LC-MS/MS ANALYSIS OF AZO DYES IN SALTED DUCK EGGS WITH FAST SAMPLE CLEAN-UP

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INTRODUCTION

Azo dyes are industrially used to stain fats, fabric and plastics and are banned from food products as they can be converted to carcinogenic amines and other metabolites[1]. Sudan I-IV have been named as unclassifiable to their carcinogenicity to humans (Group 3) by the International Agency for Research on Cancer[2] (IARC).

The dyes occured in eggs as a result of poultry having been fed with feed containing the dyes to obtain intense stained egg yolks. As Sudan dyes are lipophilic and egg yolk contains high amounts of lipids, a purification from this matrix is challenging. Additionally, phospholipids from egg yolk can interfere with mass spectrometrical detection by ion suppression.

A specialty in Asia is salted duck egg, produced by long storage of raw eggs in brine or salted charcoal, resulting in a matrix with high phospholipid and high salt content. The analysis of this matrix has been challenging and to our knowledge data showing robustness of mass spectrometrical detection of Sudan dyes in salted eggs over a couple of hundred injections is not available. Here we show a method to handle this challenging analysis, providing a starting point for further method development for the analysis of egg yolks in food safety testing.

METHODS

UPLC Conditions

LC system:	Waters ACQUITY UPLC® H-Class with FTN
Column:	ACQUITY BEH C18 2.1 x 100 mm, 1.7 µm
Column temp.:	45°C
Auto-sampler temp.:	18°C
Injection volume:	2 µL
Flow rate:	0.4 mL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile/ methanol (1/1, *v/v*)
Prime:	0.1% formic acid in water
Wash:	0.1% formic acid in acetonitrile
Seal wash:	10% methanol in water
Gradient (curve 6):	Initial: 20% B, 0.50 min: 60% B, 5.00 min 100% B, 9.0 min: 100% B, 9.01 min: 20% B, 12 min 20% B

MS Conditions

MS system:	Xevo® TQ-S micro
Ionization mode:	ESI+
Capillary voltage:	2.75 kV
Desolvation temp:	500 °C
Desolvation gas flow:	900 L/Hr
Source temp.:	150 °C

SAMPLE PREPARATION

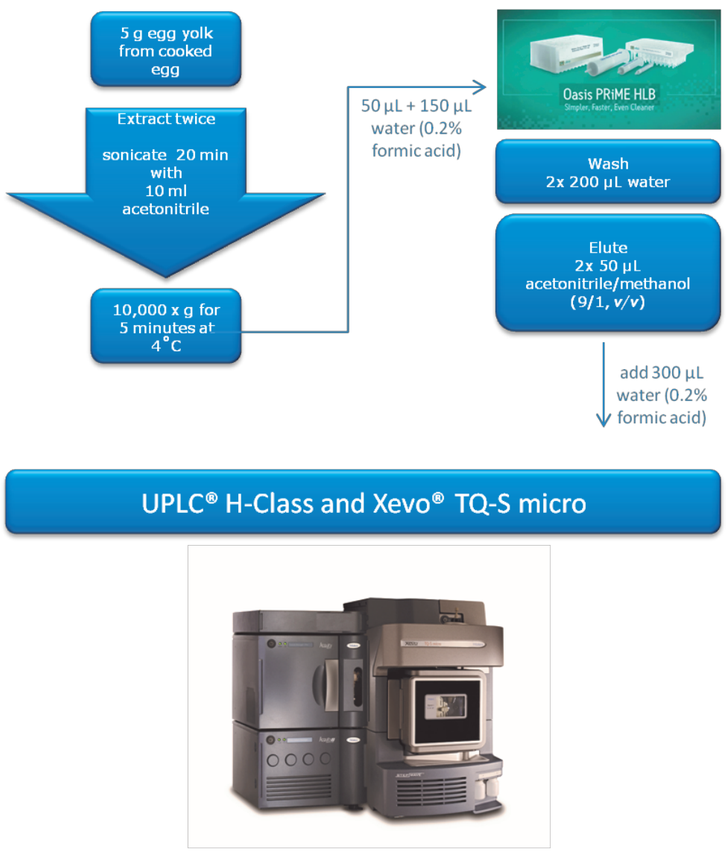

Figure 1. Workflow for the sample preparation of egg yolk extract with the Oasis® PRiME HLB µElution plate. Formic acid and water were added before applying the sample on the column to precipitate proteins and after the elution to adjust the pH to the UPLC conditions.

DETECTION OF PHOSPHOLIPIDS

Phospholipids were detected in MRM mode with the above mentioned instrument settings using a cone voltage of 20 kV and a collision energy of 30 eV. All analyzed compounds shared the fragment with *m/z*: 184.40. The precursor ions had the following *m/z*-ratios: 496.40; 520.40; 522.40; 524.40; 704.40; 758.40; 760.40; 784.40; 786.40; 806.40; and 808.40.

RECOVERY AND REPRODUCIBILITY

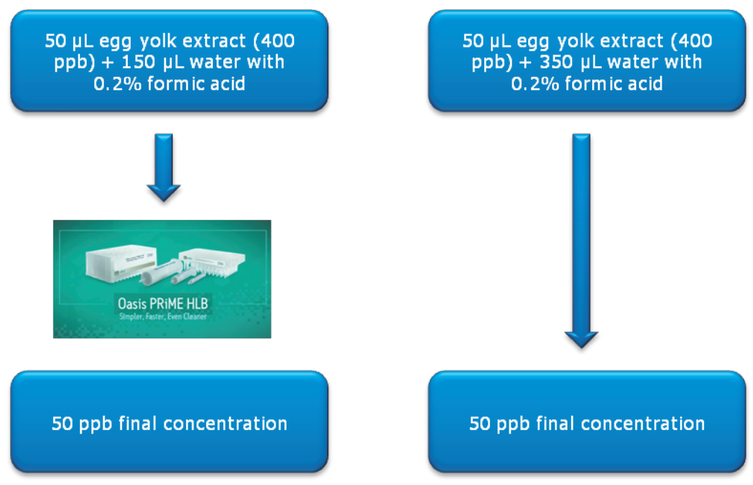

Figure 2. Experimental set-up for the investigation of recovery and reproducibility of azo dyes from salted duck egg yolk extract.

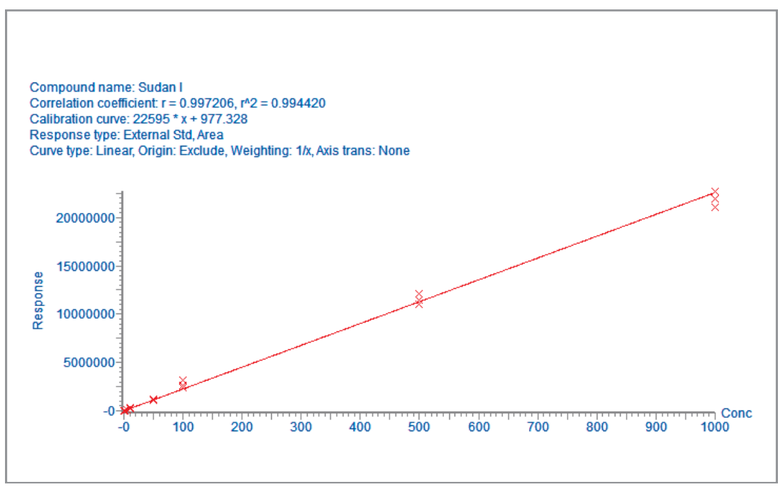

Figure 3. Calibration curve of Sudan I in matrix from 0.5-1000 ppb.

RESULTS

Name	CAS number	Precursor [*m/z*]	CV [V]	Product [*m/z*]	CE [V]	RT [min]
Dimethyl yellow	60-11-7	226.07	8	76.93	22	4.63
				120.01	32	
Sudan I	842-07-9	249.07	2	92.94	22	5.21
				127.97	26	
Sudan I-d_5	752211-63-5	254.07	2	97.94	22	5.21
				127.97	26	
Sudan II	3118-97-6	277.13	16	120.86	20	6.09
				105.96	42	
Sudan III	85-86-9	353.10	20	76.93	28	6.50
				91.83	36	
Orange G	1936-15-8	215.07	4	93.00	18	3.64
				121.96	16	
Para Red	6410-10-2	294.07	28	155.99	16	4.79
				127.91	30	
Rhodamine B	81-88-9	443.20	2	355.19	68	4.00
				341.14	68	
Sudan IV	85-83-6	381.17	20	90.98	28	7.13
				105.82	38	
Sudan IV-d_6	1014689-18-9	387.17	20	90.98	28	7.13
				105.82	38	
Red 7B	6368-72-5	380.15	10	183.11	14	6.90
				114.99	46	
Red G	1229-55-6	279.10	6	122.93	16	5.14
				107.96	32	
Black B	4197-25-5	457.20	10	246.39	24	6.88
				142.00	38	

Table 1: MRM transitions, cone voltages (CV), collision energies (CE) and retention times (RT) of the acquired dyes on TQ-S micro in ES+. Rhodamine B is a polar dye, which does not belong to the azo dye group and was chosen to investigate the suitability for of the method for polar compounds.

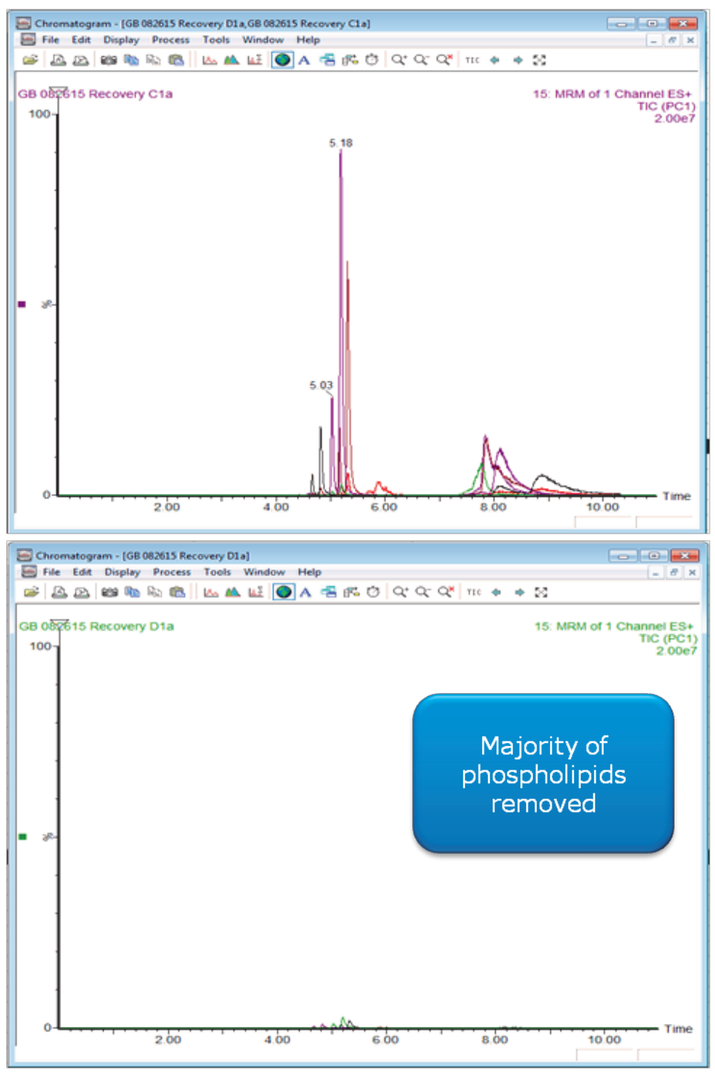

Figure 3. TIC overlay of detected phospholipid MRM transitions. Upper image unpurified egg extract, which was diluted with 50 µL ACN/MeOH (9:1) and 300 µL water with 0.2% formic acid, lower image: egg extract purified with Oasis® PRiME HLB µElution plate.

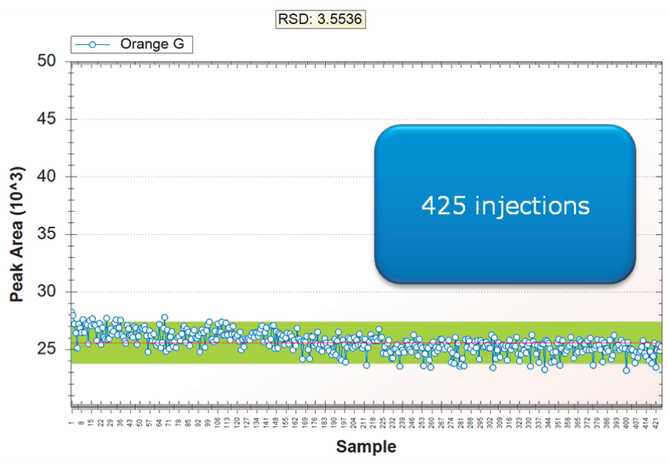

Figure 4. Peak area of consecutive 425 injections of post spiked matrix after Oasis® PRiME HLB µElution plate clean-up sample with a concentration of 10 ppb of eleven dyes in matrix (2µL). Data shown for Orange G. Data processing with Trendplot® function in TargetLynx® XS software. The RSD for the same data set for retention times was 0.05%.

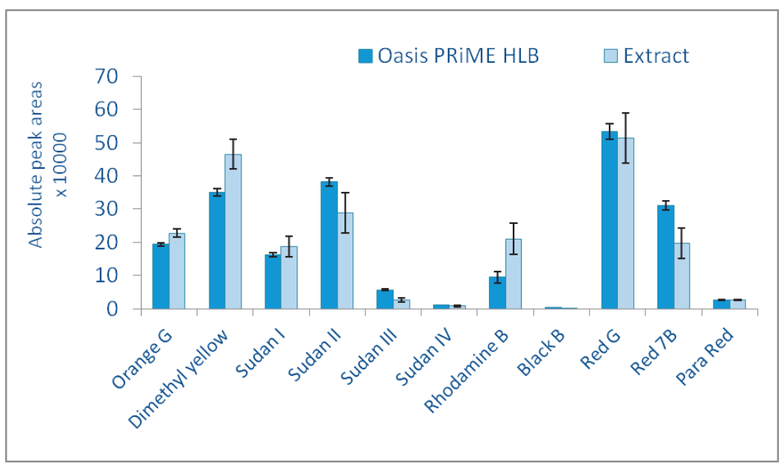

Figure 5. Recovery and reproducibility of dyes using the Oasis® PRiME HLB µElution plate compared to unpurified salted duck egg yolk extract. Standard deviation of the mean (n=7).

Limit of detection

Limit of detection (LOD) in parts per billion (ppb) of the investigated Sudan dyes. LOD is the lowest calibration point which gave a signal to noise ratio of at least 3 (S/N>3): Sudan I: 0.5; Sudan II: 0.5; Sudan III: 1.0; Sudan IV: 5; Orange G: 0.05; Dimethyl Yellow: 0.05; Red G: 0.50; Para Red: 0.50; Red 7B: 0.50; Rhodamine B: 0.05; Black B: 50.00.

DISCUSSION

A method for the analysis of azo dyes in salted duck egg yolk using Oasis® PRiME HLB µElution plate has been developed which enables the simultaneous clean-up of 96 samples.
As no conditioning or equilibration steps and no drying of the eluate are required, the sample preparation can be carried out much faster than conventional solid phase extraction protocols. Through simultaneous removal of phospholipids and salts which could interfere with mass spectrometrical detection, LOD values in the low ppb range can be achieved.

The new approach of cooking of the eggs prior to extraction has been found to advantageous over using the uncooked egg yolk and hence azo dyes are heat stable it is not expected to affect the detection of the analytes. The ratio of water and acetonitrile extract as well as the pH and temperature influence the precipitation of proteins in the sample. Adjusting the water content close to the initial UPLC conditions as well as keeping the temperature of the auto-sampler to room temperature were found to be critical parameters of the sample preparation to maintain system performance.

To our knowledge no robustness data over several hundred LC-injections of matrix containing egg yolk extract has been published previously. A combination of sample preparation, low injection volume and active removal of neutrals through the Stepwave off-axis ion source technology on the Xevo® TQ-S micro contribute to the high robustness of the method achieving lower LOD values for Sudan I-IV compared to another UPLC-MS/MS method (10 µL injection volume, LOD 10 µg/kg) with a simpler sample preparation on an older system[3].

CONCLUSION

- **Purification of salted duck egg extracts with Oasis® PRiME HLB µElution plate allows fast phospholipid removal and desalting with high robustness**
- **Quantification with high sensitivity**
- **Promising starting point for further method development with Oasis® PRiME HLB product range**

References

1. Sudan dyes: are they dangerous for human health?, Fonovich TM, Drug and Chemical Toxicology, 2013 Jul;36(3):343
2. IARC Monographs on the evaluation of carcinogenic risks to humans, Overall evaluation of carcinogenicity, An updating on IARC Monograph Volumes 1-42, Supplement 7, World Health Organisation, International Agency for Research on Cancer, Paris, 1987 March
3. Determination of Sudan Red I–IV in Duck Egg Yolk Using Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry, Zhenxia DU, Shuqi SUN, Chinese Journal of Chromatography 2007 Sept; 25(5):705 –710

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