Analysis of Malachite Green in Fish Samples

Hubert P.O. Tang & Dr. Jacky Y.Y. Choi
Chemists
Food & Environmental Hygiene C Section
Government Laboratory
14 September 2005

MG & LMG

- **Part A**
  - **Introduction**
  - **Sample Preparation**
- **Part B**
  - **Instrumental Analysis**
  - **Results**
  - **Quality Assurance**
- **Discussion**

Introduction

- **Malachite / *ˈmæləkaɪt***
  - **a decorative green stone**
  - 孔雀石
  - **“ch” / *t ʃ* ; e.g. Channel / *t ʃ ænl***
  - **Chaos / *ke as***

Introduction

- **Malachite green (MG)**
  - **direct dyeing silk, wool, leather, etc**
  - **Biological stain**
  - **Acid base indicator (Green at pH 2; colorless at pH 14)**
  - **Effective fungicide & parasiticide in fish**

Introduction

- **Malachite green**
  - 孔雀石綠
  - **Bis[p- (dimethylamino) phenyl]phenylmethylium**
  - **Cationic triphenylmethane dye**
  - **C.I. Basic Green 4 / 42000**
  - **$C_{23}H_{25}N_2^+$**
  - **Mol wt 329.464**
  - **Mainly as oxalate or chloride**

Introduction

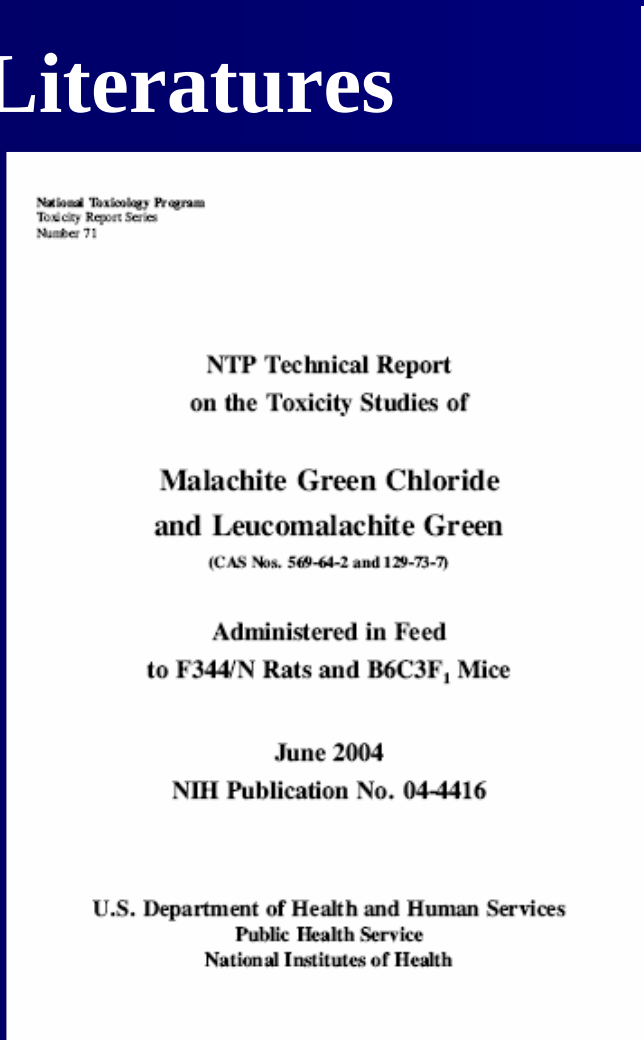
- **Chemical Structure**

Introduction

- Toxicity of MG
  - May cause liver tumors in mice
  - Carcinogenicity for MG in humans still unknown
  - Not yet evaluated by JECFA or IARC

Introduction

- Literatures



National Toxicology Program
Toxicity Report Series
Number 71

NTP Technical Report
on the Toxicity Studies of

Malachite Green Chloride
and Leucomalachite Green
(CAS Nos. 569-64-2 and 129-73-7)

Administered in Feed
to F344/N Rats and B6C3F$_1$ Mice

June 2004
NIH Publication No. 04-4416

U.S. Department of Health and Human Services
Public Health Service
National Institutes of Health

ELSEVIER

Aquatic Toxicology 66 (2004) 319–329

AQUATIC TOXICOLOGY

www.elsevier.com/locate/aquatox

Review

Toxicological effects of malachite green

Shivaji Srivastava[a], Ranjana Sinha[a,b,*], D. Roy[a]

[a] Department of Zoology, S.M.M. Town Post-Graduate College, Ballia 277001, India
[b] Central Inland Capture Fisheries Research Institute, Barrackpore 743101, WB, India

Received 16 April 2002; accepted 29 September 2003

Abstract

This review summarizes the wide range of toxicological effects of malachite green (MG), a triarylmethane dye on various fish species and certain mammals. MG is widely used in aquaculture as a parasiticide and in food, health, textile and other industries for one or the other purposes. It controls fungal attacks, protozoan infections and some other diseases caused by helminths on a wide variety of fish and other aquatic organisms. However, the dye has generated much concern regarding its use, due to its reported toxic effects. The toxicity of this dye increases with exposure time, temperature and concentration. It has been reported to cause carcinogenesis, mutagenesis, chromosomal fractures, teratogenicity and respiratory toxicity. Histopathological effects of MG include multi-organ tissue injury. Significant alterations occur in biochemical parameters of blood in MG exposed fish. Residues of MG and its reduced form, leucomalachite green have been reported from serum, liver, kidney, muscles and other tissues as also from eggs and fry. Toxicity occurs in some mammals, including organ damage, mutagenic, carcinogenic and developmental abnormalities. However, despite the large amount of data on its toxic effects, MG is still used as a parasiticide in aquaculture and other industries. It is concluded that the potential of alternative parasiticides, like humic acid, chlorine dioxide and Pyoxie, should be explored to replace MG. Until then, MG should be used with extreme care at suitable concentrations and at times when the temperature is low. Removal of residual MG in treatment ponds should also be considered.

Keywords: Malachite green; Aquaculture; Toxicity; Parasiticide; Fish

1. Introduction

Malachite green (MG) is an extensively used biocide in the aquaculture industry world-wide. It is highly effective against important protozoal and fungal infections (Hoffman and Meyer, 1974; Alderman, 1985; Schnick, 1988). Basically, it works as an ectoparasiticide: it has also been used to control skin flukes and gill flukes. Aquaculture industries have been using malachite green extensively as a topical treatment by bath or flush methods without paying any attention to the fact that topically applied therapeutants might also be absorbed systemically and produce significant internal effects. On the other hand, it is also used as a food colouring agent, food additive, a medical disinfectant and anthelminthic as well as a dye in silk, wool, jute, leather, cotton, paper and acrylic industries (Culp and Beland, 1996). However, malachite green has now become a highly controversial compound due to the risks it poses to the consumers of treated fish (Alderman and

* Corresponding author.
E-mail address: ranasinha1969@yahoo.co.uk (R. Sinha).

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doi:10.1016/j.aquatox.2003.09.008

Introduction

- **Other literatures**
  - **Canadian Food Inspection Agency (2005, July 6). Update on the CFIA monitoring activities for malachite green http://www.inspection.gc.ca/english/corpaffr/newcom/2005/20050706e.shtml**
  - **National Toxicity Program (2005). TR-527 Toxicity and carcinogenesis studies of malachite green chloride and leucomalachite green in F344/N rats and B6C3F1 mice (Feed studies) http://ntp.niehs.nih/gov/index.cfm?objectid=070B74C5-C780-E272-6721383F7A1113BC**

Introduction

- **Leucomalachite green (LMG)**
  - 無色(隱色)孔雀石綠
  - **Reduced form of MG**
  - **Persistent metabolite in tissue**
  - **$C_{23}H_{26}N_2$**
  - **Mol wt 330**

Introduction

- **Chemical Structure**

Introduction

- **Regulations**
  - **Codex not yet established food safety standards for MG in food**
  - **Not allowed in US, EU (including UK), Canada, Japan, Thailand, Singapore as veterinary drug on food animals, aquaculture or fish for human consumption**
  - **Recently included in the Harmful Substances in Food Regulation in Hong Kong**

Introduction

- **Regulations**
  - **"Zero tolerance" in Hong Kong**
  - **Minimum Required Performance Limit (MRPL) for residue of MG and LMG of 2 micrograms/kg (Commission Decision 2004/25/EC)**

Designed to harmonise the analytical capabilities of EU member states

Introduction

- Methods published in literatures
  - Sample extraction
    - by acidic buffer and acetonitrile
    - presence of antioxidants or enzyme inhibitor
    - may contain ion-pairing reagents

Introduction

- Methods published in literatures
  - SPE Clean-up
    - Cationic exchange SPE (SCX or PRS)
    - Alumina (Neutral)

Introduction

- Methods published in literatures
  - Separation
    - LC (major)
      - C18, CN, phenyl-hexyl columns
    - GC (minor)
      - for LMG only

Introduction

- Methods published in literatures
  - Detection
    - LC post column oxidation and visible detection (620nm)
    - Visible for MG (620nm) or fluorescence for LMG (Ex=265nm and Em=360nm)
    - APCI- or ESI- MS/MS

Sample Preparation

- **Sample Extraction**

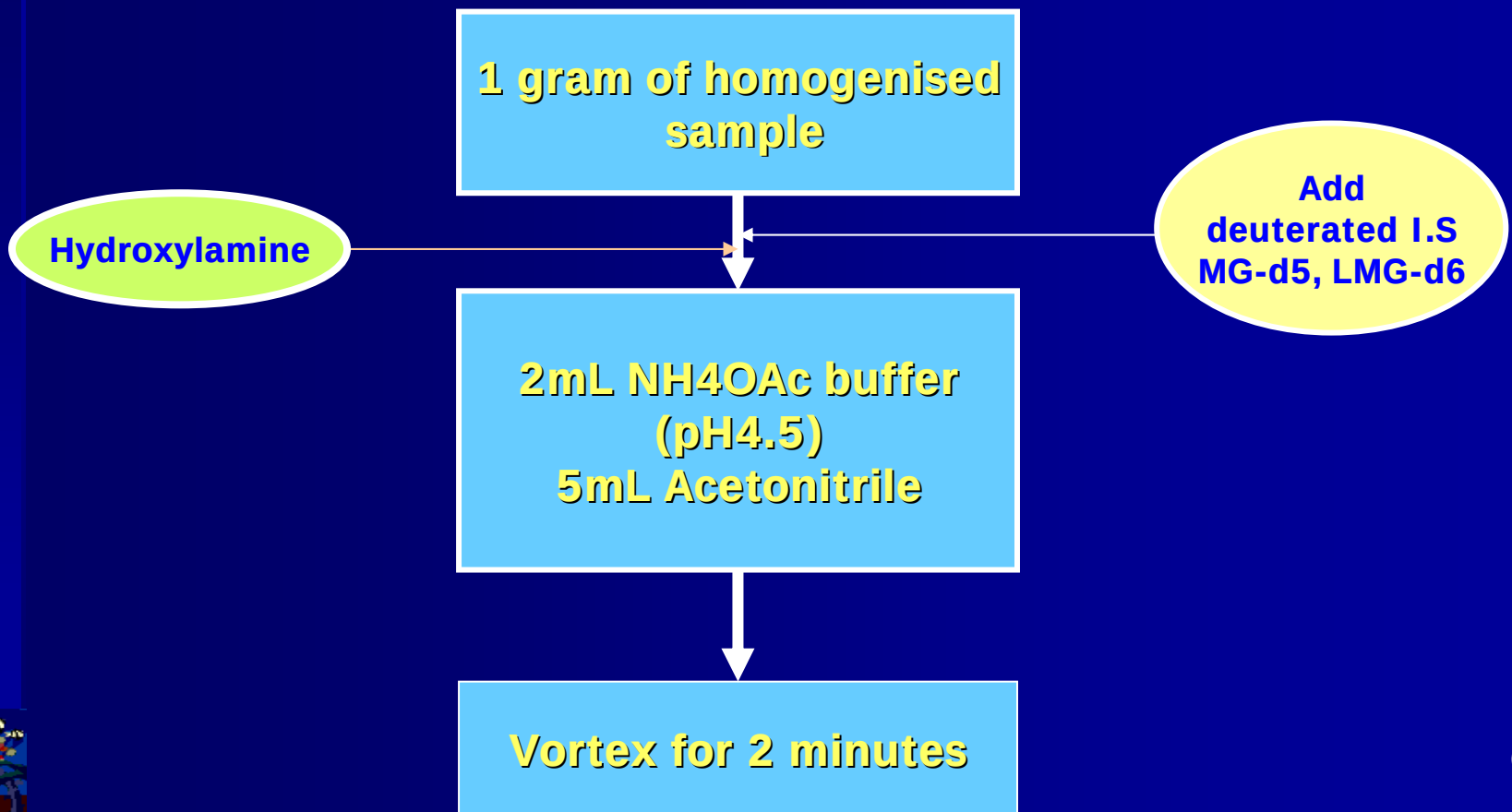
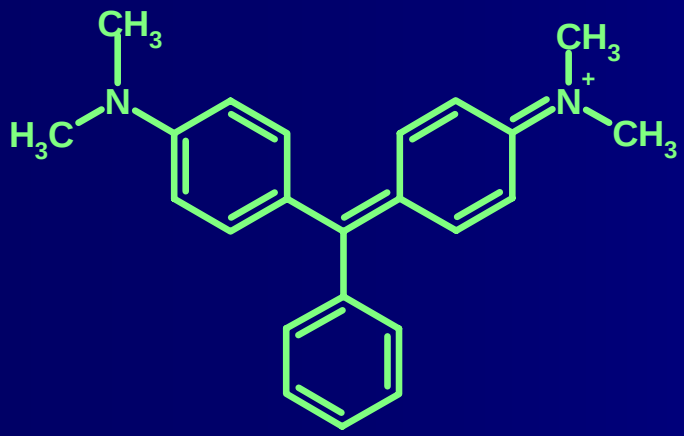


Photo-oxidation of LMG



MG

[H]

[O]

Photo-oxidation

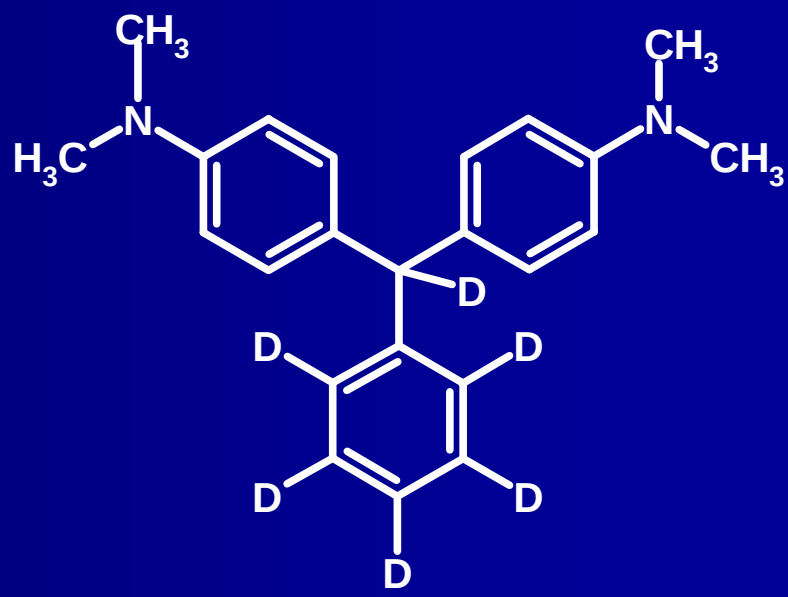
LMG

Photo-oxidation of LMG

- **Anti-oxidants**
  - **Hydroxylamine hydrochloride**
  - **L(+)-ascorbic acid**
- **Oxidase inhibitor**
  - **N,N,N’,N’-tetramethyl-1,4-phenylene diamine (TMPD)**

Internal Standards

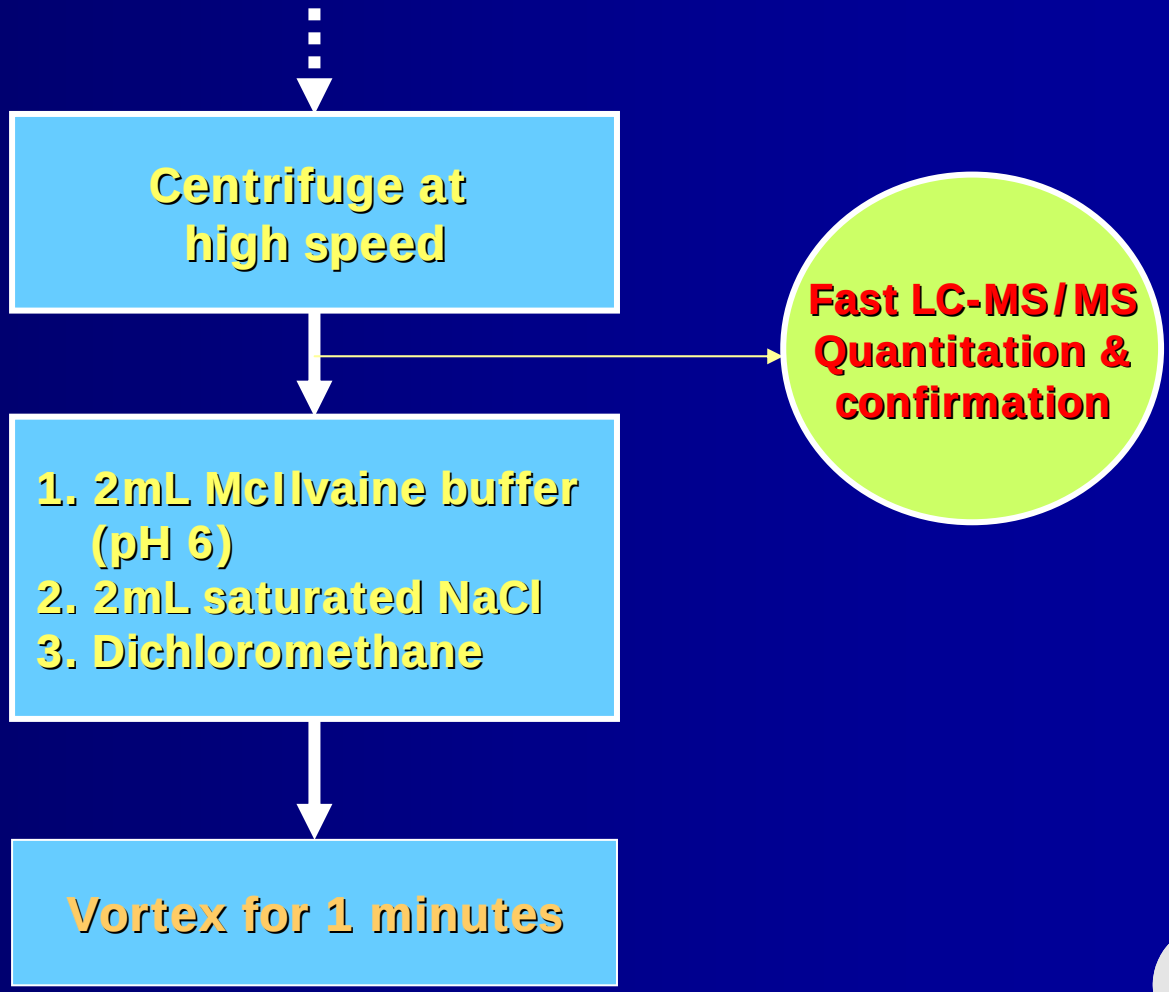
Malachite green-d5



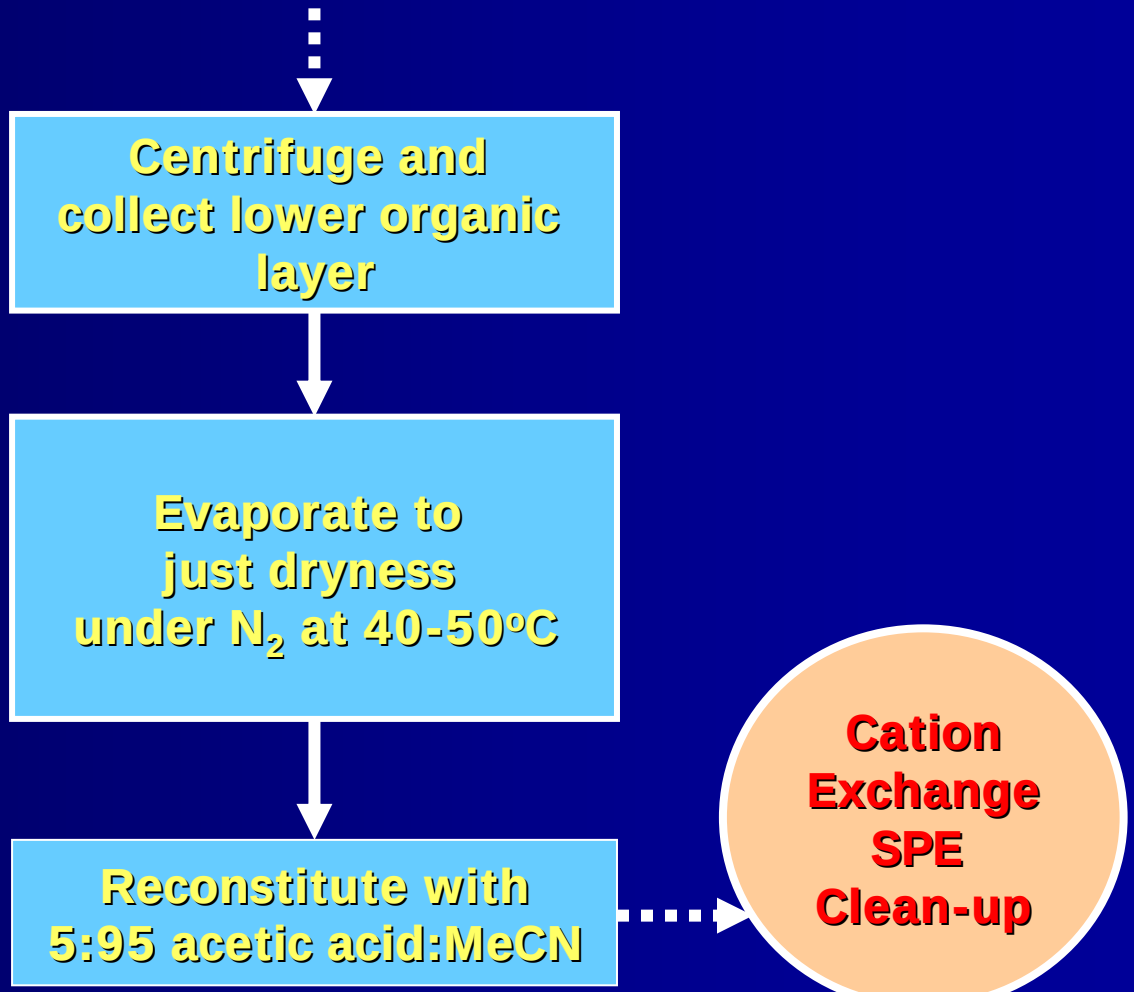
Leucomalachite green-d6

Source: WITEGA Laboratorien Berlin (www.witega.de)

Sample Preparation (cont'd)



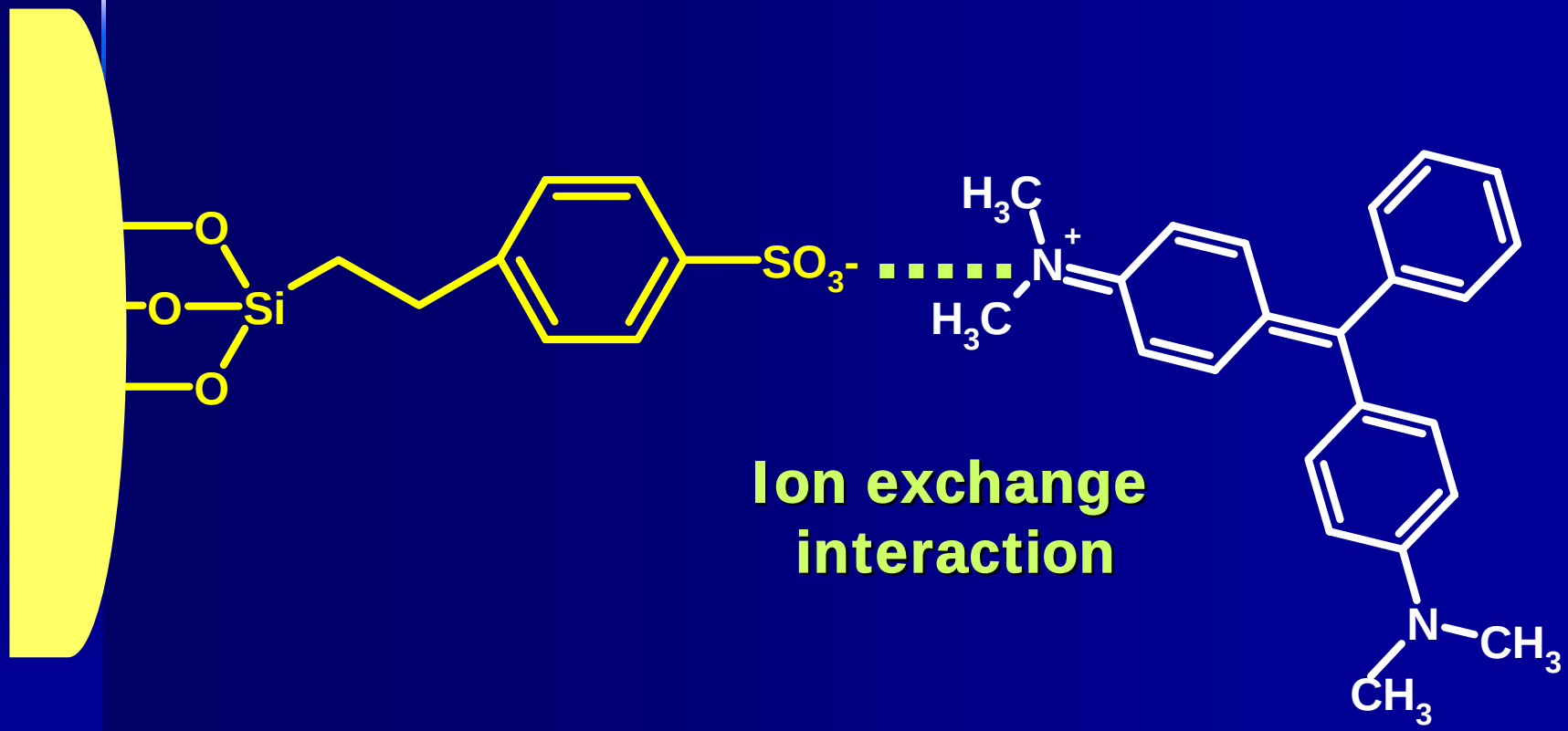
Sample Preparation (cont'd)



Solid Phase Extraction

- **SPE cartridge**
  - **Merck SCX (500mg, 3mL)**
  - **Ion exchange capacity : 0.75meq/g**
  - **Others:**
    - **e.g. Bakerbond SCX (500mg, 3mL); Strata SCX (500mg, 3mL); Oasis MCX (60mg, 3mL), etc.**

Solid Phase Extraction

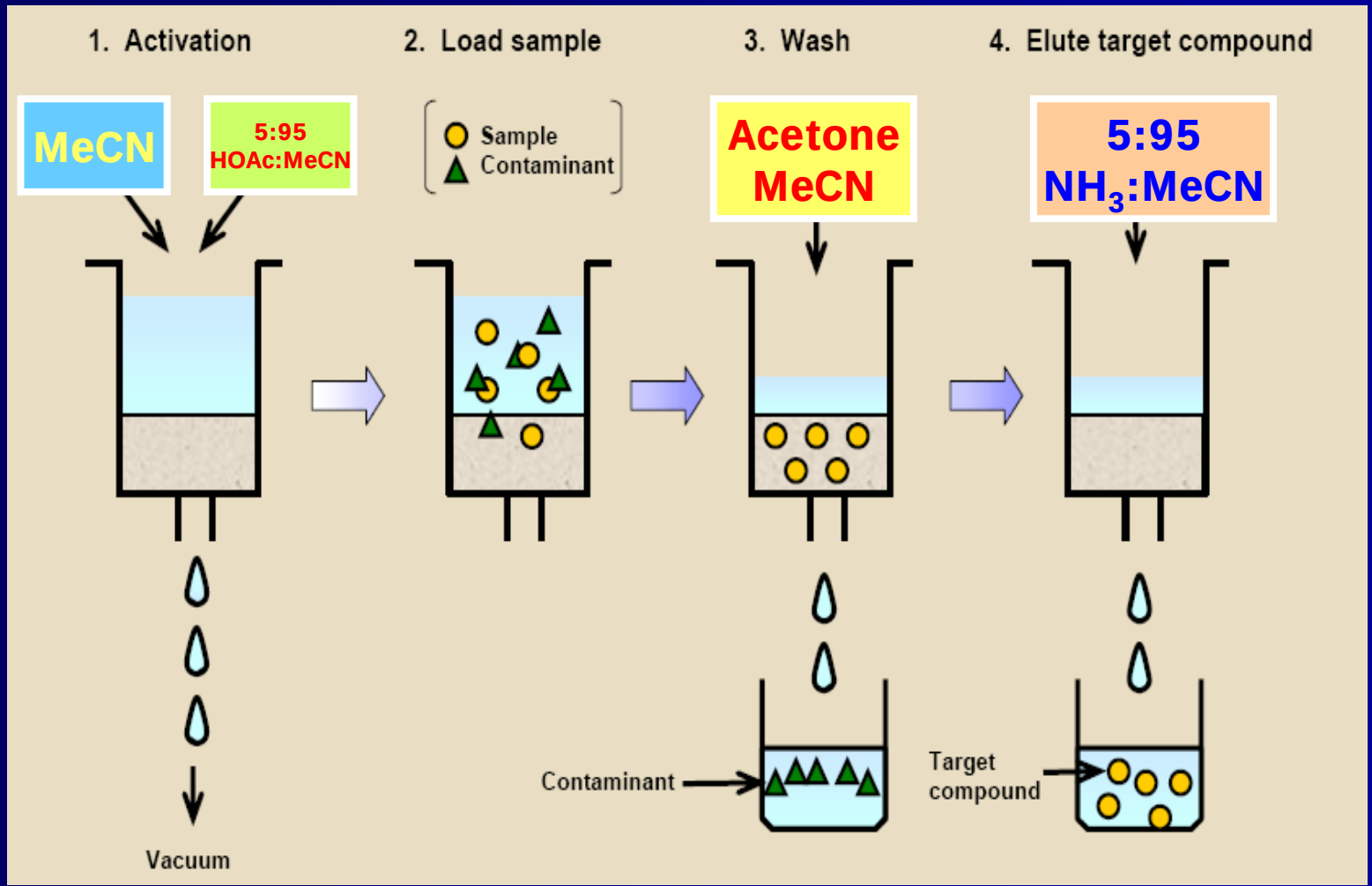


Ion exchange interaction

Solid Phase Extraction

- Four major steps
  - Conditioning
  - Sample loading
  - Washing
  - Eluting

Solid Phase Extraction



Solid Phase Extraction

- **Sorbent Mass**
  - **Mass of solute equivalent to 5% sorbent mass i.e. 5mg of TOTAL solute mass per 100mg sorbent**
- **Loading/ Eluting flow rate**
  - **↓ flow rate ↑ time for sorbent-analyte interaction (slow kinetics)**
    - **~ 1 mL/min or 1 drop per second**

Solid Phase Extraction

- **Wash volume**
  - 5 to 10 sorbent bed volumes
  - usu. 125µL per 100mg of sorbent
- **Dry**
  - Critical when elution solvents not miscible with wash solvent
  - No overdrying $\Rightarrow \downarrow$ analyte recovery
- **Elution**
  - 4-8 times sorbent bed volumes

MG & LMG

- **End of Part A**