Quantitation and Identification of 13 Azo-dyes in Spices using LC-MS/MS

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Overview

This application note describes a new and simple method including extraction, HPLC separation and MS/MS detection for the analysis of 13 different azo-dyes in spices using Multiple Reaction Monitoring (MRM) on a 3200 QTRAP® LC/MS/MS System. The developed method is available as an iMethod™ test for Cliquid® Software which can be used for the analysis and automatic reporting. In addition the results of a study of ion suppression in various spice matrices comparing quantitation with solvent standards, matrix matched standards and standard addition are presented. Standard addition gave highest accuracies while quantifying azo-dyes in extracts of spices.

Introduction

The International Agency for Research on Cancer (IARC) classified azo-dyes as potential carcinogenic substances. After oral uptake azo-dyes can be reduced to amines which are classified as partially carcinogenic substances. As a result various azo-dyes are banned as food additives and maximum residue levels exist in several countries.

Methods described in literature apply GC-MS, LC-UV, LC-MS and LC-MS/MS to analyze azo-dyes.[1] Extensive sample preparation is typically necessary to achieve required limits of quantitation (10 µg/kg).

Spices are very complex, concentrated and variable matrices and matrix effects (ion suppression or ion enhancement) can be very strong and can depend on the origin of the spice sample. Ideally, isotopically labeled internal standards of all azo-dyes should be used to improve accuracy of detection in unknown samples, but such internal standards are not available. Three different possibilities to quantify unknown samples (calibration with solvent standards, calibration with matrix matched standards and standard addition) were investigated. The results were compared regarding their accuracy when analyzing azo-dyes in different spice matrices.

Figure 1 illustrates theoretical calibration curves obtained using these three different procedures.

In general, when using a calibration curve the signal intensity of an unknown sample is compared to an external set of standard samples.

These standards can be prepared in solvent or in matrix. The smaller slope of the calibration curve with matrix matched standards in comparison to solvent standards in Figure 1 indicates ion suppression effects.

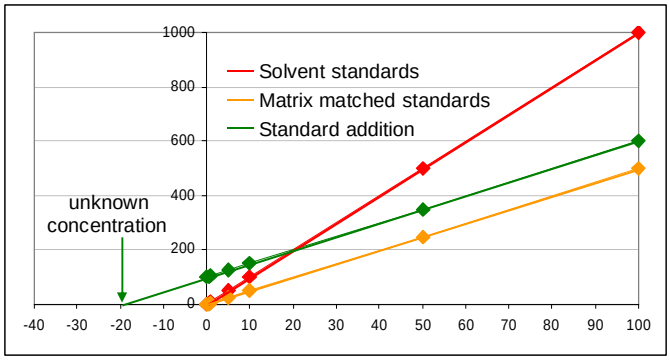

Figure 1. Calibration curves using solvent standards, matrix matched standards, and standard addition

When using standard addition, defined concentration(s) of pure standards are added to aliquots of the unknown sample. These standards, along with an aliquot which does not contain any added standard, are analyzed. The resulting calibration curve is extrapolated and the absolute value of the intercept with the concentration axis determines the concentration of the target compound in the unknown sample as shown in Figure 1. Generally, standard addition requires more time for analysis because one calibration curve per unknown sample and per analyte has to be prepared. However, standard addition can be used to solve the matrix effect problem because all analytes are quantified in the matrix itself.

Experimental

Chemicals

Solvents, reagents and dye standards were obtained at highest available purity from Sigma-Aldrich (dye content 80-98%). Internal standards (D_5-Sudan I and D_6-Sudan IV) were obtained from WITEGA laboratories (Berlin, Germany). Stock solutions were prepared in acetonitrile freshly due to degradation of some azo-dyes. Solvent standards were diluted in the starting mobile phase.

Spice Samples

Spice samples were purchased on local markets in India (Garam Masala), Korea (Red Chili), and Egypt (Saffron) and analyzed by LC-MS/MS. Not one of the 13 investigated azo-dyes was detected in the selected spice samples.

Matrix matched standards were prepared in Garam Masala extract. In addition every matrix was spiked with known concentrations of a mix of azo-dyes prior to analysis. These samples were used to investigate standard addition.

Sample Preparation

The goal was to develop a generic sample preparation procedure that is easy extendable to other emerging azo-dyes.

1. Weigh 1 g of homogenized sample (multiple times for standard addition).
2. Add 20 µL of internal standard solution (1 µg/mL of D_5-Sudan I and D_6-Sudan IV).
3. Add standard solution(s) in case of standard addition.
4. Add 10 mL of acetonitrile.
5. Shake for 10 min.
6. Add 10 mL of water.
7. Shake and centrifuge (or filtrate) before injection.

HPLC

The goal was to develop a flexible HPLC method to separate a variety of emerging dyes. A gradient of 30 min was chosen to allow sufficient separation of analytes from matrix components. This method can be shortened easily, but matrix effects might increase significantly. No HPLC conditions could be identified for the separation of the two isomeric dyes Sudan IV and Sudan Red B, although various columns (C_8 and C_{18}), mobile phases (water, methanol, acetonitrile), buffers (ammonium formate, ammonium acetate, formic, and acetic acid), and pH values were investigated.

An Agilent 1100 HPLC system with binary pump (without static mixer), well plate autosampler, and column oven was used. A Phenomenex LUNA 5u C8,150x2 mm column and a gradient of eluent A: water + 0.2% formic acid + 2 mM ammonium formate and eluent B: water/acetonitrile (10/90) + 0.2% formic acid + 2 mM ammonium formate was used at a flow rate of 300 µL/min. Details of the gradient are given in Table 1. The column oven temperature was set to 30°C. A volume of 50 µL of each sample was injected.

Table 1. HPLC gradient

Step	Total Time (min)	A (%)	B (%)
0	10	80	20
1	15	0	100
2	29	0	100
3	30	80	20

MS/MS

A 3200 QTRAP® LC/MS/MS System equipped with Turbo V™ Source and Electrospray Ionization (ESI) probe was used. ESI was found to be suitable for the ionization of azo-dyes. The ion source temperature (450°C) was optimized for the highest sensitivity of Orange II and Para Red, the two compounds showing lowest sensitivity in positive polarity. Two MRM transitions were monitored per analyte to allow quantitation and identification using ion ratios (Table 2).

Two additional MRM transitions were detected for Sudan IVand Sudan Red B to allow differentiating between both co-eluting and isomeric compounds.[2]

Table 2. MRM transitions, retention times (t_R), of detected azo-dyes and signal-to-noise (S/N) of the qualifier MRM transition at a concentration of 10 ng/mL

Analyte Name	CAS	Q1 (amu)	Q3-1 (amu)	Q3-2 (amu)	Q3-3 (amu)	Q3-3 (amu)	t_R (min)	S/N at 10ng/mL
Dimethyl Yellow	60-11-7	226.1	120.1	105.1	-	-	14.5	980
Fast Garnet GBC	97-56-3	226.1	91.1	107.1	-	-	13.5	300
Orange II (positive)	633-96-5	329.1	156.0	128.0	-	-	13.0	30
Orange II (negative)	633-96-5	327.0	171.0	80.0	-	-	13.0	220
Para Red	6410-10-2	294.1	156.1	128.1	-	-	14.2	300
Rhodamine B	81-88-9	443.2	399.1	355.1	-	-	8.7	10600
Sudan I	842-07-9	249.1	93.0	156.1	-	-	15.0	500
Sudan II	3118-97-6	277.1	121.1	106.1	-	-	16.6	1090
Sudan III	85-86-9	353.1	197.1	128.1	-	-	17.4	200
Sudan IV	85-83-6	381.1	224.1	225.1	143.1	104.1	18.8	80
Sudan Orange G	2051-85-6	215.1	93.1	122.1	-	-	11.8	310
Sudan Red 7B	6368-72-5	380.2	183.1	115.1	-	-	18.9	1860
Sudan Red B	3176-79-2	381.2	224.1	225.1	156.1	134.1	18.8	140
Sudan Red G	1229-55-6	279.1	123.1	108.1	-	-	14.7	1910
D5-Sudan Red I		254.1	156.0	-	-	-	14.9	-
D6-Sudan Red IV		387.1	106.0	-	-	-	18.7	-

Results and Discussion

Standard chromatograms in positive and negative polarity using Electrospray Ionization are given in Figures 2 and 3. Orange II had ~10 times higher sensitivity in negative polarity.

The method developed provides enough sensitivity to detect all 13 azo-dyes at required concentration of 10 µg/kg in matrix. This is indicated by Signal-to-Noise ratios (S/N) calculated using 3x standard deviation (Table 2). The complete range of linearity was not of interest for this study. Only the range from one order below to one order above the level of 10 µg/kg was investigated. The following quality control parameters were observed: r^2>0.99 with accuracy between 90-110% for each concentration and %CV<15% at 1 µg/kg and <5% at 10 µg/kg (n=3).

Matrix effects and how to compensate them using different calibration procedures were investigated. A comparison of accuracies based on calibration with solvent standards, matrix matched standards and standard addition is summarized in Table 3. Values ~100% indicate that no matrix effects occur or that they were compensated completely. These results indicate that ion suppression varies strongly depending on the spice matrix. Using a calibration curve based on solvent standards did not provide sufficiently accurate data when analyzing spices. A calibration curve based on matrix matched standards provided more accuracy and can be used when matrices of similar composition have to be analyzed. But standard addition provided the best accuracy and is highly recommended if a broad range of complex matrices, such as different spices, have to be analyzed.

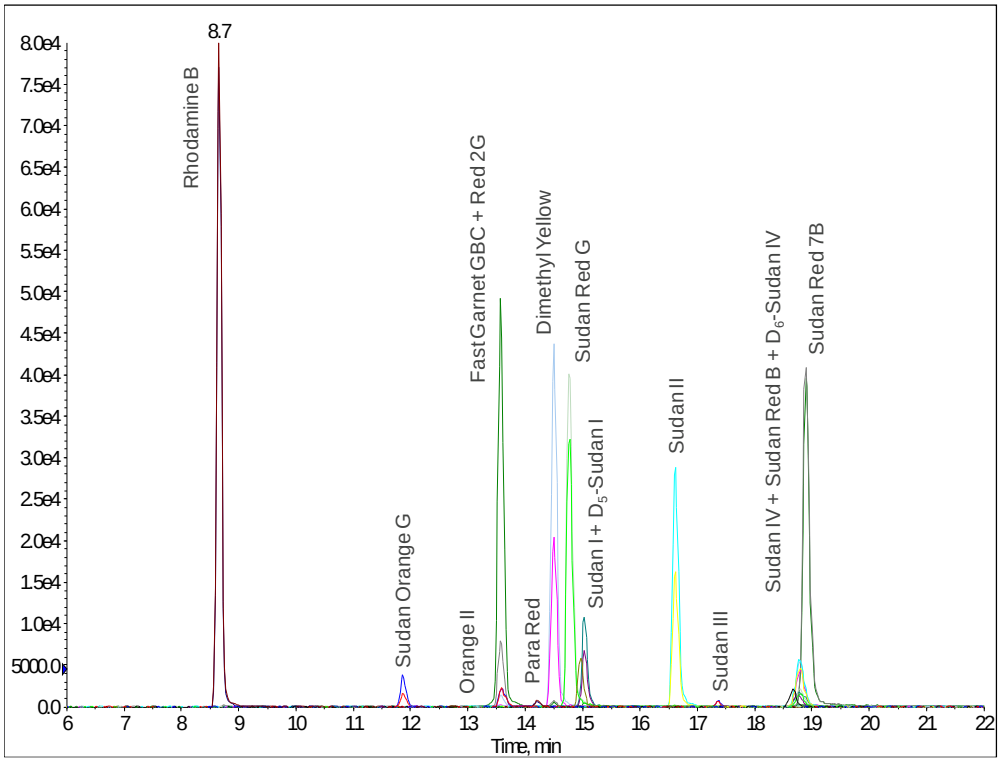

Figure 2. Detection of 13 selected azo-dyes in positive polarity

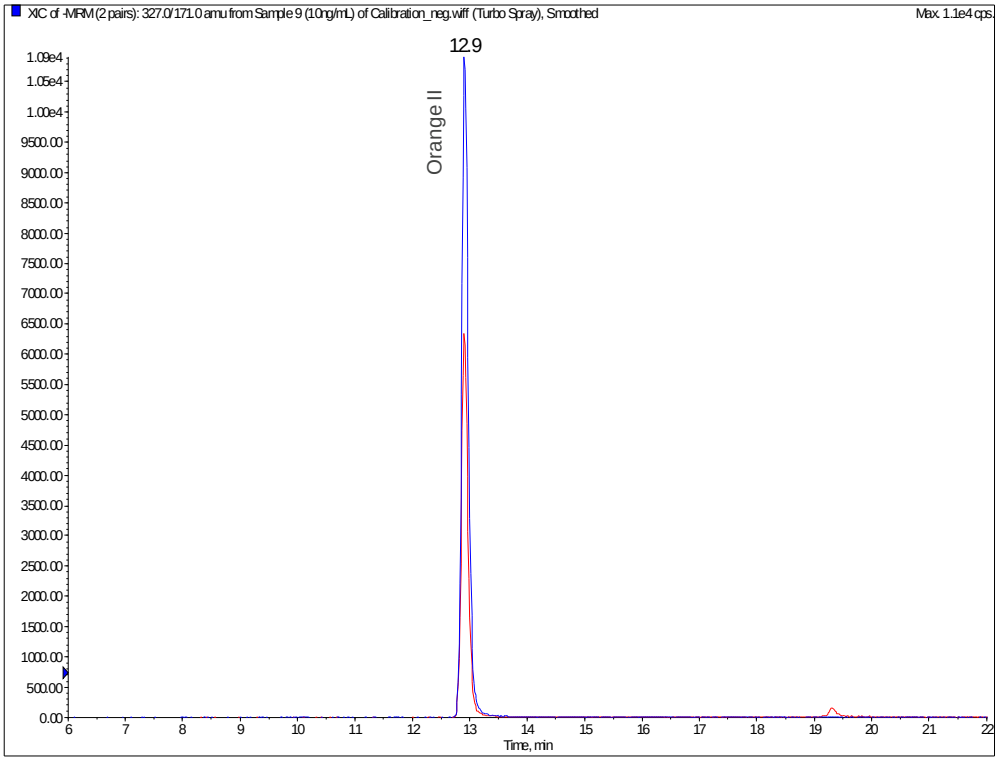

Figure 3. Detection of Orange II in negative polarity

Table 3. Accuracy of quantifying azo-dyes in 3 different spice matrices using calibration with solvent standards, matrix matched standards (prepared in Masala extract), and standard addition

	Solvent Standards			Matrix Matched Standards (Prepared in Masala Extract)			Standard Addition		
Analyte Name	**Masala**	**Chili**	**Saffron**	**Masala**	**Chili**	**Saffron**	**Masala**	**Chili**	**Saffron**
Dimethyl Yellow	10%	43%	20%	96%	288%	143%	96%	97%	95%
Fast Garnet GBC	36%	67%	51%	97%	195%	143%	97%	99%	94%
Orange II (positive)	25%	24%	29%	101%	101%	150%	101%	82%	95%
Rhodamine B	52%	44%	47%	101%	73%	95%	101%	89%	104%
Sudan I	47%	77%	48%	100%	176%	105%	100%	91%	110%
Sudan II	35%	44%	34%	97%	126%	104%	97%	94%	108%
Sudan III	66%	80%	53%	97%	133%	81%	97%	98%	111%

The colors represented in this table reference the data from the calibration curves in Figure 1.

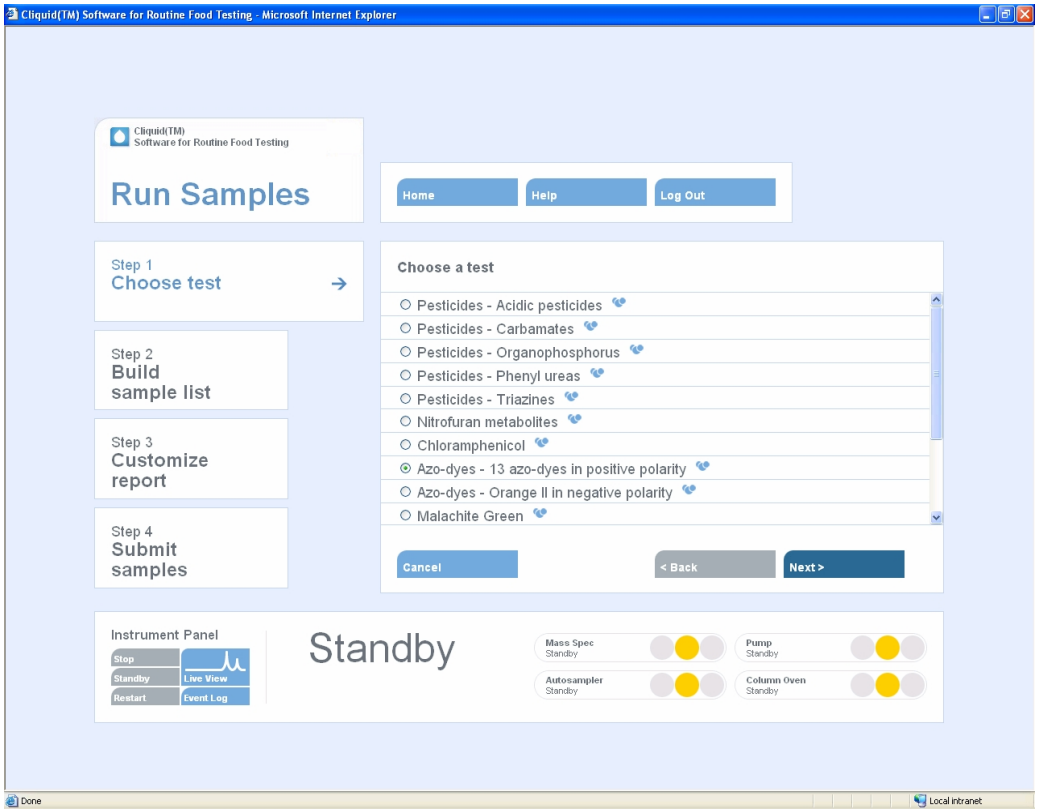

Figure 4. Cliquid® Software; easy-to-use LC-MS/MS software with preconfigured iMethod™ Tests and automatic reporting

Cliquid® Software and iMethod™ Tests

Cliquid® Software was specifically developed for LC-MS/MS analysis in routine food testing laboratories. The software provides an easy-to-use interface with a four step wizard to perform sample analysis and automatic report generation. These four steps include choosing a test to perform, building the sample list, customizing reporting options, and submitting the samples for analysis. The developed method for the analysis of azo-dyes in spices is available as an iMethod™ Test.

Screenshots illustrating the wizard and example reports generated when analyzing unknown contaminated spice samples are shown in Figure 4 and 5.

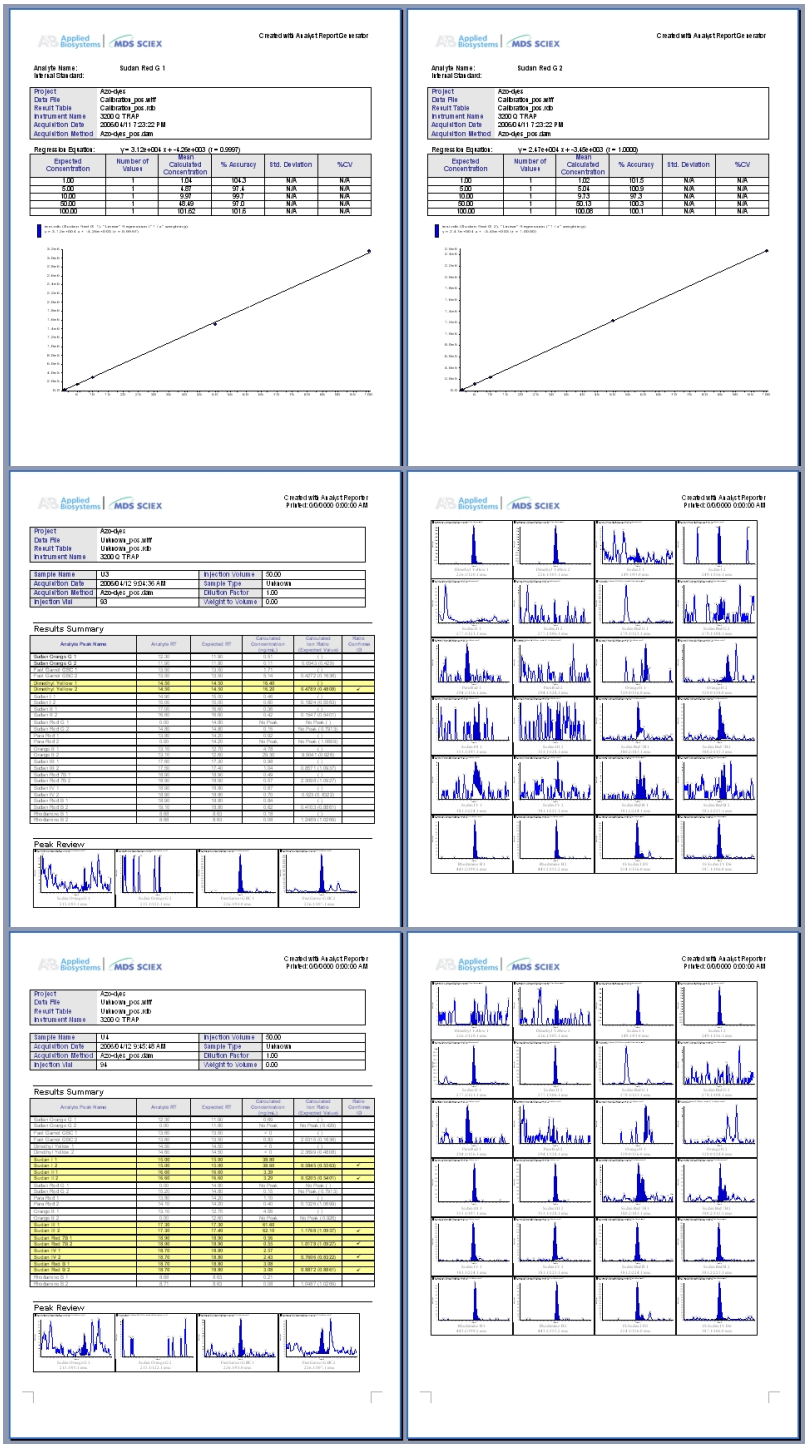

Figure 5. Example reports generated automatically by Cliquid® Software showing calibration curves, statistical information of accuracy and reproducibility, and detected azo-dyes in unknown samples with highlighted analytes when identified by MRM ratio

Summary

A new analytical procedure was developed to determine 13 azo-dyes, which are of high priority in many European and Asian countries, by simple solvent extraction and LC-MS/MS analysis.

Ion suppression varied strongly from matrix to matrix. Thus, standard addition is recommended to quantify dyes in spices due to a lack of isotopically labeled internal standards. The detection of two MRM transitions per compound is needed to match regulatory requirements.

Cliquid™ Software is easy-to-use software focusing on the typical workflow from LC-MS/MS analysis to automatic report generation. The described method for the analysis of azo-dyes in spices is available as an iMethod™ Test.

References

1 Lutz Hartig et al.: 'Detection of 6 Sudan Dyes, Dimethyl Yellow and Para Red in Spices and Sauces with HPLC/MS/MS' poster presented at ASMS conference on Mass Spectrometry (2005) San Antonio, Texas, USA

2 André Schreiber et al.: 'Accuracy of quantitation using external and internal calibration to analyze dyes in extracts of spices' poster presented at ASMS conference on Mass Spectrometry (2006) Seattle, Washington, USA

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