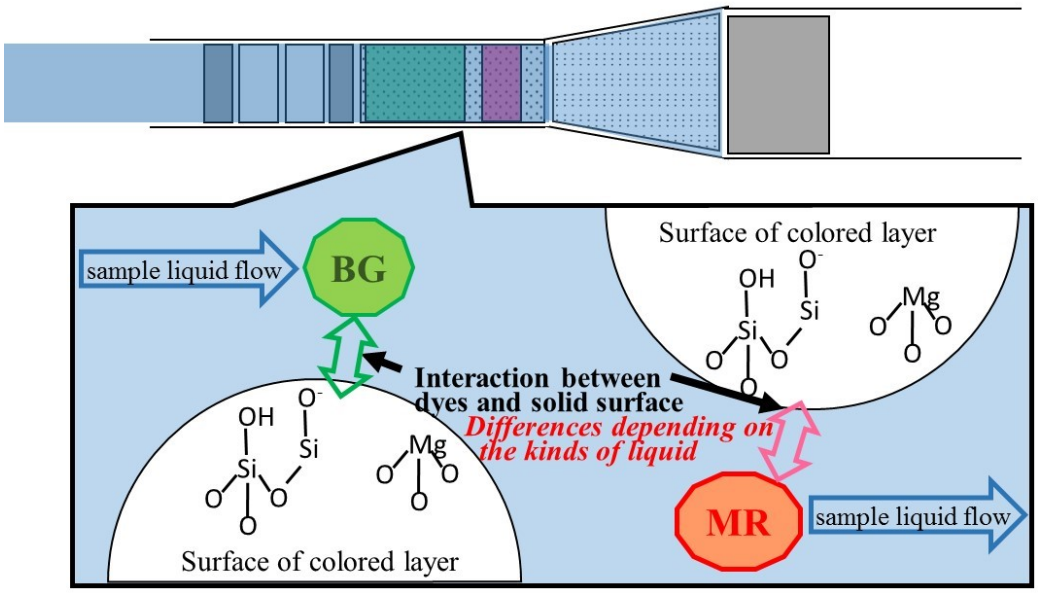


Fig.2-3 Discrimination by interaction between dyes and solid surface

As shown in Table 2-1, the coloring patterns of colored layer brought about by BG can be classified into 4 groups; colored blue only at front portion of colored layer 1 (for water, acetone, and acetonitrile), colored blue over whole part of colored layer 1 (for methanol and ethanol), colored light blue (for 2-propanol), and without coloring by BG (for ethyl acetate and toluene). Eight kinds of liquid samples were classified into 4 groups based on the coloring pattern of colored layer 1 by BG.

The adsorption property of MR was also dependent on the nature of samples. As shown in Table 2-1, within the groups with the same coloring patter by BG (a group including water, acetone, acetonitrile and a group including ethanol, methanol) showed clearly different coloring pattern by MR, and each could be discriminated. In the case of methanol, adsorption of MR was not observed at colored layer 1 and 2. MR reached

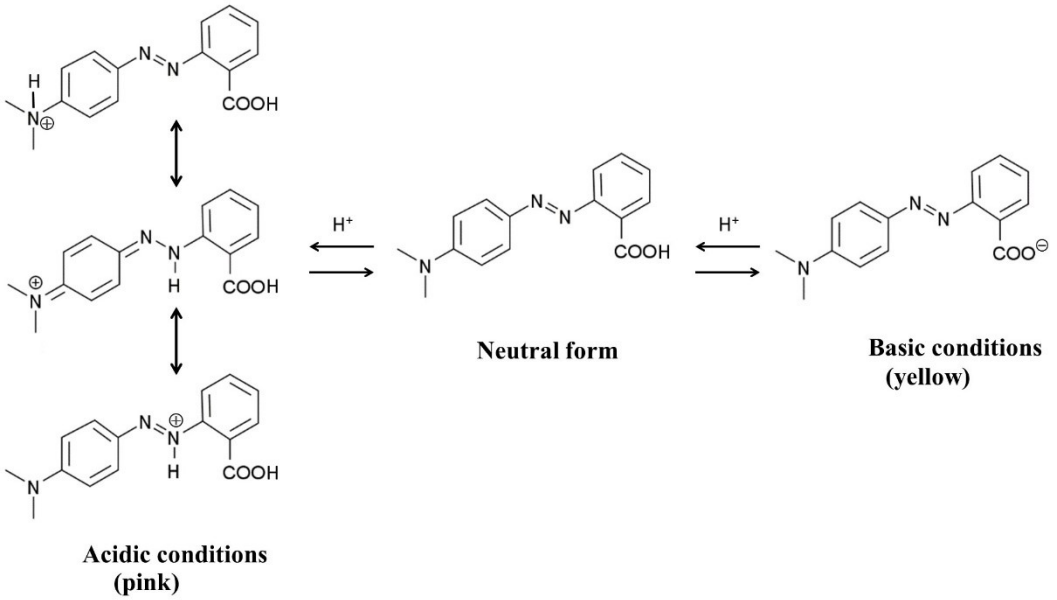
colored layer 3, which was colored by the methanol solution of MR. Ethyl acetate and toluene, categorized to a group where BG did not dissolve, could be discriminated because only ethyl acetate could dissolve MR and colored layers were colored by MR. In this way, the combination of coloring patterns for two dyes made it possible to discriminate all the sample liquids examined.

BG and MR can be separated in the pipette according to their different adsorption properties to colored layers. This difference makes it easy to observe the behavior of each dye, and this separation process contributes to the discrimination ability of the proposed method.

2.3.3 Discrimination by the color change of the dye adsorbed on solid surface

The color of MR is pink in acidic conditions and is yellow in basic conditions. MR is commonly used as a pH indicator for aqueous solution based on this color changing property [2-4]. The structures of MR in acidic and basic conditions are shown in Scheme 2-2. Additionally, MR is also a useful chemical in various applications utilizing its color changing mechanism. For example, it is utilized as a molecular recognition indicator [5], and for the measurement of solid surface acidity [6]. At the colored layer 2, MR showed different color between sample liquids, as shown in Table 2-1. For water, for example, MR showed pink, while for acetone, it showed yellow. This difference is considered to be due to the difference of adsorption point on the florisil surface; the adsorption point of MR was dependent on the characteristics of sample liquids. To investigate the contribution of sample liquids, the following experiment was conducted. Florisil was suspended in acetone solution of MR, and acetone was gradually dried accompanied with observation of the surface color of florisil. In the presence of acetone, florisil surface was yellow. With the removal of acetone, the color of florisil surface

changed to pink.



Scheme 2-2 Structures of MR in acidic and basic conditions

MR adsorbed florisil surface was evaluated using microscopic spectrometer for three conditions; dry, water containing, and acetone containing conditions. As shown in Fig. 2-4, spectra for dry (a) and water containing (b) conditions were similar each other, but in presence of acetone, a spectrum was different from former two spectra. From these examinations, it is considered that on dry and water containing conditions, MR adsorbs preferentially onto acid sites and MR colored pink [7]. In the presence of acetone, inversely, acetone can preferentially adsorb onto acid sites because of its electron donating property [8]. MR cannot adsorb onto the acidic site, resulting in yellow color, as shown in Fig.2-5. In this mechanism, adsorption site of MR on florisil surface differs between sample liquids. This phenomenon based on the nature of sample liquids contributes to discrimination of samples.

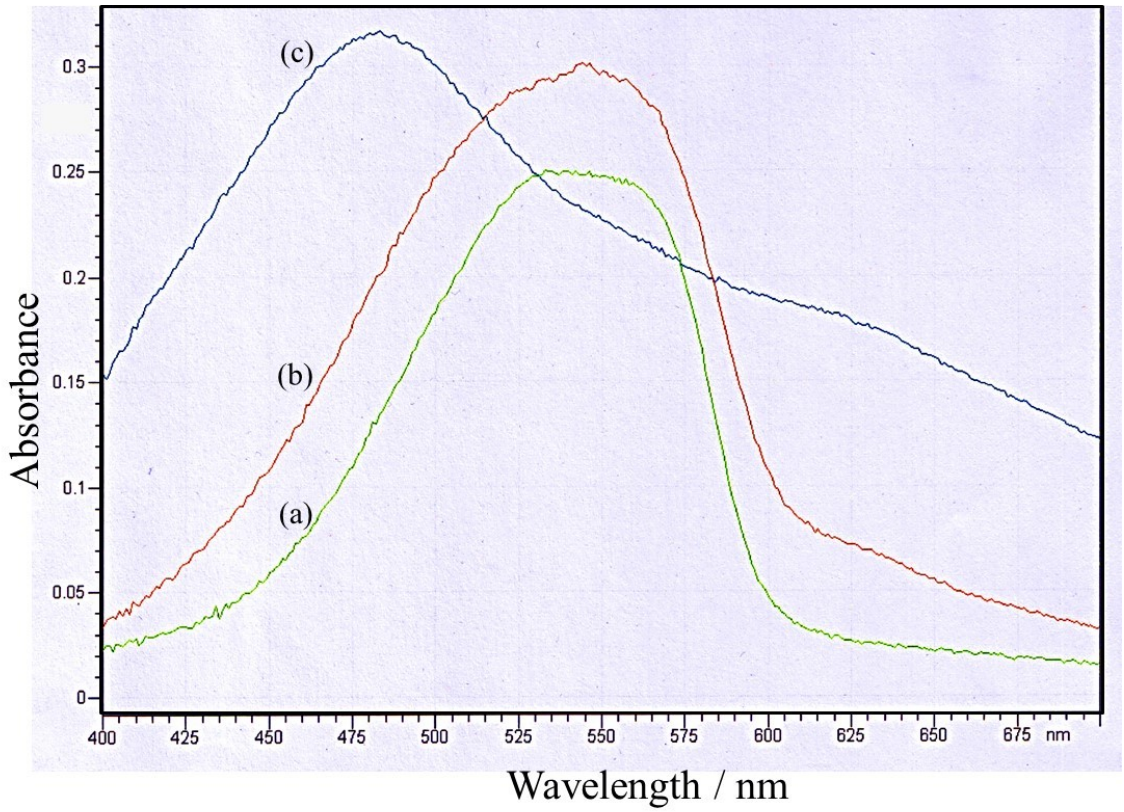


Fig. 2-4 Absorption spectra of MRon florisil surface; measured in the absence of solvent (a), and in the presence of water (b), and acetone (c)

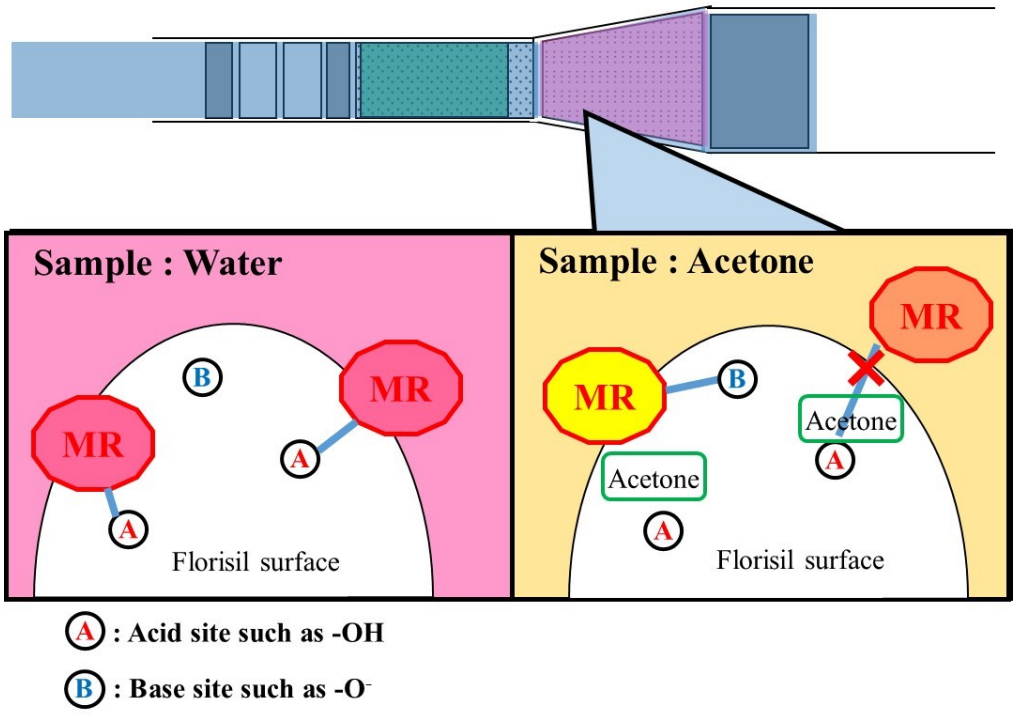


Fig. 2-5 Mechanism of color change of MR on florisil surface.

2.3.4 Comparison to thin-layer chromatography

Coloring behavior of dyes in the presented pipette was compared with the developed profile in thin-layer chromatography (TLC). Thin-layer chromatograms of BG and MR were obtained using each sample liquid as an eluent. To evaluate the coloring behavior of dyes in pipette, *D* value was defined as follows;

$D = l/L$.

Here, *L* is the whole length of the colored layers 1-3, and *l* is length of colored part with dyes from the tip of the colored layer 1. The value l_{BG} and l_{MR}, the length of colored part for BG and MR, respectively, were measured, and *D* value for each dye, D_{BG} and D_{MR}, was determined as shown in Fg.2-6.

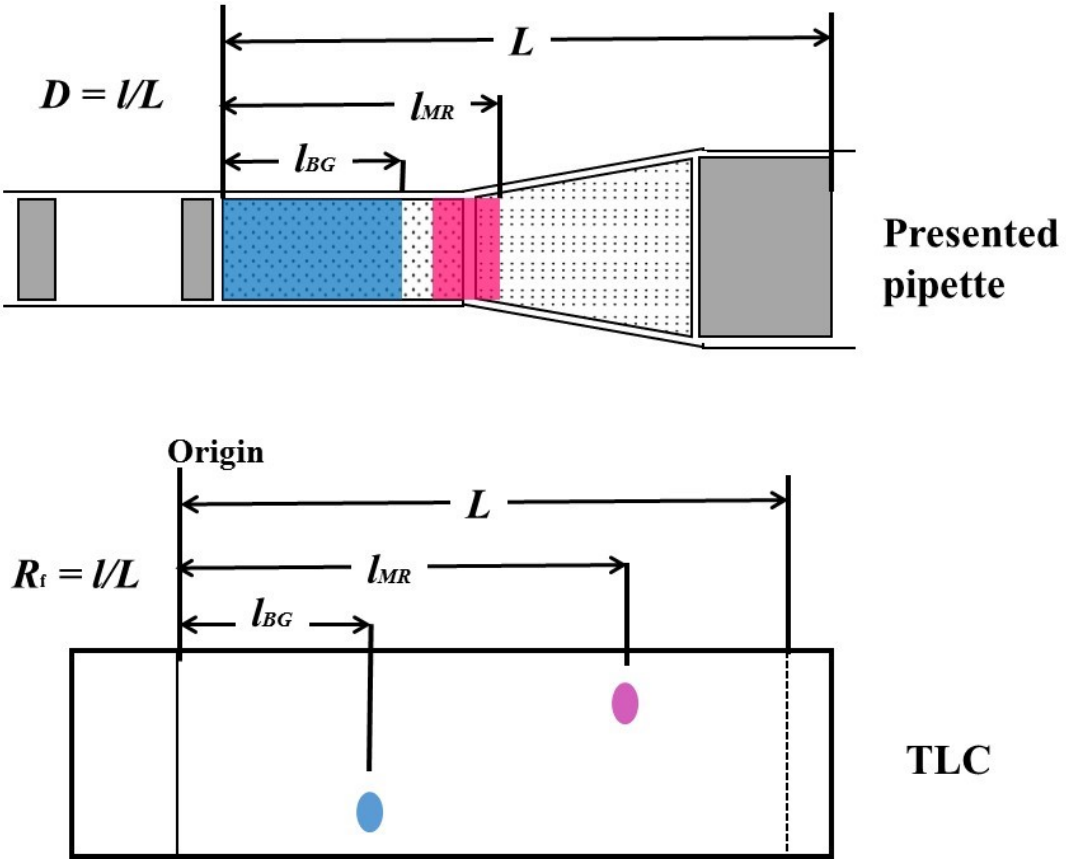


Fig. 2-6 Definitions of the *D* value and R_f value.

For each liquid sample, relationships between D values and R_f values obtained from TLC, are shown in Fig. 2-7. There is positive correlation for BG (R^2=0.885), but there is no correlation for MR.

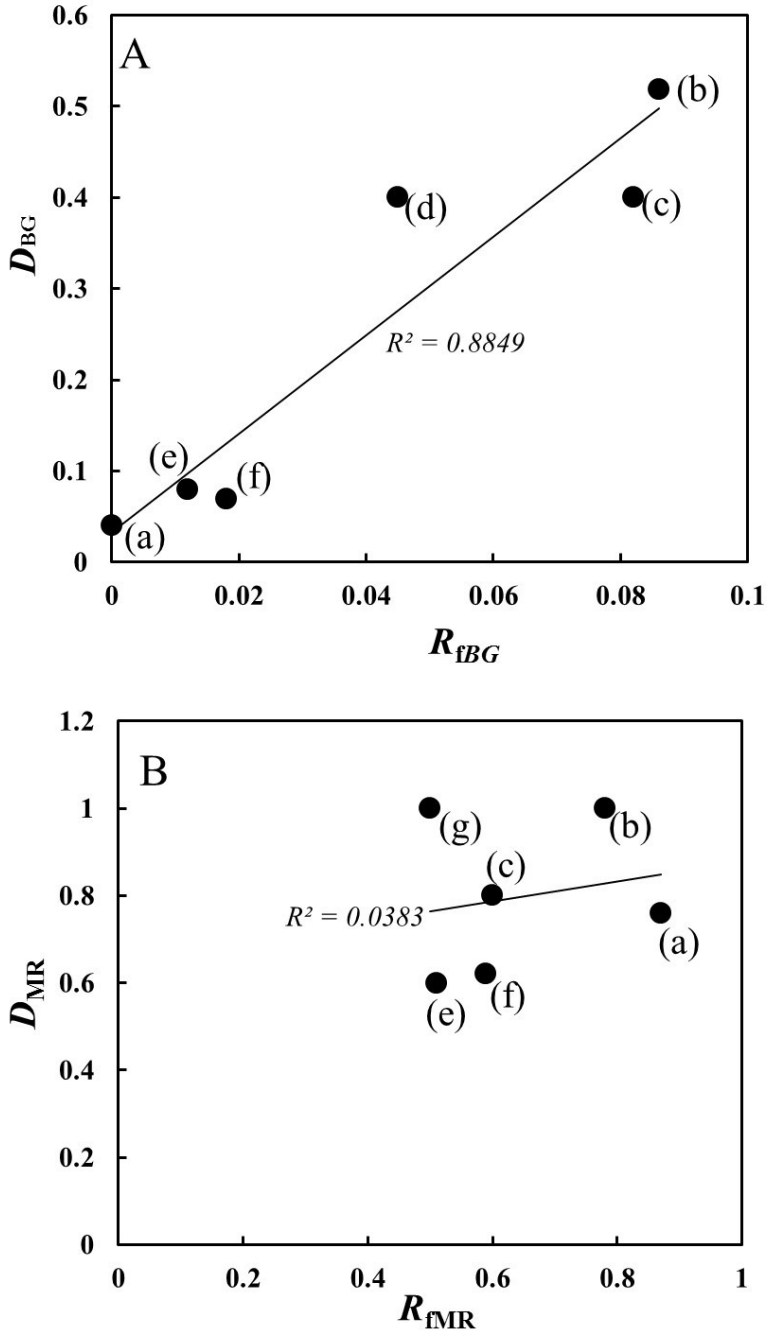


Fig. 2-7 R_f vs D plot for BG (A) and MR (B).
(a): water; (b): methanol; (c): ethanol; (d): 2-propanol [1]; (e): acetone; (f): acetonitrile; (g): ethyl acetate. (g) in A and (d) in B are not shown because their D values were not measured.

In TLC analyses, R_{fBG} are smaller than R_{fMR}. In pipette experiments, the color of BG was observed at the front potion. BG adsorbs strongly onto silica gel and coloration by BG occurred mainly at the colored layer 1 (silica gel) in the pipette. The correlation between the results for BG in the pipette, and TLC experiments is probably due to the fact that the adsorbent material in TLC is silica gel. This correlation leads to the conclusion that the effect of sample liquid on the interaction between dye and solid surface contributes to coloring behavior of BG in pipette. On the other hand, the interaction between MR and silica gel is relatively weak. In the pipette, MR reaches directly florisil or PP for some sample liquids. Therefore, it is reasonable that the results from the pipette experiments show no correlation with TLC data, since there is large difference in conditions.

2.4 Conclusion

A novel and simple discrimination method of liquid sample is developed. The only aspiration of the sample into the pipette enables visual discrimination of samples clearly within 10 seconds. The proposed method can be considered as a rapid and easy on-site discrimination method for liquid samples.

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Chapter 3

Development of an optical sensing method for acid solution using Oil Red O supported by florisil

3.1 Introduction

Unknown liquid samples are often left in public places or at crime scenes. To presume the hazardous nature of these liquid samples is important for police officers and people nearby to ensure their safety. Concentrated acid solutions are especially hazardous materials and frequently used in crimes. For this reason, a rapid, safe and inexpensive field sensing method for concentrated acid solutions is needed. Although various kinds of optical sensors for low-concentration acid samples such as pH indicators have been developed [1-3], a sensor that is applicable to high-concentration acid samples has yet to be developed.

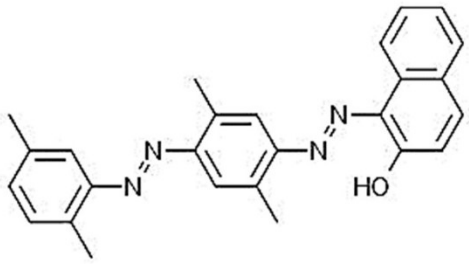
For field optical sensing, utilization of dyes has several advantages, such as simplicity and easy handling. In chapter 2, we have established a pipette-based optical discrimination method using dyes and solid substances that can discriminate some organic solvents [4]. In screening studies for the development of novel field sensing methods, 1-[[4-[(2,5-dimethylphenyl)azo]-2,5-dimethylphenyl]azo]naphthalen-2-ol, called Oil Red O (ORO, Scheme 3-1), changes its color against acids with extremely high concentration, and the color changing behavior is modified when the dye is supported by a florisil surface.

ORO is mainly used in biological studies to stain neutral lipids and cholesteryl esters in cells [5]. Color change phenomena of azo dyes are widely known, such as azo and hydrazone tautomeric equilibriums [6-9]. To our knowledge, however, an application of

ORO as an indicator of the nature of liquids has not been developed.

Florisil is a coprecipitate of silica and magnesia, which is used as a polar absorbent in column chromatography and thin-layer chromatography. It is also used in the preparation of samples, such as biological, environmental, and pharmaceutical before chromatographic analysis [10, 11].

In this chapter, the author presents a novel optical indicator for concentrated acid solutions utilizing the color change phenomena of ORO supported by florisil. The color change behavior of ORO is modified by adsorbing it on the florisil surface, which leads to the extension of the applicable concentration range.



Scheme 3-1 Structure of Oil Red O.

3.2 Experimental

3.2.1 Reagents and chemicals

Oil Red O, ethanol, formic acid, sulfuric acid, silica gel and florisil (60 - 100 mesh) were purchased from Wako (Japan), and alumina (Activated, Neutral, Activity: I) was purchased from MP Biomedicals (USA). All reagents were used as received.

For measurement of absorption spectra of ORO in solution, the stock ethanol solution of ORO with concentration of 1.0 mg/10 mL was prepared. Then, 200 μL of dye solution and 2 mL of acid solution were mixed followed by UV-Vis measurement.

The ORO-florisil, ORO-silica gel and ORO-alumina samples were prepared as follows. Ethanol solution of ORO with concentration of 1.0 mg / 100 mL was prepared as a stock solution. To the ORO stock solutions (2.04, 6.12, 10.2 and 20.4 mL) was added 1 g of adsorbents. The suspensions were heated at about 363 K in a water bath to evaporate ethanol and ORO-florisil, ORO-silica gel and ORO-alumina samples obtained. For ORO-florisil samples, the ratios of ORO to florisil were 0.5, 1.5, 2.5 and 5.0×10^{-4} mol/g. For the ORO-silica gel and ORO-alumina sample, the ratio of ORO to each support materials was 2.5×10^{-4} mol/g.

3.2.2 Apparatus

Absorption spectra were measured with a JASCO V-670 spectrophotometer (Jasco Corp., Japan). Diffuse reflectance spectra were measured with a JASCO V-670 spectrophotometer (Jasco Corp., Japan) equipped with a JASCO ISN-470 integrating sphere system (Jasco Corp., Japan).

3.2.3 Procedure

Absorption spectra of the dye solution were obtained against distilled water as background. For visual observation, 0.1 mg of ORO-florisil or ORO-silica gel sample was put on PARAFILM®, and acid solution was dropped on it. For measuring diffuse reflectance spectra, acid solutions were added to 0.1 mg of ORO-florisil or ORO-silica gel samples in a powder sample holder. Diffuse reflectance spectra of ORO-florisil or ORO-silica gel samples with acid solutions were obtained against ORO free florisil or silica gel as background. The diffuse reflectance spectra were transformed according to the Kubelka-Munk model. The diffuse reflectance spectra can be expressed as the Kubelka-Munk function, *K/S*, by the following equation [12-14]:

$K/S = (1\text{-}R)^2/2R$

The K and S values are called the adsorption and scattering coefficients, respectively. R is the reflectance of the sample over a background reflectance.

3.3 Results and discussion

First, UV-Vis spectra of ORO in acid solutions were measured. Figure 3-1 shows UV-Vis spectra of ORO in various concentrations of sulfuric acid solutions. When the dye was added to water (0 mol/L of sulfuric acid), the absorbance is low since ORO was not completely dissolved. But except for its absorbance, almost the same spectral profile as the spectra of ORO in 1 - 4 mol/L of sulfuric acid was obtained. For more than 5 mol/L of sulfuric acid, the absorption maximum shifted from 510 to 605 nm with an increase in sulfuric acid concentration.

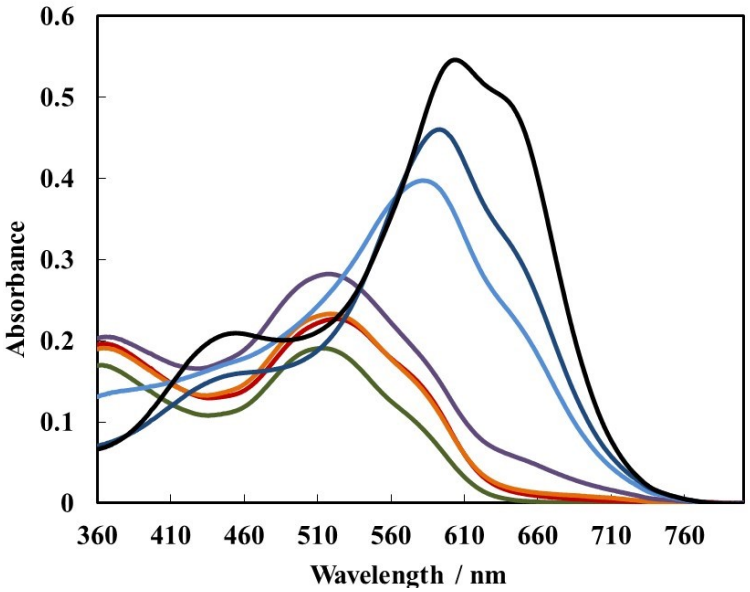


Fig. 3-1 UV-Vis spectra of ORO dissolved in various concentrations of sulfuric acid. Green line for 0 mol/L of sulfuric acid; red line for 2 mol/L of sulfuric acid; orange line for 4 mol/L of sulfuric acid; purple line for 5 mol/L of sulfuric acid; light blue line for 6 mol/L of sulfuric acid; blue line for 7 mol/L of sulfuric acid; black line for 8 mol/L of sulfuric acid.

When ORO was supported on a florisil surface, the color change behavior differed from in solution. Against 0 - 1 mol/L of sulfuric acid, the color of the ORO-florisil sample changed from pink to bluish purple. As shown in Fig. 3-2, the color change was distinguished by visual observation in the concentration range lower than 0.1 mol/L of sulfuric acid.

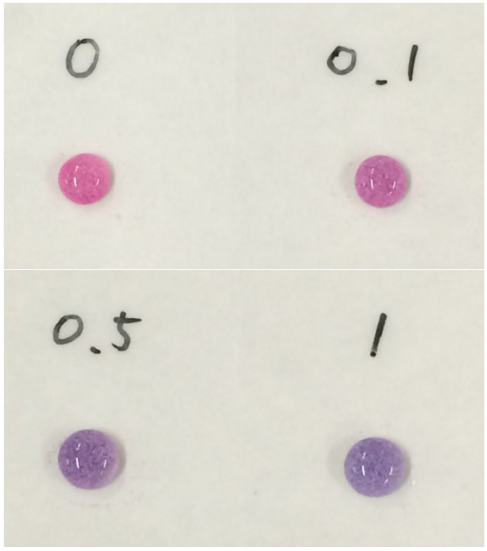


Fig. 3-2 Photograph of ORO-florisil samples in the presence of 0 ~ 1 mol/L of sulfuric acid solutions. Numbers attached on each sample indicate the concentration of sulfuric acid (mol/L).

Figure 3-3 shows UV-Vis spectra of ORO-florisil samples in the range between 0 and 1 mol/L of sulfuric acid. The absorption at 545 nm was diminished, while the absorption at 655 nm was enhanced with the increase of the sulfuric acid concentration. It is noteworthy that the color of dye changes against a far lower sulfuric acid concentration than in solution. Moreover, the shapes of spectra against sulfuric acid solutions are different from those in Fig. 3-1.

For more concentrated sulfuric acid, the ORO-florisil sample showed a clearly distinguishable color change as shown in Fig. 3-4.

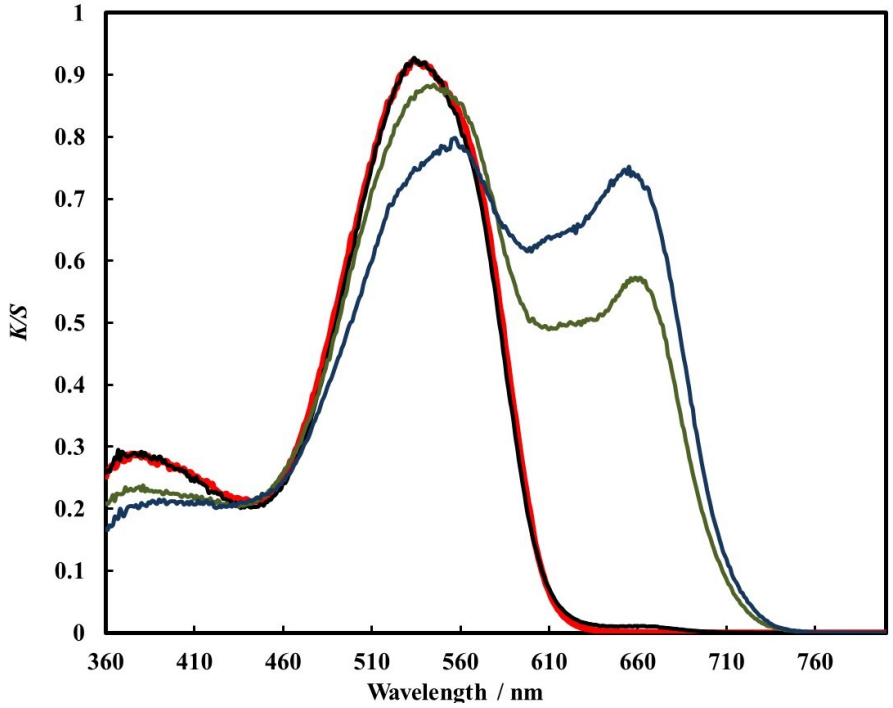


Fig. 3-3 UV-Vis spectra of ORO – florisil sample against various concentrations of sulfuric acid. Red line for 0 mol/L of sulfuric acid; black line for 0.1 mol/L of sulfuric acid; green line for 0.5 mol/L of sulfuric acid; blue line for 1 mol/L of sulfuric acid.

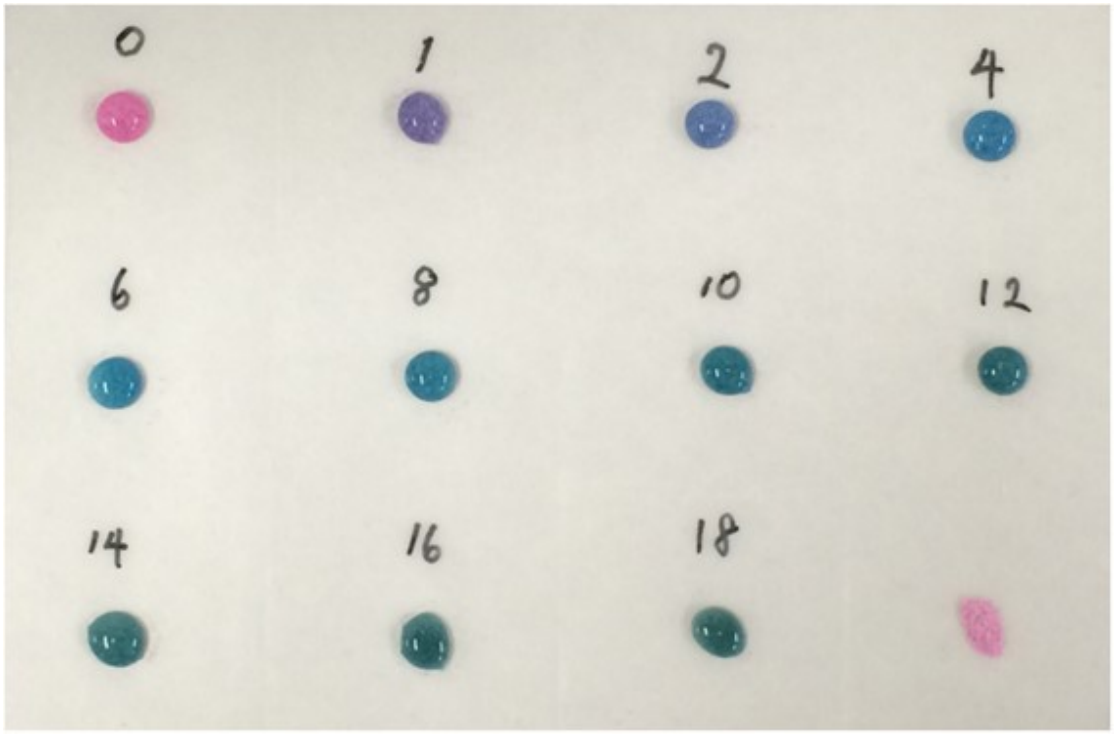


Fig. 3-4 Photograph of ORO-florisil samples in the presence of 0 ~ 18 mol/L of sulfuric acid solutions. Numbers attached on each sample indicate the concentration of sulfuric acid (mol/L). Right end of bottom column is ORO-florisil sample without the solution.

The color of ORO-florisil gradually turned from pink (against water) to a bluish color (1 – 4 mol/L) and then turned to a greenish color (4 – 10 mol/L). Against over 12 mol/L of sulfuric acid, the ORO-florisil sample showed a dark green color. The colors were independent of the volume of acid solution as long as the ORO-florisil sample was fully soaked. These findings can be applied to optical acid sensing, which can estimate the approximate concentration of sulfuric acid for an extremely wide concentration range. This interesting phenomenon may be attributed to the characteristics of the dye adsorbed on the florisil surface. In order to evaluate the specific properties of florisil, silica gel was used as a support of ORO, in place of florisil. In the case of the ORO-silica gel sample, as shown in Fig. 3-5, a color change against sulfuric acid was not observed even at 2 mol/L. Moreover, the alumina support showed the same color change pattern as the silica gel (Fig. 3-6).

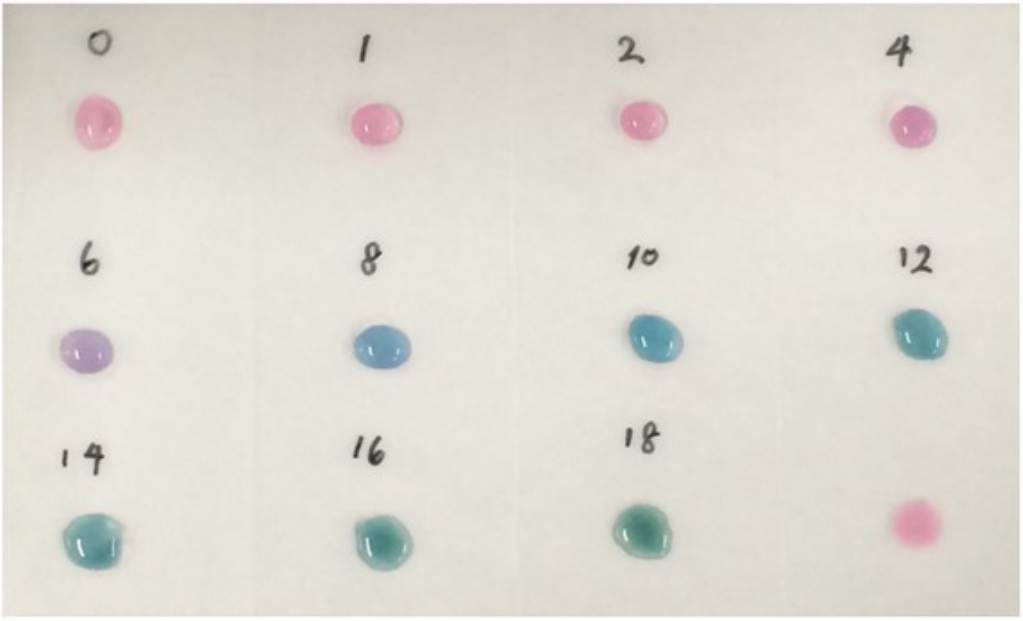


Fig. 3-5 Photograph of ORO-silica gel samples in the presence of 0 ~ 18 mol/L of sulfuric acid solutions. Numbers attached on each sample indicate the concentration of sulfuric acid (mol/L). Right end of bottom column is ORO-silica gel sample without the solution.

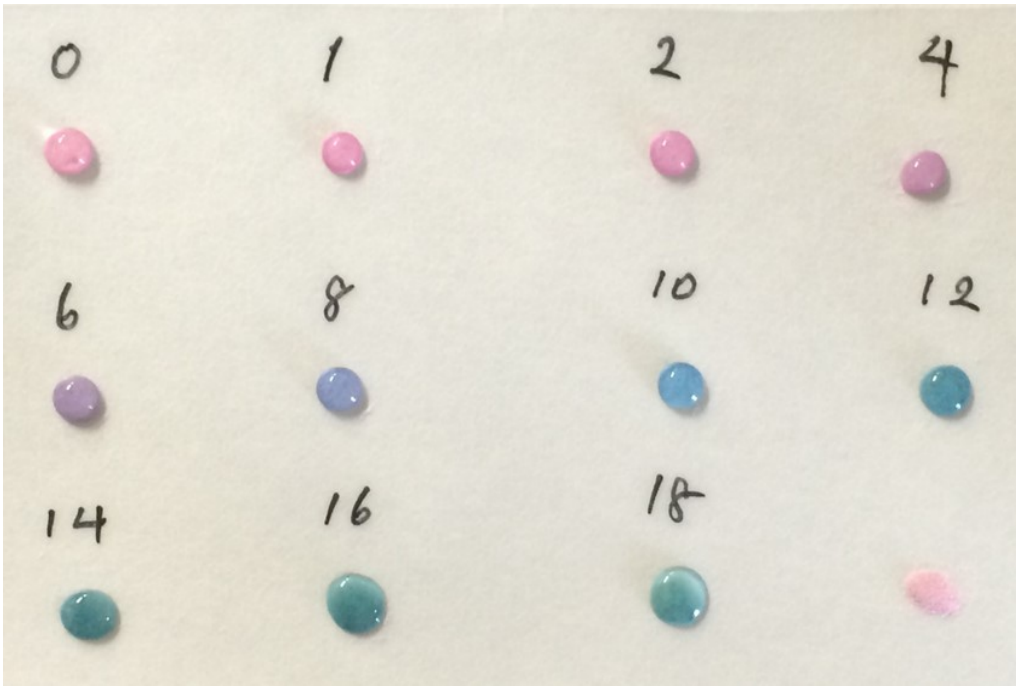


Fig. 3-6 Photograph of ORO-alumina samples in the presence of 0 ~ 18 mol/L of sulfuric acid solutions. Numbers attached on each sample indicate the concentration of sulfuric acid (mol/L). Right end of bottom column is ORO-alumina sample without the solution.

ORO changes its chromism characteristic when it is adsorbed on a florisil surface, especially in low concentration region. This result suggests that some specific sites on the florisil surface contribute to the color change behavior of ORO.

It is well-known that Lewis acid sites, Mg(II) ions, are present and they play a key role for the adsorption of compounds that have basic electron donating groups [10]. ORO includes electron donating azo groups and these functional groups may dominantly be attached to Lewis acid sites. Adsorbing to Lewis acid sites can lead to the modification of the color change property of ORO.

To elucidate the effect of the amount of ORO on the florisil surface, UV-Vis spectra for various ORO-florisil samples against 0 – 1 mol/L of sulfuric acid were measured. In this concentration range, the color change is observed only when ORO is supported by florisil. Figure 3-7 shows the dependence of the ratios of *K/S* values at 655 to 545 nm

(K/S_{655}/ K/S_{545}) on the concentrations of sulfuric acid for each ORO-florisil sample. The fact that the lower the amount of ORO on the florisil, the higher the value of K/S_{655}/ K/S_{545}, was observed when the same concentration of sulfuric acid solution was added. This phenomenon leads to the following consideration: ORO is preferentially adsorbed on the Lewis acid site and its color changes against sulfuric acid. As the amount of dye increases, the ratio of the dye adsorbed on the other sites, where their color does not change against sulfuric acid, increases. This situation results in the lower K/S_{655}/ K/S_{545} values.

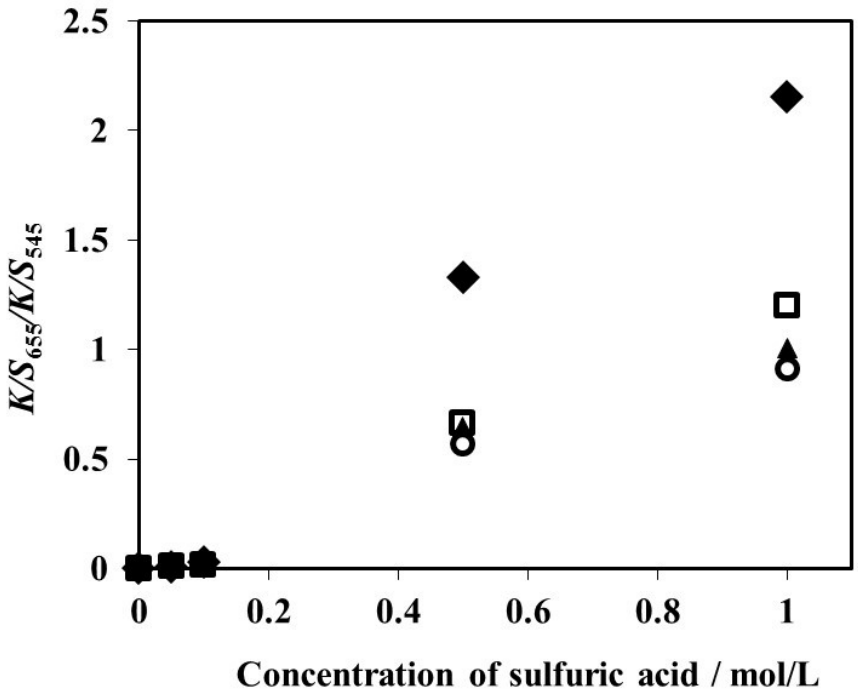


Fig. 3-7 Dependence of the ratios of K/S_{655}/ K/S_{545}values on concentrations of sulfuric acid for each ORO-florisil sample ; 0.5 (◆), 1.5 (□), 2.5 (▲), 5.0 (○)×10^{-4} mol/g respectively.

The effect of type of acid was preliminarily investigated. For nitric acid, hydrochloric acid, phosphoric acid and formic acid, the ORO-florisil sample showed a chromism characteristic as in the case of sulfuric acid even when the concentration of each acid

solution was 1 mol/L, as shown in Fig. 3-8. On the other hand, acetic acid did not alter the color of the ORO-florisil sample regardless of the level of concentration.

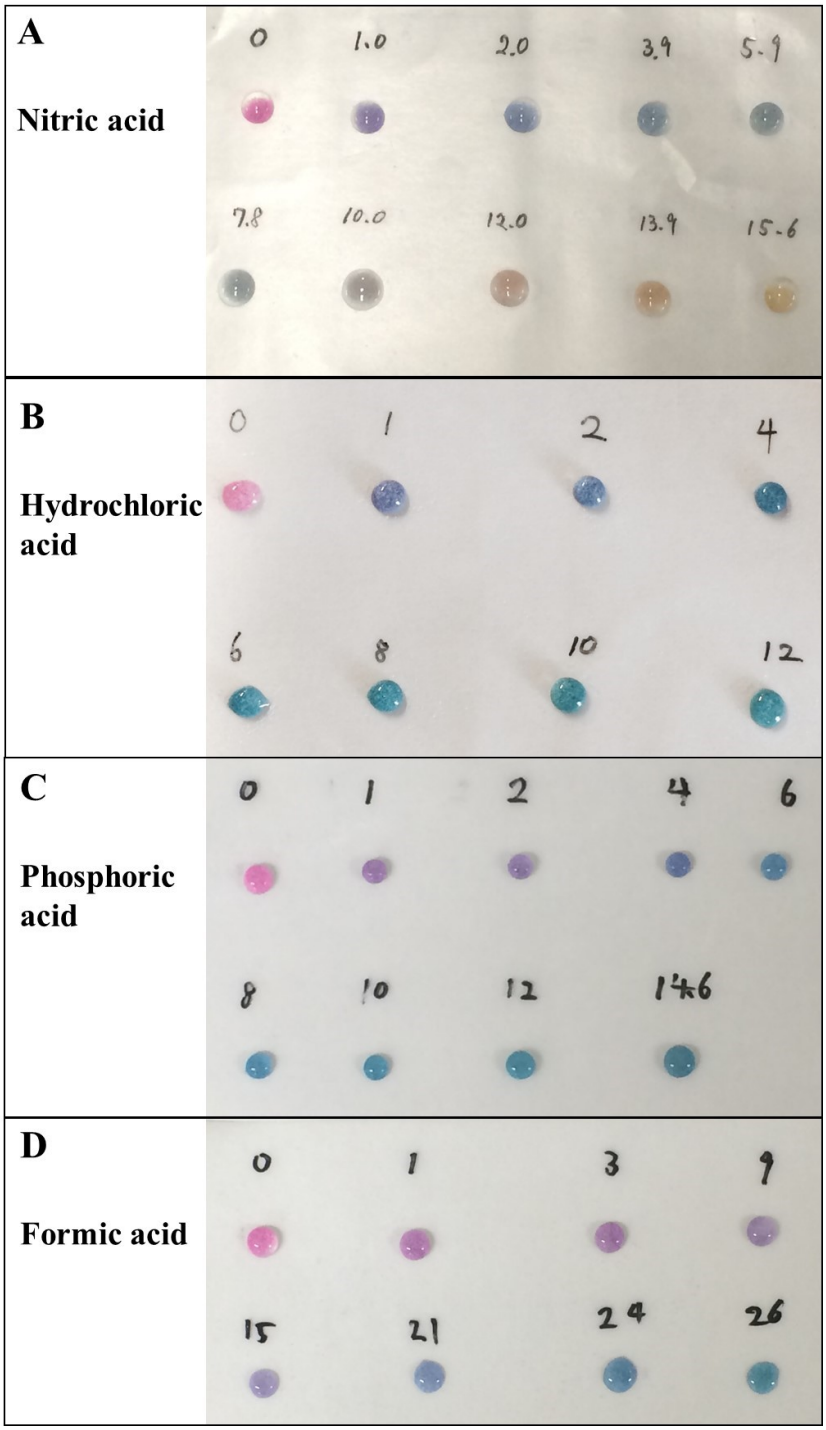


Fig. 3-8 Photographs of ORO-florisil samples in the presence of various concentrations of acid samples. A : Nitric acid; B : Hydrochloric acid; C : Phosphoric acid; D : Formic acid. Numbers attached on each sample indicate the concentration of acid samples (mol/L).

3.4 Conclusion

In this chapter, characteristic color change phenomena of ORO supported by florisil were discovered, and its utilization for a novel optical indicator for concentrated acid solutions was described. It was found that ORO changes its color against concentrated sulfuric acid. The color change behavior of ORO was modified when it was supported by florisil, which leads to the extension of the applicable concentration range. These findings can be utilized for an optical sensing method of sulfuric acid. Consequently, it enabled us to estimate the approximate concentration of sulfuric acid in an extremely wide concentration range. Although additional research is needed to investigate the origin of the color change mechanism of ORO-florisil samples, the proposed method is applicable to sensing of high concentration acid solutions for which comercially available indicators are not suitable.

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Chapter 4

Investigation of color change property of Sudan III against concentrated sulfuric acid in acetonitrile and its quantification

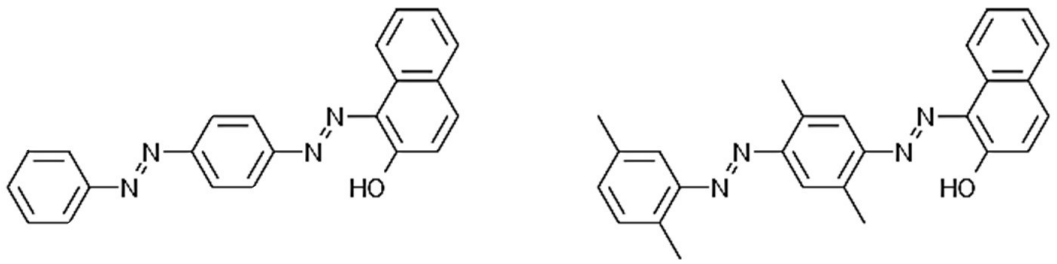
4.1 Introduction

Concentrated sulfuric acid is widely used in chemical laboratories and industrial plants. In industry, it is used in a variety of fields, such as catalysts for the production of high-octane-number trimethylheptanes [1] and biodiesel [2, 3] and solvents for the production of fibers with high mechanical strength [4], for example. On the other hand, concentrated sulfuric acid is an extremely hazardous chemical, and it sometimes causes fatal injuries [5]. In the criminal fields, it is abused in some assaults [5], clandestine methamphetamine manufacture [6], or in the production of counterfeit gas oils in Japan [7, 8]. Although the titration method has been generally utilized for the quantification of concentrated sulfuric acid, it is not suited to high-throughput analyses because it is time-consuming and requires a special technique and apparatus. In criminal fields, there are difficulties in analyzing sulfuric acid: 1) rapidity is required; 2) the amount of available sample is usually small; 3) the sample concentration is unpredictable before analysis. Therefore, a rapid and simple analytical method for the quantification of small amounts of concentrated sulfuric acid is strongly desired in forensic analyses, and it will also be helpful in industrial fields for quality checks. To date, however, an efficient method has not yet been developed.

Some sensing methods for concentrated acids using the color change of dyes have already been reported. For example, Wang *et al*. investigated acid-sensing applications of 9-(cycloheptatrienylidene)fluorine in the range of 0 - 4 mol/L [9]. Chapter 3 described on an optical sensing method using Oil Red O supported by florisil, which enables one

to predict the approximate concentration of sulfuric acid in the range of 0.5 - 12 mol/L by visual observation [10]. In our previous method, however, it is difficult to discriminate concentrations over 12 mol/L; also, the mechanism for the color change of Oil Red O has not been understood.

A hydrophobic bisazo dye, Sudan III (C.I. solvent red 23), has a simpler structure than does Oil Red O (scheme 4-1). We found that these dyes show similar color-change properties against concentrated sulfuric acid. Because of the presence of four methyl groups, Oil Red O has isomeric structures, which leads to difficulties in the elucidation of its color-change mechanism. Due to its structural simplicity, the author chooses Sudan III to investigate the color-change mechanism. Sudan III is known as a kind of Sudan dye, which is mainly used for staining lipids or the coloring of waxes, oils, cosmetics and foods [11-14]. In this chapter, the color-change behavior of Sudan III against sulfuric acid in acetonitrile was investigated. UV-Vis and ^{1}H-NMR spectrometries provided information about the color-change mechanism. Using this color-change phenomenon, the author proposes a new method for the quantification of a small amount of concentrated sulfuric acid. The method requires only a small amount of the sample, 0.04 mL, and enables rapid quantification.



Sudan III　　Oil Red O

Scheme 4-1 Structures of Sudan III and Oil Red O.

4.2 Experimental

4.2.1 Reagents and chemicals

Sudan III, organic solvents (acetonitrile methanol, ethanol, 2-propanol, acetone, dimethyl sulfoxide), acids (sulfuric acid, hydrochloric acid, hydrofluoric acid, nitric acid, phosphoric acid, formic acid and acetic acid) were purchased from Wako (Japan). Sodium hydroxide was purchased from Kanto Kagaku (Japan). For ^{1}H-NMR measurements, deuterated acetonitrile and tetramethylsilane purchased from Wako (Japan) were used as a solvent and an internal reference, respectively. All reagents were used as received. For quantification, the concentration of sulfuric acid was determined to be 17.99 mol/L by titration using a 0.54 mol/L of NaOH aqueous solution.

4.2.2 Apparatus

^{1}H-NMR spectra were measured with a JEOL AL300 spectrometer (JEOL Ltd., Japan). UV-Vis spectra were measured with a JASCO V-670 spectrophotometer (JASCO Corp., Japan) using a quartz cell with a path length of 10 mm.

4.2.3 Procedure

For ^{1}H-NMR measurements, 0.6 mg of Sudan III was brought to deuterated acetonitrile containing 0.01 vol% of tetramethylsilane (TMS). Solutions with or without sulfuric acid were transferred to NMR tubes with a 5 mm inner diameter.

The proton spectra were recorded at 303.0 K with accumulation time of 32. The TMS signal was used as a reference signal. Data were processed and analyzed with Delta software (JEOL Ltd., Japan).

For UV-Vis measurements, solutions of Sudan III were prepared as follows. A stock

solution was prepared by dissolving 2.5 mg of Sudan III in 50.0 mL of acetone. Then 0.40 mL of the stock solution was heated in glass vials to evaporate acetone. Acetone was chosen as the solvent of the stock solution because of the good solubility of Sudan III and easiness of evaporation. To investigate the color-change property of Sudan III, each organic solvent was added to the vial so as to dissolve the dye powder; some amount of acids were then added. The final volume of each solution was adjusted to 5.0 mL. To quantify the sulfuric acid, 5.0 mL of acetonitrile was added to the vial to yield the acetonitrile solution of Sudan III. Then 0.04 mL of sulfuric acid with various concentrations was added to the dye solution, followed by the measurement. UV-Vis absorption spectra were recorded using acetonitrile without any dye as a background. All measurements were carried out at room temperature (approximately 298 K).

4.3 Results and discussion

4.3.1 NMR spectroscopic study

To elucidate the color-change mechanism of Sudan III against sulfuric acid, ^{1}H-NMR measurements were carried out. By visual observation, the orange color of a deuterated acetonitrile solution of Sudan III turned to dark blue when approximately 2 μL of sulfuric acid was added. The ^{1}H-NMR spectra of a deuterated acetonitrile solution of Sudan III without or with sulfuric acid are shown in Figs. 4-1a and 4-1b, respectively. Referring to the literature [14-16], the observed peaks were assigned as follows. Resonances observed between 6.5 – 9 ppm are due to aromatic protons. A relatively broad resonance at 16.08 ppm in Fig. 4-1a is due to the azo – hydrazone tautomeric proton. Resonances due to aromatic protons did not change after the addition of sulfuric acid. On the other hand, a resonance due to the tautomeric proton was immediately

broadened after the addition of sulfuric acid (sulfuric acid concentration of the solution, 0.04 vol%), as shown in Fig. 4-1b. This indicates that a rapid hydrogen exchange occurred between sulfuric acid and the tautomeric proton of Sudan III. Although this result does not give any direct information about the color-change mechanism, it suggests that a proton exchange or protonation in the azo or hydroxyl group is the key role of the color-change phenomenon. When other acids, such as formic acid and acetic acid, were added in place of sulfuric acid, no change was observed for a resonance at 16.08 ppm. This suggests that there is almost no interaction between the acids and the tautomeric proton of the dye.

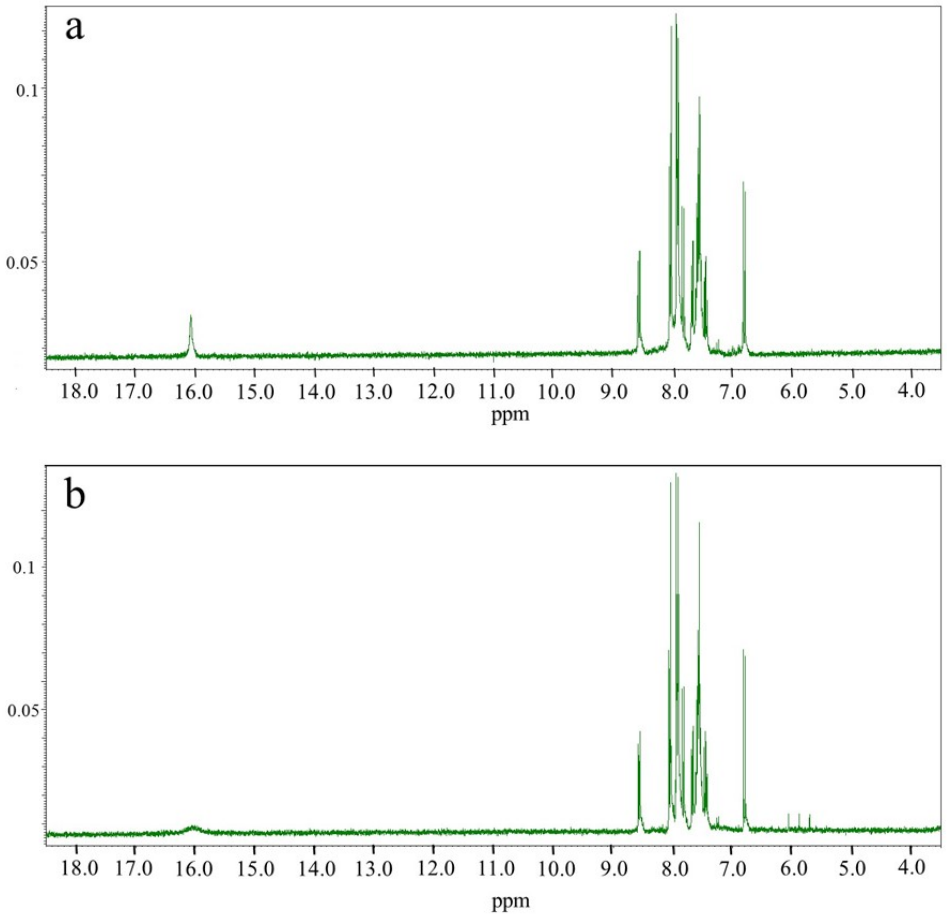


Fig. 4-1 ^{1}H-NMR spectra of deuterated acetonitrile solution of Sudan III without (a) or with (b) sulfuric acid.

4.3.2 Color change property of Sudan III

A series of observations of the color-change property of Sudan III for various volumes of sulfuric acid were carried out. When 0 - 0.1 mL of sulfuric acid was added to an acetonitrile solution of Sudan III, the color of the solution changed clearly from orange to blue upon the addition of sulfuric acid. Figure 4-2 shows the UV-Vis spectra of acetonitrile solutions of Sudan III without and with various amounts of sulfuric acid. For an acetonitrile solution of Sudan III without sulfuric acid, absorption peaks at 505 and 350 nm were observed. Upon the addition of sulfuric acid, a peak at 611 nm was observed. Absorbances at 505 and 350 nm were decreased, while the absorption at 611 nm was enhanced along with the increase of the amount of sulfuric acid. The isosbestic point was observed at 535 nm, which suggests the presence of the two inter converting species in this system. When a few drops of water were added to the blue solution, the color changed back to orange reversibly.

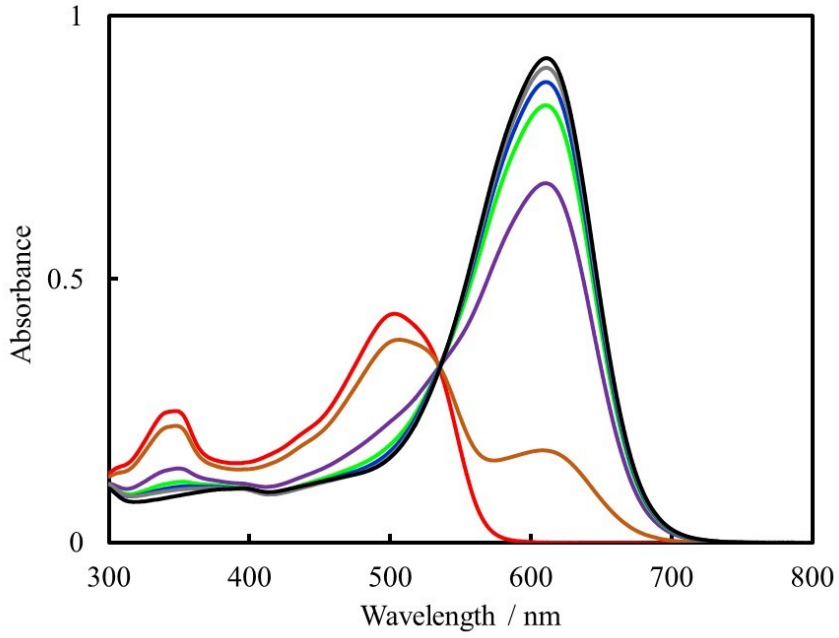


Fig. 4-2 UV-Vis spectra of acetonitrile solutions of Sudan III with the addition of various volumes of sulfuric acid. Red, 0 mL; Orange, 0.004 mL; Purple, 0.01 mL, Green, 0.02 mL; Blue, 0.03 mL; Gray, 0.04 mL; Black, 0.1 mL of sulfuric acid were added.

When the other organic solvents, such as methanol, ethanol, 2-propanol, acetone, dimethyl sulfoxide, were used, in place of acetonitrile, a color change did not occur upon the addition of 0.04 mL of sulfuric acid. These observations suggest that the hydroxyl, ether, or ketone group including oxygen atom interfered with the interaction between the dye and sulfuric acid. When acids other than sulfuric acid, such as hydrochloric acid, hydrofluoric acid, nitric acid, phosphoric acid, formic acid and acetic acid, were added to the acetonitrile solution of Sudan III, no acid caused the color-change of the solution as sulfuric acid even as high concentration as we can purchase as shown in Fig. 4-3. These facts indicate that the presented system, the acetonitrile solution of Sudan III, is the most suitable for observing the color-change phenomenon.

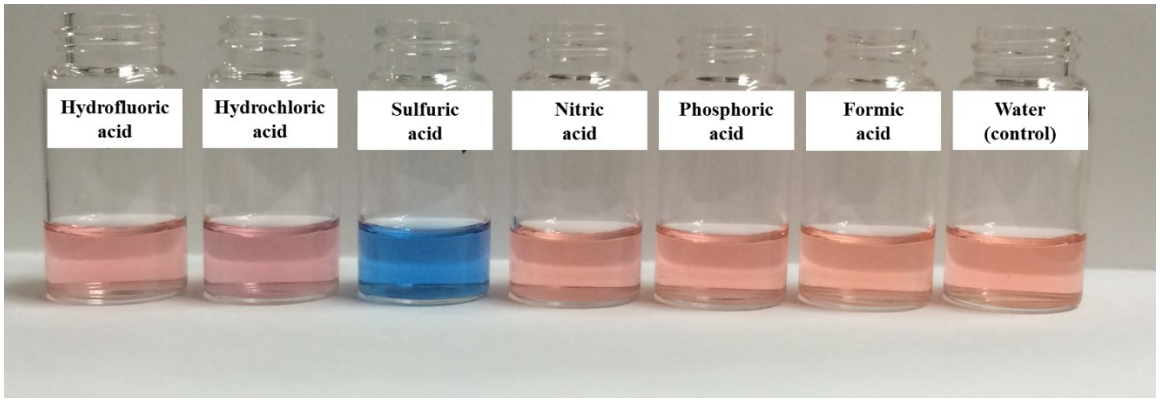


Fig.4-3 Photograph of acetonitrile solution of Sudan III with addition of various kinds of acids.

When the aqueous solution of sodium hydroxide with a concentration of 2 mol/L was added to the acetonitrile solution of Sudan III, the UV-Vis spectrum (shown in Fig. 4-4) was different from the spectra upon the addition of sulfuric acid, as shown in Fig. 4-2. This observation leads to the conclusion that the color-change mechanism of Sudan III against sulfuric acid is due to protonation of the dye by sulfuric acid.

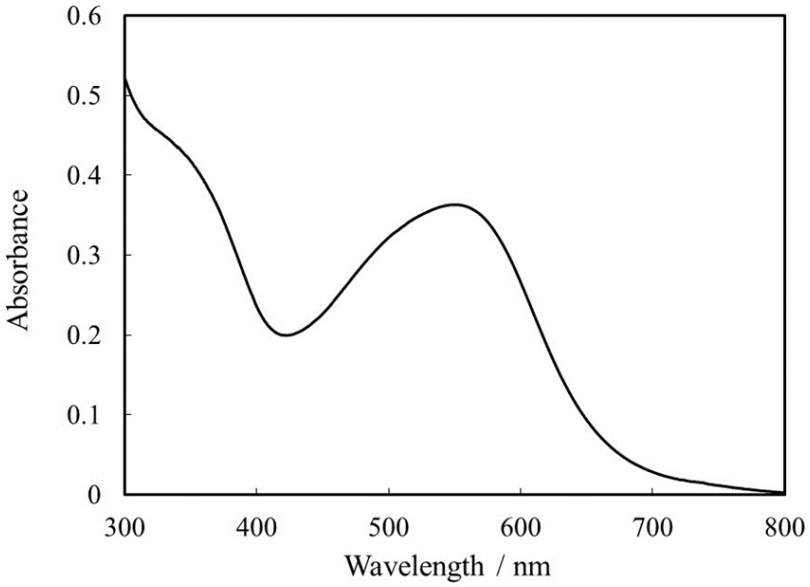


Fig.4-4 UV-Vis spectrum of acetonitrile solutions of Sudan III with the addition of an aqueous solution of sodium hydroxide (2 mol/L).

4.3.3 Quantitative evaluation

The relationship between the absorbance at 611 nm and the sulfuric acid concentration of the solution is shown in Fig. 4-5. The absorbance reaches a plateau at over 0.36 mol/L of the sulfuric acid concentration. A protonated form of Sudan III has an absorption maximum at 611 nm; on the other hand, an unprotonated form of the dye does not have any absorption at this wavelength.

Then, the absorption at 611 nm reflects the concentration of the protonated form of the dye directly. An HPLC analysis indicated that the reagent includes about 2.2% of some impurity. Then, the molar absorbance constant of the protonated form of the dye at the absorption maximum is estimated to be $\varepsilon = 8.29\times10^{4}$ L mol^{-1} cm^{-1} by the Lambert - Beer law, using the initial concentration of the dye (1.11×10^{-5} mol/L) and the absorbance value at the plateau region.

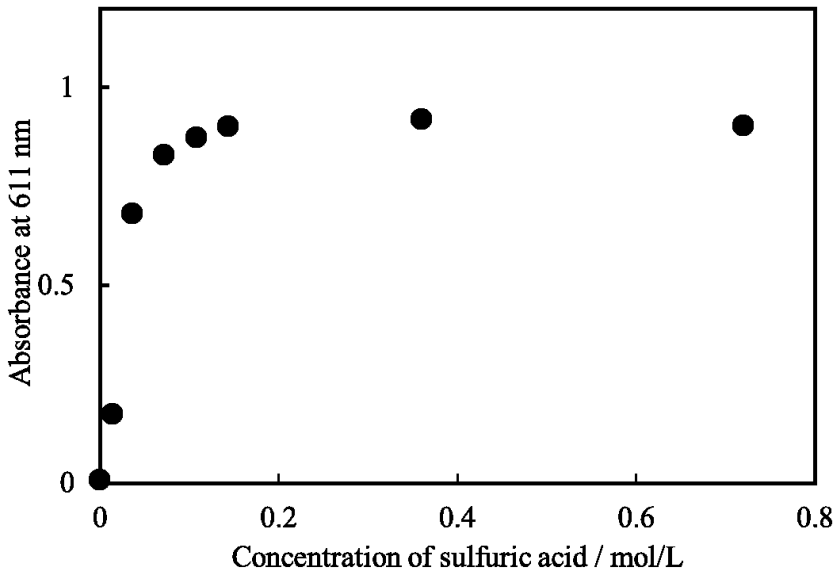


Fig. 4-5 Relationship between the absorbance at 611 nm and the sulfuric acid concentration of the solution.

4.3.3.1 Effect of water content

Arnett and Douty have shown that in a non-aqueous solvent (sulfolane) system, small quantities of water reduce the acidities of a sulfuric acid solution [17]. The presented system looks similar to this combination, since acetonitrile is also the aprotic solvent, and this system is a non-aqueous system. Therefore, the influence of the water content was estimated. The absorbance at 611 nm of the dye solution with a constant sulfuric acid concentration (0.144 mol/L) containing various amounts of water was measured; the relationship between the absorbance and the amount of water is shown in Fig. 4-6 . The absorbance at 611 nm, corresponding to the protonated form of the dye, decreased as the water content increased, even though the concentration of the dye and sulfuric acid was kept constant. This phenomenon suggests that water plays the role of a base competing with the dye for protons from the sulfuric acid in this system, which is governed by the same mechanism as that which Arnett and Douty proposed.

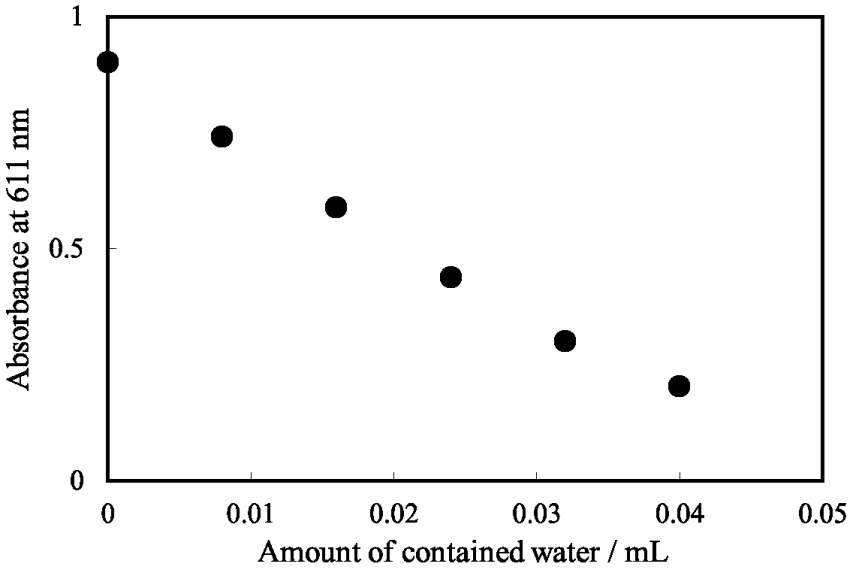


Fig. 4-6 Relationship between the absorbance at 611 nm of the dye solution with constant sulfuric acid concentration (0.144 mol/L) and the water content.

4.3.3.2 Quantification of sulfuric acid sample

From the observation described above, the amount of water that is initially contained in sulfuric acid can also affect the protonation degree of the dye. Figure 4-7 shows the UV-Vis spectra of acetonitrile solutions of Sudan III with the addition of a constant volume (0.04 mL) of sulfuric acid with various concentrations (the concentration was adjusted by water). Since the spectrum profiles are the same as those in Fig. 4-2, the involved species are the same in case the water content increases.

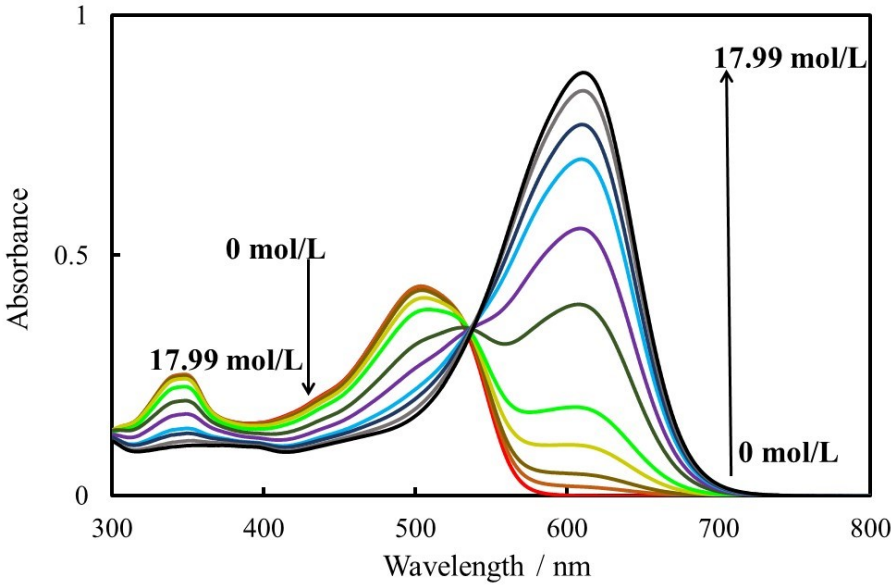


Fig. 4-7 UV-Vis spectra of acetonitrile solutions of Sudan III with the addition of a constant volume (0.04 mL) of sulfuric acid sample with various concentrations.

Relationships between the absorbance at 611 nm and concentration of sulfuric acid or water in the solution are shown in Fig.4-8. The absorbance at 611 nm enhanced with the increasing of the concentration of sulfuric acid in the solution (Fig. 4-8 a). The plot, however, shows the sigmoidal relationship different from that in Fig. 4-5. The relationship between the absorbance at 611 nm and water concentration (Fig. 4-8 b) is different form Fig. 4-6. These differences suggest that both factors, sulfuric acid concentration and water concentration, should be taken into consideration in order to understand the behavior in this system. Sulfuric acid samples used in this system contains some amount of water, which should play a key role on the protonation degree of the dye by competing with the dye for protons. In the region of the lower sulfuric acid concentration, larger amount of water disturbs the protonation of dye. Inversely, as the increase of sulfuric acid concentration, the contribution of water decreases because the amount of water decreases. Sigmoidal relationship should be due to this mechanism.

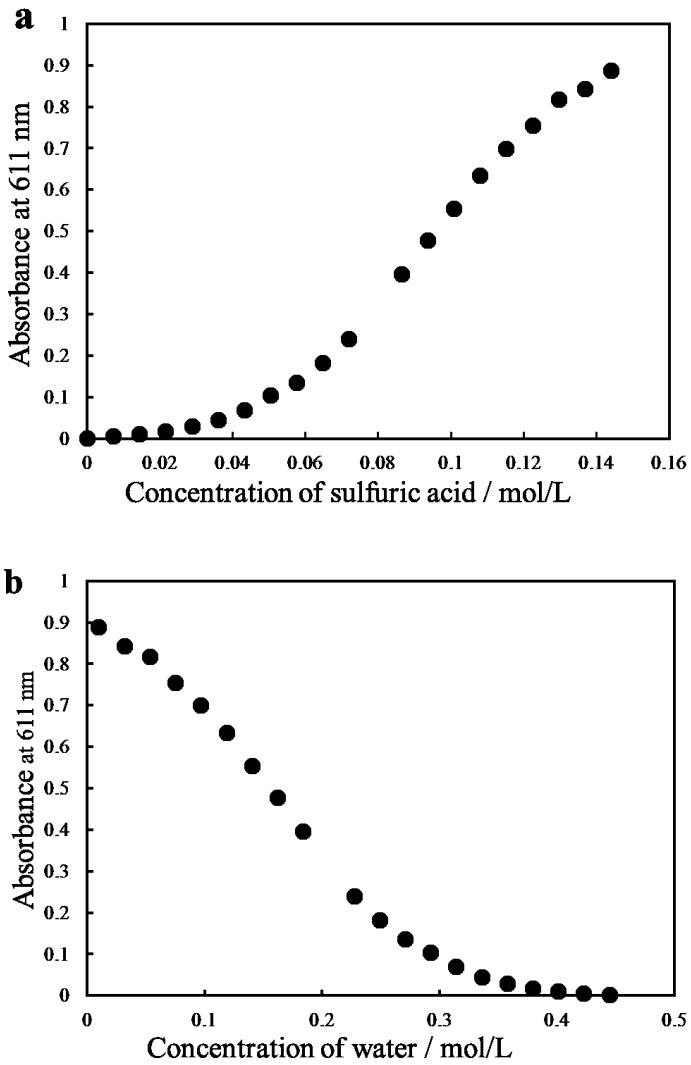


Fig. 4-8 Relationship between the absorbance at 611 nm and the concentration of sulfuric acid (a) and water (b).

Figure 4-9 shows the relationship between the absorbance at 611 nm and the concentration of the acid sample. The plots exhibit a sigmoidal relationship. This is resulted from the mechanism described above. Therefore, the absorbance at 611 nm reflects the ratio of sulfuric acid and water contents. Described observation indicates a possibility that the proposed system has a potential for the quantification of samples containing sulfuric acid. As shown in Fig. 4-9, a liner correlation is not obtained when the protonation degree of the dye is plotted with the concentration of sulfuric acid

samples.

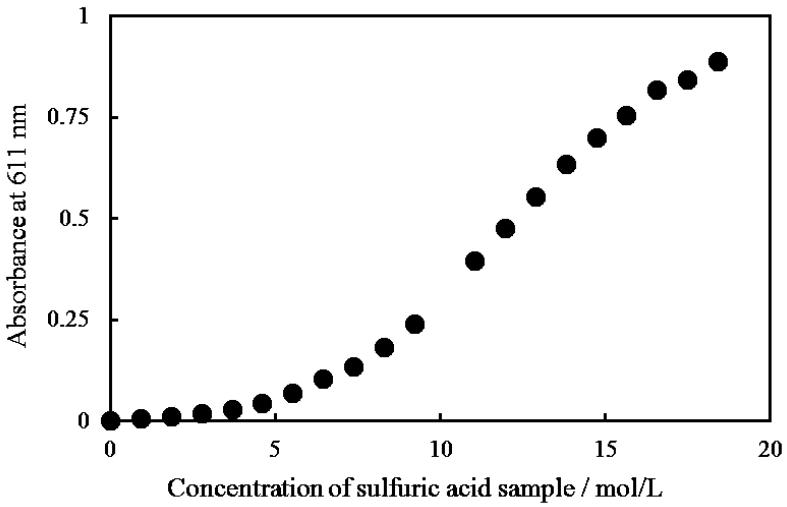


Fig. 4-9 Relationship between the absorbance at 611 nm and the concentration of sulfuric acid samples.

To quantify the sulfuric acid concentration, the Hammett acidity function is introduced to the present system. The Hammett acidity function, H_0, is a parameter of the protonation ability of acid in a non-ideal solution, and is defined as follows [18]: in the equilibrium as given by the Eq. (4-1), the dissociate constant, $K_{\mathrm{BH^+}}$, of the conjugate acid $\mathrm{BH^+}$ of base B is given by Eq. (4-2) in terms of activity a:

$$\mathrm{BH^+} \leftrightarrows \mathrm{B} + \mathrm{H^+} \tag{4-1}$$

$$K_{\mathrm{BH^+}} = \frac{a_{\mathrm{B}}\ a_{\mathrm{H^+}}}{a_{\mathrm{BH^+}}} \tag{4-2}$$

Taking logs and substituting a for the concentration C and the molar activity coefficient, f, Eq. (4-2) gives Eq. (4-3), and H_0 is defined by Eq. (4-4):

$$\mathrm{p}K_{\mathrm{BH^+}} = \log\frac{C_{\mathrm{BH^+}}}{C_{\mathrm{B}}} - \log a_{\mathrm{H^+}}\frac{f_{\mathrm{B}}}{f_{\mathrm{BH^+}}} \tag{4-3}$$

$$H_0 = -\log a_{\mathrm{H^+}}\frac{f_{\mathrm{B}}}{f_{\mathrm{BH^+}}} \tag{4-4}$$

In the present system, Sudan III corresponds to the base, B and is signified as 'Dye'. Eq. (4-3) can be converted into Eq. (4-5) using H_0:

$$\log\frac{C_{\mathrm{DyeH^+}}}{C_{\mathrm{Dye}}} = -H_0 + \mathrm{p}K_{\mathrm{DyeH^+}} \tag{4-5}$$

Herein, $C_{\mathrm{DyeH^+}}$and C_{Dye} represent the concentrations of the protonated form of the dye and the unprotonated form of the dye, respectively. The ionization ratio, $C_{\mathrm{DyeH^+}}/C_{\mathrm{Dye}}$, is experimentally available from UV-Vis spectroscopic measurements while monitoring the absorbance at 611 nm, signified as A_{611}. Since the unprotonated form of the dye has no absorption, A_{611} is in proportion to $C_{\mathrm{DyeH^+}}$, and $C_{\mathrm{DyeH^+}}/C_{\mathrm{Dye}}$ can be described in Eq. (4-6) using A_{611} and the plateau value, A_∞, observed in Fig.4-5:

$$\frac{C_{\mathrm{DyeH^+}}}{C_{\mathrm{Dye}}} = \frac{A_{611}}{A_\infty - A_{611}} \tag{4-6}$$

Since $\mathrm{p}K_{\mathrm{DyeH^+}}$ is constant, the ionization ratio varies as a function of H_0. If the H_0 value depends on the concentration of added sulfuric acid, some correlation between $\log(C_{\mathrm{DyeH^+}}/C_{\mathrm{Dye}})$ and the concentration of added sulfuric acid samples should be obtained. As shown in Fig. 4-10a, relationship between $\log(C_{\mathrm{DyeH^+}}/C_{\mathrm{Dye}})$ and the concentration of added sulfuric acid samples shows a non-liner correlation under 4 mol/L and over 16 mol/L. On the other hand, within a range of 4.5-15.3 mol/L, a liner correlation was observed, as shown in Fig. 4-10b.

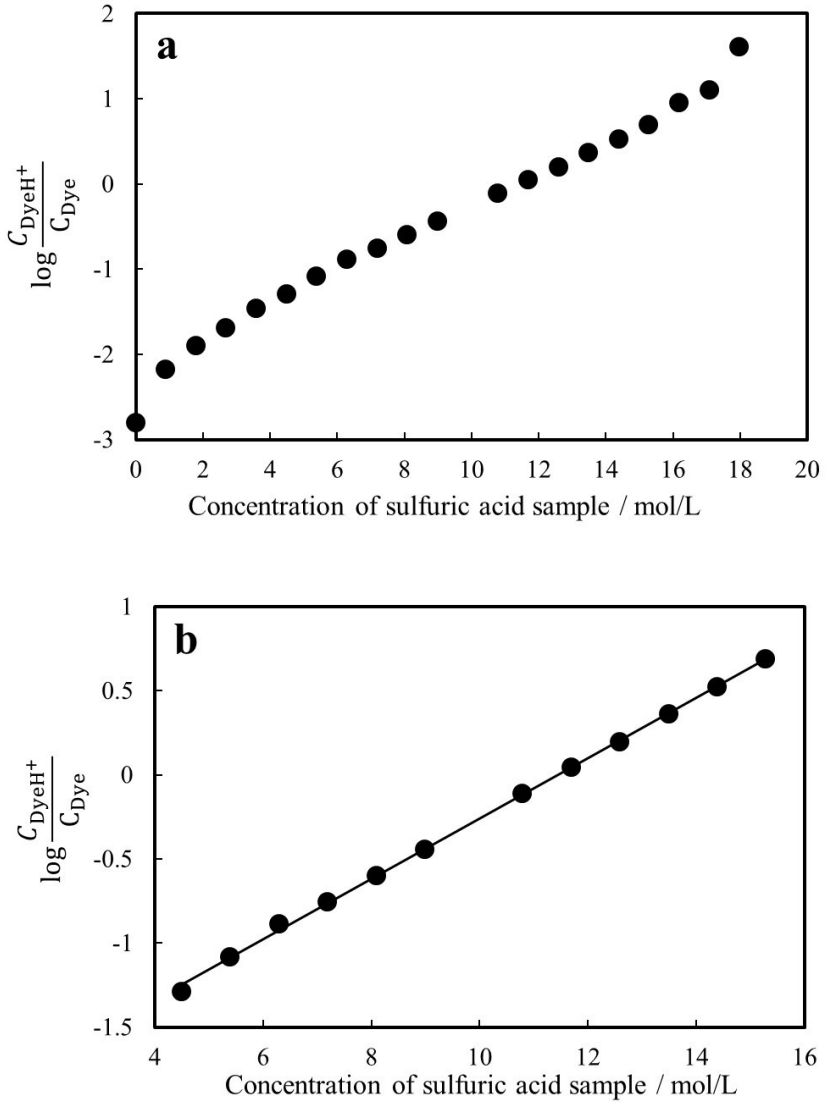


Fig. 4-10 Relationship between $\log(C_{\mathrm{DyeH^+}}/C_{\mathrm{Dye}})$ and the concentration of sulfuric acid samples. (a), overview; (b), magnified view of within a range of 4.5-15.3 mol/L of sulfuric acid samples.

To utilize this liner part for the quantification of a higher concentration sulfuric acid sample, the sample concentration was apparently decreased by the addition of a little volume of water to the dye solution initially. When the sulfuric acid sample was added to the acetonitrile solution of Sudan III containing 0.8 vol% of water, the plot of $\log(C_{\mathrm{DyeH^+}}/C_{\mathrm{Dye}})$ vs the concentration of added sulfuric acid sample shows a liner correlation within a range of 6.5-17.99 mol/L (17.99 mol/L of sample is not diluted

sample). The liner calibration curve, $y = 1.713x - 2.2065$, was obtained in this concentration range; it showed an excellent liner correlation ($R^2 = 0.999$). In this way, the initial addition of water to the dye solution enabled us to quantify the concentration of the concentrated sulfuric acid. The proposed method was applied to the quantification of three sulfuric acid samples with unknown concentrations. Samples were also analyzed by the titration method. As shown in Table 4-1, the analytical results obtained by the proposed method almost agreed with the titration results.

Table 4-1 Comparison of the quantification results of concentration of sulfuric acid samples obtained from the proposed method and titration

Sample	Proposed method[a] (mol/L)	Titration (mol/L)
A	17.19±0.86	16.98
B	15.35±0.69	15.37
C	11.14±1.61	11.27

a. $n = 3$, mean±RSD*(%)*.

4.4 Conclusions

In this chapter, the color-change phenomenon of Sudan III against sulfuric acid is described concerning an acetonitrile solution. It is shown that the color-change phenomenon is due to protonation, and that the system is applicable to the quantification of sulfuric acid by introducing the Hammett acidity function. The advantages of the proposed quantification method are that it can be used for the rapid and easy quantification of concentrated sulfuric acid. It requires quite small sample amount, 0.04 mL. This method can be helpful, especially in forensic analysis and industrial quality checks.

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Chapter 5

General conclusion

In this thesis, the author aim for development of novel on-site testing method of unknown liquid samples which is complementary to existing methods. To accomplish the aim, colorimetric approach was chosen and dyes were utilized as indicators.

In chapter 2, a novel on-site discrimination method for unknown liquid samples have been developed. A novel method, “Pipette for discrimination”, is a rapid and safe method for the field discrimination of various liquid samples. The pipette is composed of two dye layers (using two dyes, Brilliant Green and Methyl Red) and three colored layers (silica gel, florisil and polypropylene). When liquid samples applied the pipette, various color patterns of the colored layers were observed. The pipette performs mainly based on three principals; 1) the differences of the solvation properties of two dyes against the various liquid samples, 2) the differences of the interaction between dyes and solid surface with the presence of various liquid samples, 3) the color change properties of the dye when the dye is adsorbed on the solid surface. Using the pipette, 8 types of liquid samples, such as water, methanol, ethanol, 2-propanol, acetone, acetonitrile, ethyl acetate and toluene were discriminated each other. It enables visual discrimination of samples clearly within 10 seconds. The proposed method can be considered as a rapid and easy on-site discrimination method for liquid samples.

In chapter 3, characteristic color change phenomena of Oil Red O (ORO) supported by florisil was discovered and its utilization for a novel optical indicator for concentrated sulfuric acid solutions was described. The color change behavior of ORO is modified when it adsorb onto the florisil surface. When ORO is supported by silica gel, the color change property of ORO is clearly different from the case when florisil is

used as a support. These findings was applied to optical sulfuric acid sensing. Modification of the color change property by florisil surface expands the applicable concentration range. Consequently, ORO-florisil enabled to estimate the approximate concentration of sulfuric acid for an extremely wide concentration range. The proposed method is applicable for sensing of concentrated sulfuric acid solutions which comercially available indicators are not suitable.

In chapter 4, the color-change phenomenon of Sudan III, which have similar structure to ORO, against sulfuric acid is described concerning an acetonitrile solution. Solvents except for acetonitrile inhibit the color change of Sudan III against small quantity of sulfuric acid. ^{1}H-NMR and UV-Vis spectroscopic studies showed that the color-change phenomenon is due to protonation. The system is applicable to the quantification of sulfuric acid by introducing the Hammett acidity function. The advantages of the proposed quantification method are that it can be used for the rapid and easy quantification of concentrated sulfuric acid. It requires quite small sample amount, 0.04 mL. This method can be helpful, especially in forensic analysis and industrial quality checks.

Although those developed methods are in advance and need to be investigated in depth, possibilities of establishment of new on-site testing methods are shown by this research.

“Pipette for discrimination” showed a novel approach for the easy and rapid identification method for unknown liquid sample. Further evaluation, such as applicability to the mixture samples with more than two constituents, should be done, however, this method can be a new practical on-site testing method.

Oil red O supported by florisil is a quite unique sensing method and it can be applied to estimation of the concentration of concentrated sulfuric acid, which any research have not accomplished. It is expected to clarify the contribution of florisil surface to color

change phenomena of ORO.

The color change of Sudan III against small quantity of sulfuric acid is newly discovered and investigated phenomenon and by this research. Performed quantification method is attractive to quantify the concentration of concentrated sulfuric acid, especially when the available quantity of sample is limited. Although this method is difficult to use as on-site testing method directly, knowledges obtained through examinations must be helpful to develop a novel on-site testing method in future.

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[1] T. Sakurai, S. Kurata and M. Nagai, “Development of New Method for Rapid Field Discrimination of Liquid Sample using a Pipette Composed of Brilliant Green and Methyl Red”, *Bunseki Kagaku* , **2013**, *62*, 645. (Corresponds to chapter 2)

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