

# Testing of **Sudan Dyes** in General Foodstuffs



Dr. LO Man-fung, Samuel  
Chemist  
Outsourcing Management Section  
Government Laboratory, HKSAR





# Sudan Dyes in Food

- May 2003  
France informed European Union (EU) that they found Sudan I in chilli products originating in India.
- February, March /2005  
Food Standard Agency (FSA) of UK was informed that some Worcester Sauce and chilli powders had been tested positive for Sudan I. Totally 580 food products were withdrawn as a result of Sudan I contamination.

EU announced the detection limit of HPLC method is in the range of 0.5 – 1.0 ppm for Sudan I and other illegal dyes.



- November/2006

CCTV reported that non-permitted colouring matter Sudan IV was used in duck feeds to enhance the egg yolks colour. Mainland authorities found Sudan IV in some hen and duck egg samples.

The reported concentration is in ppb level.



# Hong Kong Regulation

- In accordance with the Hong Kong Regulation Chapter 132H, Colouring Matters in Food Regulations, permitted colouring matters have been listed in the Schedule 1.
- The other colouring matters, including Sudan dyes, which are not listed in the schedule will be considered as banned food dyes.



# International Testing Methods

## EU method

### **Analysis and dosage of the colorants Sudan and Bixin in chilli powder and pepper-based products**

O. Véré tout, L. Demesse, L. Szymanski

- Scope: Sudan I, II, III, IV, Sudan Orange G, Sudan Red 7B and Bixin
- Extraction solvent: Acetonitrile
- No clean-up
- Instrument: HPLC-UV with C18 column and LC-MS for confirmation.
- Quantification limits: 106 ppb.



# FSA methods



FOOD  
STANDARDS  
AGENCY

## TLC method for palm oil

- Saponification
- Extracted by ether
- TLC determination
- Detection Limits: 10 ppm for Sudan I and II and 5 ppm for Sudan III and IV

[http://www.food.gov.uk/multimedia/pdfs/mb\\_052\\_dec2004.pdf](http://www.food.gov.uk/multimedia/pdfs/mb_052_dec2004.pdf)

## HPLC methods – 145A and 145B

- For Chilli powder/Crushed chilli
- Scope: Sudan I (but it can also applied to other Sudan dyes.)
- Extraction solvent: Industrial Methylated Spirit / MeOH
- No clean-up
- HPLC-UV-VIS detection.
- Detection Limits: to be assessed in the light of the collaborative trial.

<http://www.food.gov.uk/multimedia/pdfs/sudan145a.pdf>

<http://www.food.gov.uk/multimedia/pdfs/145b.pdf>



## International Testing Methods



**Government Chemist**

Government Chemist of LGC released two reports in October 2006 about the Analysis of Illegal Dyes in Chilli Powder.

### (1) By HPLC-UV

- Scope: Sudan I, II, III, IV, Para Red, Rhodamine B and Orange II
- Extraction Solvent: Acetonitrile: Acetone 9:1
- No clean-up
- Instrument: HPLC-UV with ODS column
- Detection Limits: 0.1 – 0.8 ppm.

<http://www.governmentchemist.org.uk/Publications.aspx?m=78&amid=385>



## (2) By LC-MS/MS

- Scope: Sudan I, II, III, IV, Sudan Red B, Sudan Red G, Sudan Red 7B, Dimethyl Yellow, Para Red, Toluidine Red, Rhodamine B and Orange II.
- Extraction Solvent: Acetonitrile
- No clean-up / SPE clean-up
- Sudan I-d5 and Sudan IV-d6 are used as internal standards.
- Instrument: ABI 4000 Qtrap with Phenomenex Synergi 4u Polar column.
- Sudan Orange G, Rhodamine B and Orange II with poor recovery.
- Detection Limits: 10 ppb

*“In summary this work indicates that there is **unlikely** to be a single generic procedure that is suitable for both screening and quantification of all the dyes in any one matrix or that covers the wide range of concentration required,.....**What is more feasible is that, once information on the contaminants in the sample are known, a selected method can be chosen from several available options for the purpose of quantitative analysis.** The likelihood is that, for trace level applications in uncommon matrices, the method chosen will include both **the standard addition techniques and LC-MSMS end analysis.**”*

<http://www.governmentchemist.org.uk/Publications.aspx?m=78&amid=386>



National Standard of the People's Republic of China  
中华人民共和国国家标准

GB

GB methods – GB/T 19681-2005

Scope: Sudan I, II, III, IV.

Extraction solvent: hexane

Use neutral alumina column to clean-up

Instrument: HPLC-UV with C18 column

Detection Limits: 10  $\mu\text{g}/\text{kg}$ .

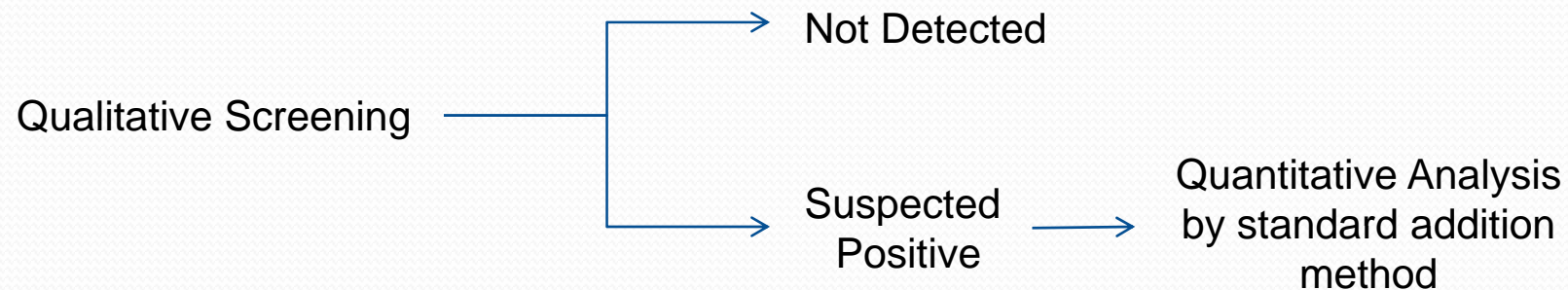
“在国标法中，处理样品的氧化铝活性柱的活度不易控制；食品种类繁多、机体复杂，如果色谱出现干扰峰，国标法也无法对其进行鉴定和确认。”

食品中苏丹红检测研究进展, 潘志明, 李忠, 王冰, 周路明,  
世界科技研究与发展, 2007年12月, 第29卷, 第6期 14—18页

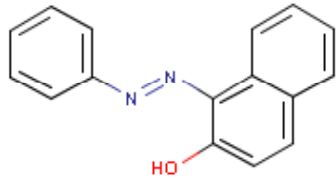


## GL method

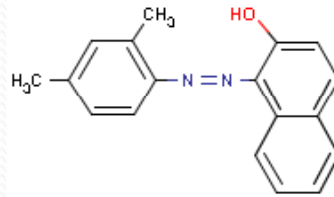
- As the concentration level found in egg is in ppb level, LC-MS/MS is used for the detection of Sudan dyes.
- Quite a number of literatures, commercial application notes and our experience indicates matrix calibration is needed due to matrix effect.
- Our approach is then to develop a fast and sensitive screening techniques and then quantify the content case by case by different techniques such matrix match calibration or standard addition method, etc.



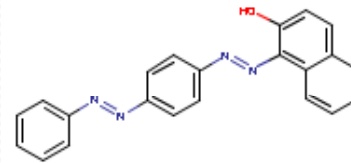
# Our Testing Parameters include:



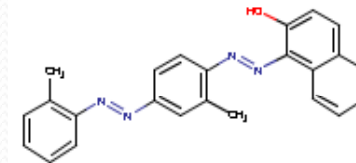
Sudan I  
1-Phenylazo-2-naphthol,  
C.I. 12055, C.I. Solvent Yellow 14.



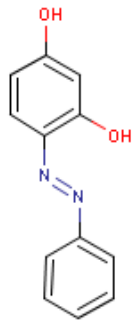
Sudan II  
Sudan Red,  
1-((2,4-Dimethylphenyl)azo)-2-naphthalenol,  
C.I. Solvent Orange 7, C.I. 12140.



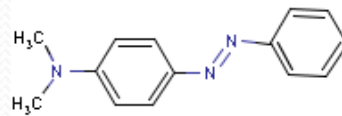
Sudan III  
1-(4-(Phenylazo)phenylazo)-2-naphthol,  
C.I. Solvent Red 23, C.I. 26100.



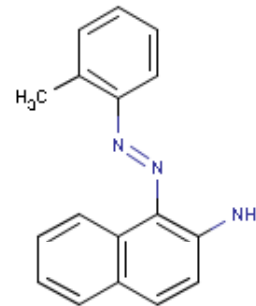
Sudan IV  
1-(2-Methyl-4-(2-methylphenylazo)phenylazo)-2-naphthol,  
Scarlet Red  
C.I. Solvent Red 24, CI 26105.



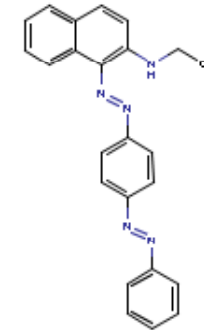
Sudan Orange G,  
4-(Phenylazo)-1,3-benzenediol,  
Oil Yellow GG,  
C.I. Solvent Orange 1,  
C.I. 11920.



Dimethyl yellow,  
4-(Dimethylamino)azobenzene  
Sudan Yellow, Sudan GG,  
Sudan Yellow GG,  
C.I. Solvent Yellow 2, C.I. 11020



Yellow OB,  
1-((2-Methylphenyl)azo)naphthalen-2-amine,  
C.I. Food Yellow 11,  
C.I. Solvent Yellow 6, C.I. 11390.



Sudan Red 7B  
N-Ethyl-1-(4-(phenylazo)phenylazo)-2-naphthylamine  
Fat Red 7B,  
C.I. Solvent Red 19, C.I. 26050.

The reporting limits: 20 ppb



# Sample Preparation

1. Weigh accurately about 5 g homogenised sample into a 50-mL centrifuge tube
2. Spike Internal Standard Solution into the sample.

For egg sample

1. Homogenise the sample thoroughly by stirring and then weigh accurately about 5 g sample into a 50-mL centrifuge tube.
2. Spike Internal Standard Solution into the sample. Put the centrifuge tube in boiling water bath and cook the sample thoroughly. Comminute the cooked egg into small pieces

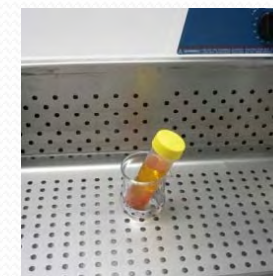


Sudan I-d5  
Sudan IV-d6



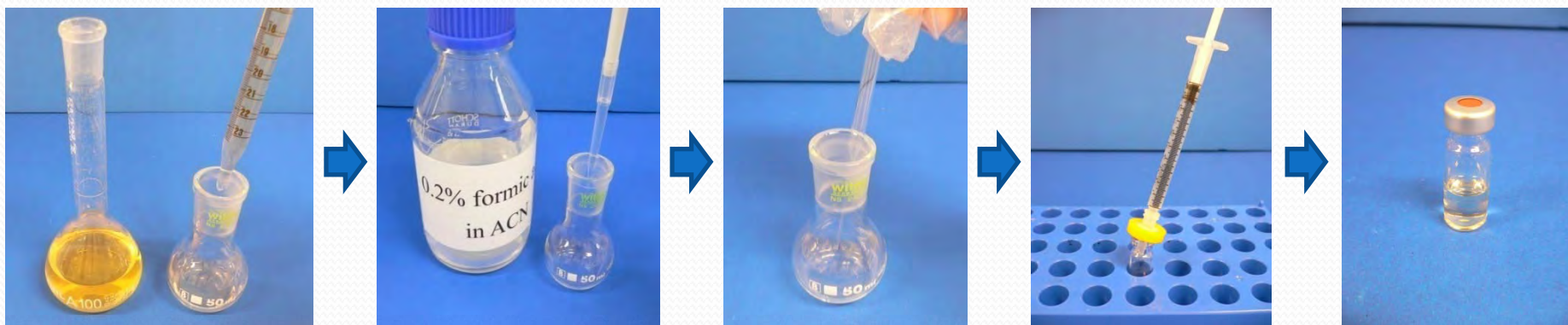
# Sample Preparation

3. Add about 40 mL acetonitrile to the centrifuge tube. Shake the content for 30 minutes and filter the solution through anhydrous sodium sulphate to a 100-mL volumetric flask.
4. Add about 40 mL acetonitrile to the centrifuge tube and then sonicate for another 60 minutes. Filter the solution through anhydrous sodium sulphate to the 100-mL volumetric flask.
5. Make up to volume with acetonitrile.



# Sample Preparation

6. Take 20 mL of extracted solution to a 50-mL round bottom flask and remove all solvent using rotary evaporator.
7. Reconstitute the residue with 1 mL 0.2% (v/v) formic acid in acetonitrile and then filter the solution to vial for LC-MS/MS determination.
8. Keep the vials in total darkness for about one and half hours before injection .



## Testing Method: HPLC condition for Sudan Dyes

- Column : Zorbax Eclipse XDB-C18 150 mm x 4.6 mm or other equivalent column.
- Mobile phase : A: 0.2% formic acid in water  
B: 0.2% formic acid in acetonitrile
- Gradient Program : A:B (30:70) to A:B(5:95) in 8 minutes
- Flow rate : 0.6 mL/min.
- Injection volume : 20  $\mu$ L



## Testing Method: Typical MS condition

MS/MS system	:	ABI 3200 Q-Trap
Ionization	:	Turbo Spray
Curtain Gas (CUR)	:	25
Ionization Voltage (IS)	:	5500
Temperature (TEM)	:	600
Gas 1 (GS1)	:	40
Gas 2 (GS2)	:	50
Collision Gas Supply (CAD)	:	Medium



## Testing Methods: HPLC condition for Sudan Dyes

Analyte	Purpose <sup>1</sup>	Parent Ion (Q1)	Daughter Ion (Q3)	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)
Sudan Orange G	D	215.1	93.2	33	7	37
	C	215.1	122.1	33	7	21
Dimethyl Yellow	D	226.1	77.1	40	7	33
	C	226.1	133.3	40	7	40
Sudan I	C	249.1	231.3	35	7	38
	D	249.1	93.3	35	7	40
Sudan I-d5 (IS) <sup>2</sup>	D	254.2	236.3	30	7	20
Yellow OB	C	262.1	130.2	36	8	50
	D	262.1	106.3	36	8	55
Sudan II	C	277.1	128.1	30	7	43
	D	277.1	121.2	30	7	25
Oil Yellow XP	D	307.2	121.2	46	11	31
	C	307.2	120.2	46	11	33
Sudan III	D	353.1	128.1	43	11	50
	C	353.1	197.1	50	11	30
Sudan Red 7B	D	380	183.2	30	6	25
	C	380	169.3	30	5	35
Sudan IV	D	381.3	106.1	50	7	50
	C	381.3	156.2	50	7	40
Sudan IV-d6 (IS) <sup>3</sup>	D	387.4	224.4	50	9	30

### 1. MRMs conditions (for reference only):

Note 1: D= Diagnostic Ion; C= Confirmation Ion.

Note 2: Internal standard for Sudan Orange G, Dimethyl Yellow, Sudan I, Yellow OB, Sudan II and Oil Yellow XP.

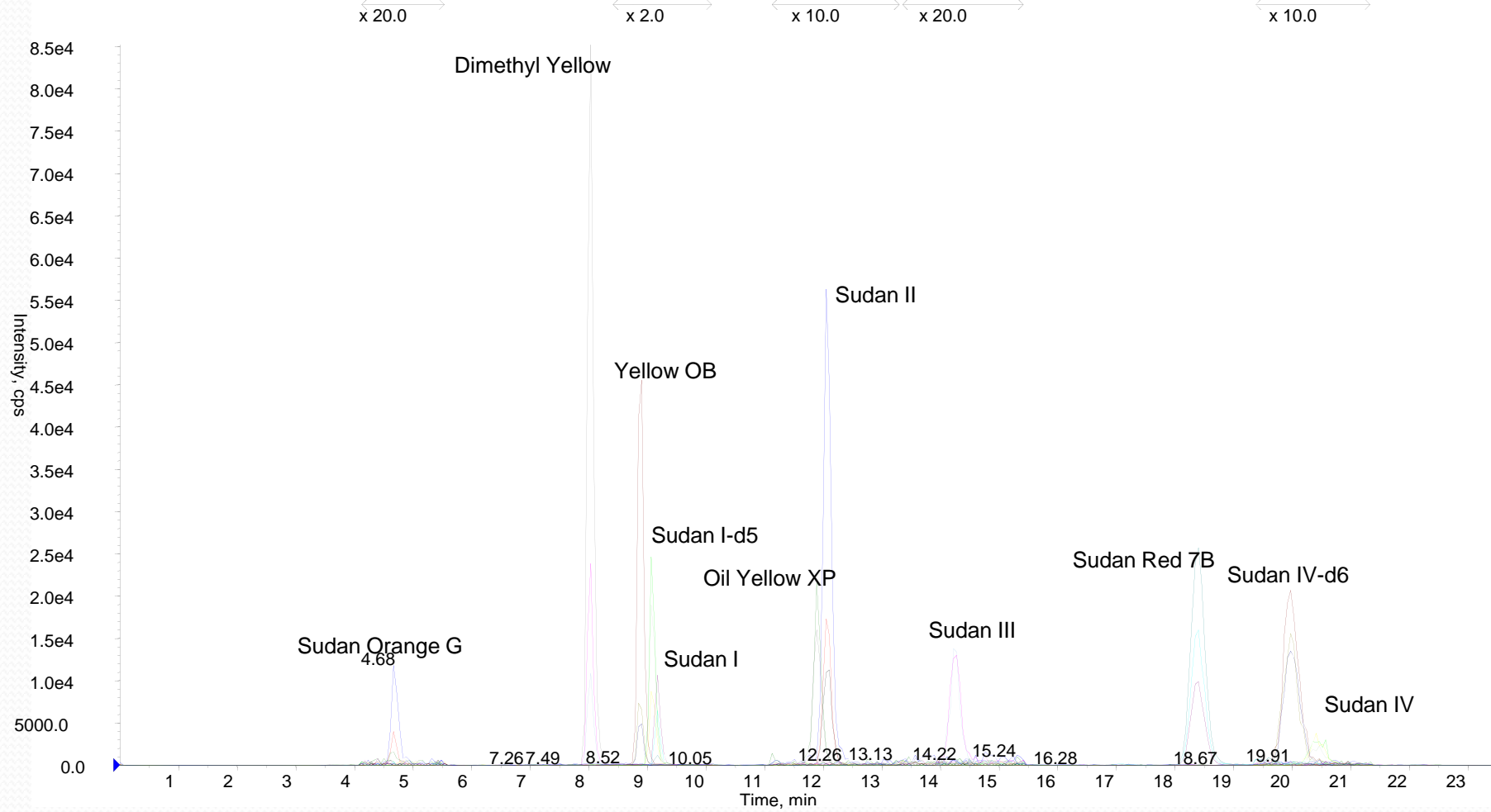
Note 3: Internal standard for Sudan III, Sudan red 7B, and Sudan IV.

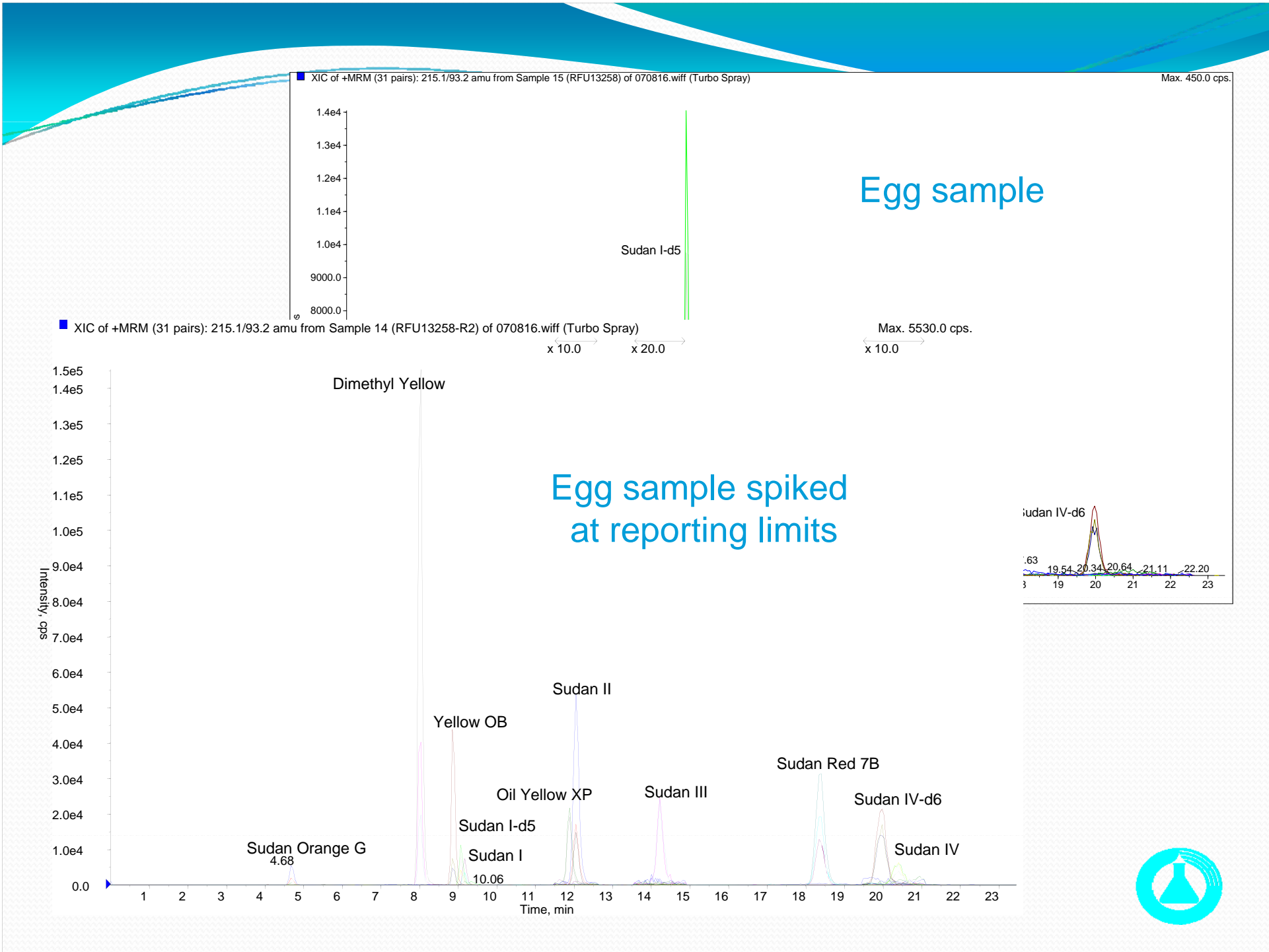


# 10 ppb standard

XIC of +MRM (31 pairs): 215.1/93.2 amu from Sample 7 (W070816Sudan1-2) of 070816.wiff (Turbo Spray)

Max. 590.0 cps.





# Result Interpretation

- Control point/Cut-off point

Peak Area/ Ratio  $<$  Control Point  $\rightarrow$  Not Detected

Peak Area/ Ratio  $>$  Control Point  $\rightarrow$  Suspected Positive



Ion Ratio Matching and  
Quantitative Determination





The control point will be determined by replicate injections of a low level standard.

(Prefer equivalents to reporting limits or lower.)

→ Control point = Peak Area Ratio x factor (F)

The value of factor (F) will be depended on the study of false positive rate and false negative rate during the validation process.

According to EU Decision 2002/657/EC  
False Negative Rate < 5% at the level of interest



## Validation Plan and Quality Control Plan

Spike 20 ppb of analytes to various matrix determine the control point and estimate the control point which giving less than 5% false negative rate.

QC plan in each batch:

- Spike 20 ppb analytes to the blank sample should be detected.
- IS peak area / signal-to-noise ratio should be greater than specified value.



# Identity confirmation

Ion ratio comparison based on the EU Commission Decision 2002/657/EC.

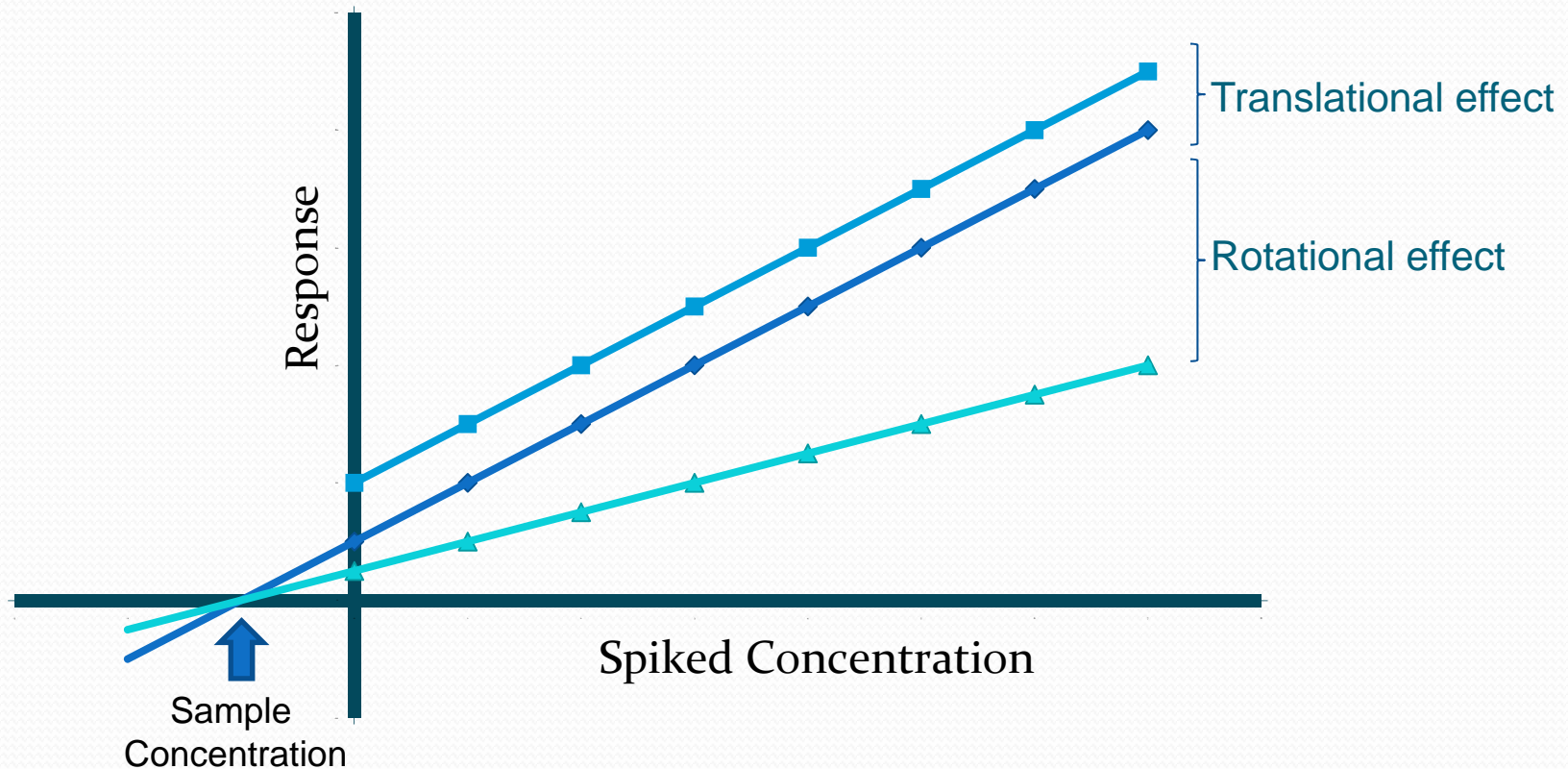
<http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2002:221:0008:0036:EN:PDF>

Average Ion-ratio based on the standards	Maximum permitted tolerances for I-ratio in sample
>50%	±20%
20%-50%	±25%
10%-20%	±30%
≤10%	±50%



# Standard Addition Method

## Eliminate rotational matrix effects

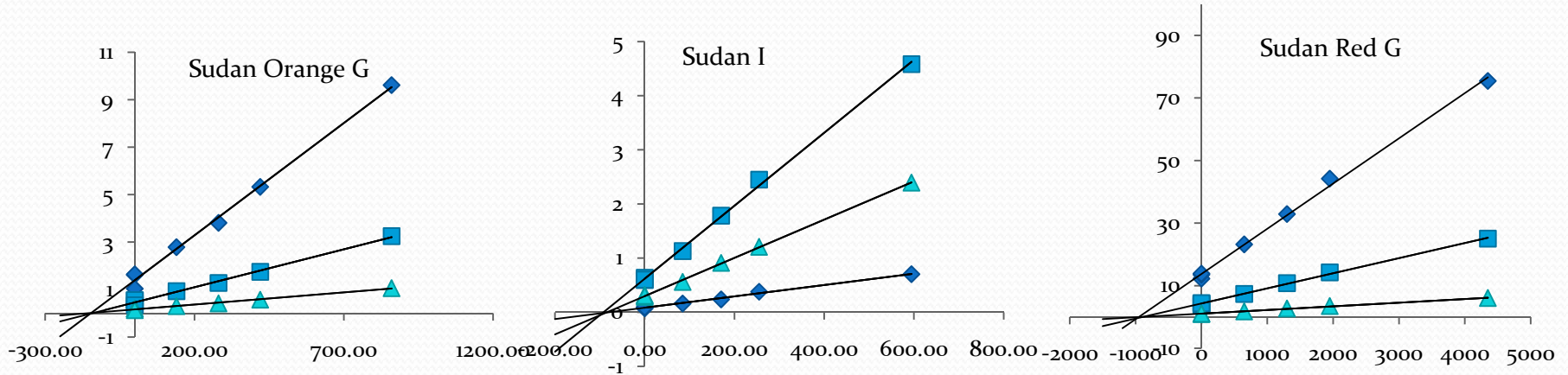


Measurement is followed by extrapolation of the calibration line to zero response



# Example: Standard Addition – FAPAS 2060

## Tabasco Sauce

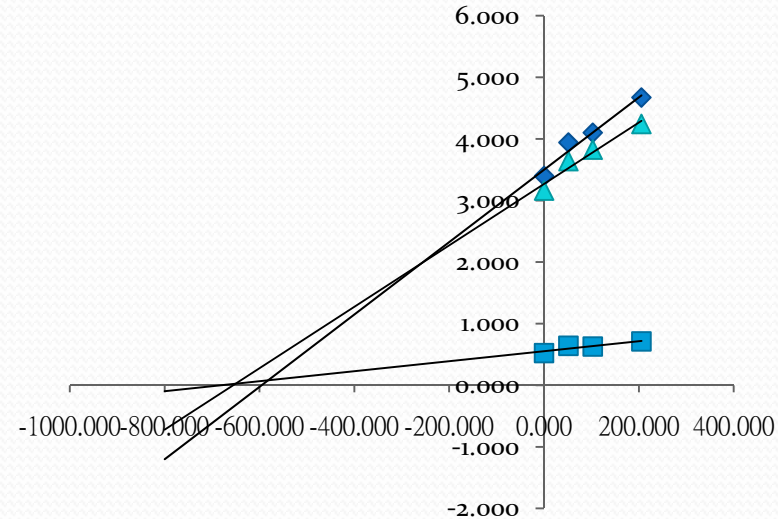
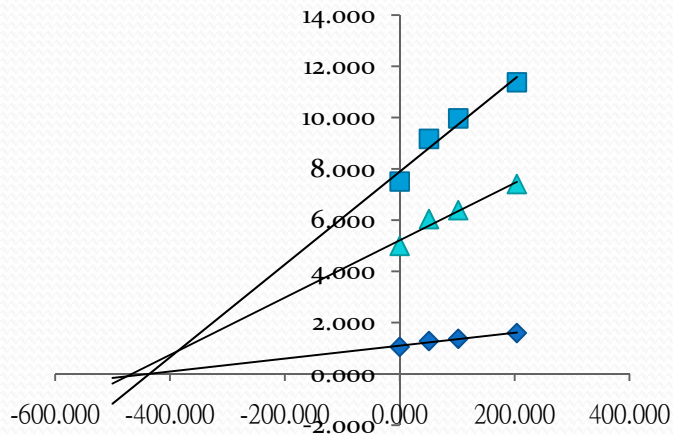


	Sudan Orange G 215.1 / 93.2	Sudan Orange G 215.2 / 122.0	Sudan Orange G 215.2 / 197.2	Sudan I 249.1 / 231.3	Sudan I 249.1 / 93.3	Sudan I 249.1 / 156.1	Sudan Red G 279.3 / 123.3	Sudan Red G 279.3 / 156.0	Sudan Red G 279.3 / 247.0
Slope	0.009	0.003	0.001	0.001	0.007	0.004	0.014	0.005	0.001
Intercept	1.392	0.457	0.161	0.084	0.619	0.297	13.732	4.310	1.068
R	0.997	0.997	0.996	0.997	0.999	1.000	0.999	0.999	0.999
Concentration of FAPAS 2060 221 (µg/kg)	147.105	143.169	157.534	79.979	91.896	84.027	950.242	891.985	914.796
Single Point Standard addition	150.43	147.56	166.75	84.24	91.52	85.88	922.65	873.92	891.37
Assigned value (µg/kg)	Detected			93.6			782		
Acceptable Range (µg/kg)				56.2-131			469-1094		



# Example: Standard Addition – FAPAS 2068

## Tabasco Sauce



	<b>Sudan I</b> 249.1 / 231.3	<b>Sudan I</b> 249.1 / 93.3	<b>Sudan I</b> 249.1 / 156.1	<b>Para Red</b> 294.1 / 156.2	<b>Para Red</b> 294.1 / 137.8	<b>Para Red</b> 294.1 / 128.2
R	0.981	0.976	0.979	0.981	0.910	0.976
Slope	0.003	0.018	0.011	0.006	0.001	0.005
Intercept	1.100	7.889	5.211	3.500	0.553	3.270
Concentration found in sample (µg/kg)	435.93	435.73	465.96	595.72	680.98	655.75
Assigned Value (µg/kg)	418.00			651.00		
Acceptable Range (µg/kg)	251-585			391-912		



# Extrapolation from Calibration Curve vs. Spike at one level

Standard Additions: myth and reality, Michael Thompson, Stephen L.R. Ellison, *Analyst* **2008**, 133, 992-997.

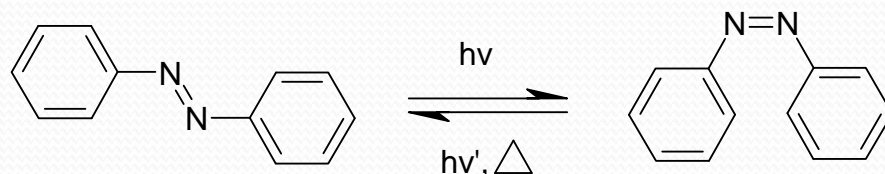
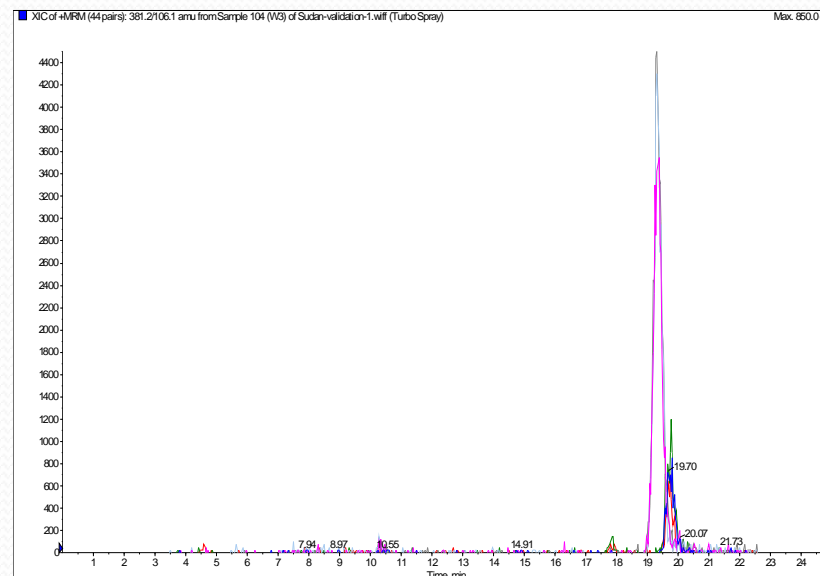
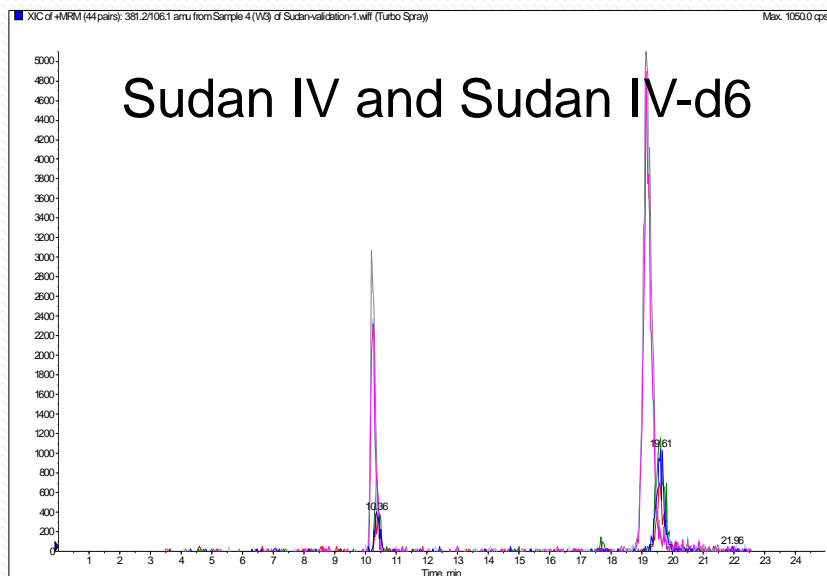
A review of interference effects and their correction in chemical analysis with special reference to uncertainty, Michael Thompson, Stephen L.R. Ellison, *Accred Qual Assur* **2005**, 10, 82-97.

## Their Recommendations

- Effective Linear over the whole of required concentration range
- No Translational Effect
- Use only one level of added analyte with repeated measurement
- At least four times the concentration of the analyte if possible.



# Note: Early peaks of Sudan III and Sudan IV.



## *E-Z isomerization*

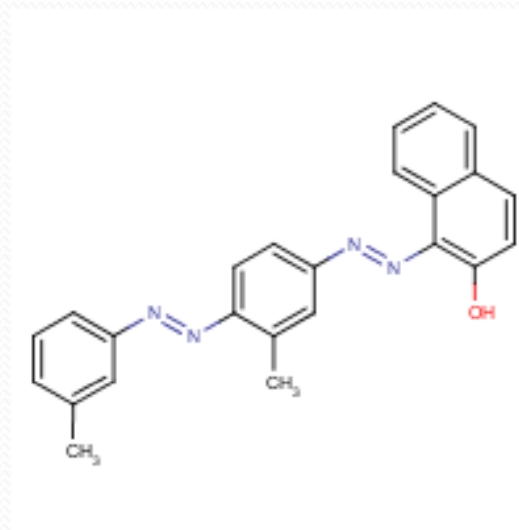
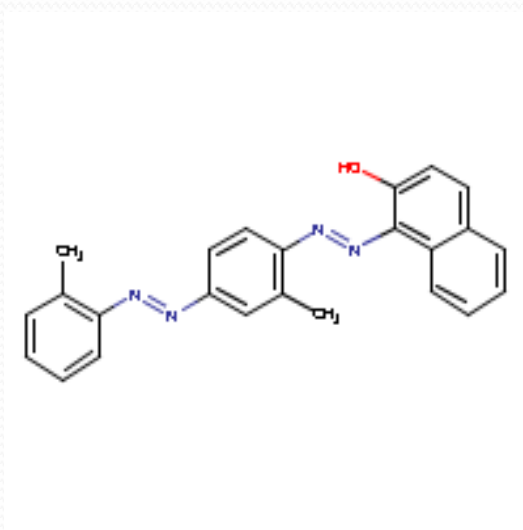
“Fast peaks” in chromatograms of Sudan dyes,  
Kadi Molder, Allan Kunnapas, Koit Herodes, Ivo Leito, *J Chromatogr A* **2007**, 1160, 227-234.

Analysis of Illegal Dyes in paprika Powder by LC-MS/MS,  
Statutory Analysis Government Chemist Program, Ad Hoc Project 1, Oct 2006, LGC/GC/2007/006.



## Note: Confirmation of Standard Identity

### Sudan IV and Sudan Red B



Same molecular mass  
Same daughter ions  
Very close retention time



# Development of the Solid Phase Extraction method

Preliminary Trial for some curry powder samples

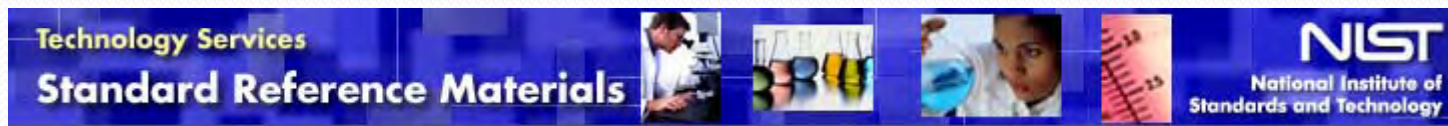
Extract the dyes as normal  
20 mL of the extract + 5 mL hexane + 25 mL 12.5% methanol in 200 g/L  
NaCl solution  
Shake and centrifuge

Use Silica SPE column, 500mg/4.0 mL  
Condition with 3 mL diethyl ether  
Condition with 6 mL hexane  
Load 2 mL of the hexane layer to column  
Wash with 3 mL hexane  
Elute with 5 mL 10% acetone in hexane

.  
. .  
. . .



# Certified Reference Materials



## SRM 2037 - Solvent Red 24 Diesel Fuel Dye



**BW3524 Sudan I**  
**BW3525 Sudan II**  
**BW3526 Sudan III**  
**BW3527 Sudan IV**  
**BW3538 Sudan Red 7B**



# Proficiency Test



Qualitative or Quantitative Determination of Illegal Dyes

<http://www.fapas.com>

Government Laboratory will organize a proficiency test for Sudan Dyes in chilli powder in Dec 2009 (tentatively)



This presentation is for your reference only.  
If you have any questions / discussion, you can e-mail to  
[mflo@govtlab.gov.hk](mailto:mflo@govtlab.gov.hk)

Thank you

