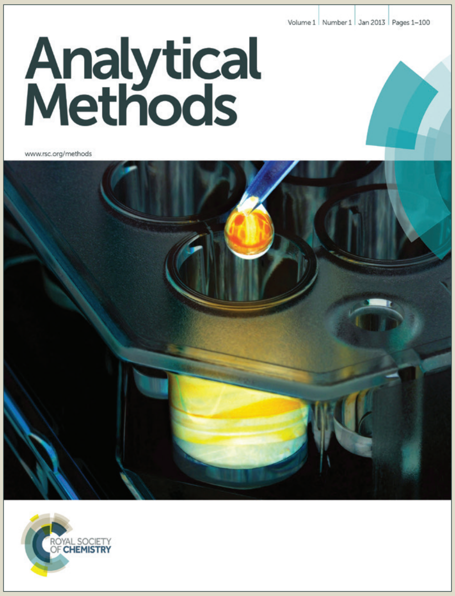
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods

Journal Name RSCPublishing

ARTICLE

Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

Rapid analysis of four Sudan dyes using direct analysis in real time-mass spectrometry

Ze Li, Yi-Wei Zhang, Yi-Ding Zhang, Yu Bai, Hu-Wei Liu*

A simple direct analysis in real time-mass spectrometry (DART-MS) method was developed for rapid determination of four Sudan dyes (I-IV) in chili powder. Simple liquid extraction by hexane without further clean-up was used for sample preparation. DART parameters were systematically optimized to achieve the best detection performance. DIP-it sampler was used for automatic sampling. Matrix effect was measured by comparing the limit of detection (LOD) in matrix solution with that in pure organic solution. Eventually, the identification of the Sudan dyes was confirmed by MS/MS results and LOD for four analytes in matrix solution was ～0.5 μg/mL. The method showed good linearity with correlation coefficients (R^2) greater than 0.99 for concentrations ranging from 1 to 20 μg/mL. The whole analytical process could be completed within 15 minutes with good recoveries (88-116%) and satisfactory repeatability (<26%, *n*=3).

Introduction

Sudan dyes are a family of synthetic organic oza-compounds, which are abundantly used by industry for coloring. However, some human foodstuff such as chili powder and chili sauces were reported to be illegally adulterated with Sudan dyes a few years ago.[1, 2] That is very harmful to human health because molecules with diazo bonds could cause cancer.[3] Due to their potential danger, many countries have banned the use of most azo-dyes in human consumption products. Actually, the European Food Safety Authority (EFSA) has declared Sudan dyes suspected carcinogens and classified as group 3 compounds. Additionally, the European Union fixed an action to set a limit of 0.5 mg/kg for Sudan dyes in foodstuff (Commission Directive 2006/33/EC).[4] Several methods for the determination of Sudan I-IV have been published in the past years,[2 1, 5-10] mostly were based on liquid chromatography (LC) with photometry or mass spectrometry (MS) detector, which is time-consuming due to chromatography separation and complicated sample preparation. Micellar electrokinetic chromatography (MEKC)[10] and the enzyme-linked immunosorbent assay (ELISA)[11] have also been proposed. Furthermore, some efficient sample pre-preparation methods[12-14] such as molecularly imprinted solid-phase extraction (MISPE), SPE and dispersive liquid-liquid microextraction (DLLME) were introduced to obtain better sensitivity. Similarly, complex sample pre-preparation procedures were labour-intensive. Therefore, fast and sensitive methods for the identification and quantification of Sudan dyes in food have been extensively desired.

Ambient mass spectrometry (AMS) is a novel ionization technique first proposed by G. Cooks in 2004.[15] The main advantage of this method was that it facilitated MS analysis under ambient conditions without or with a little sample preparation and separation. Direct analysis in real time (DART) is a representative ion source of AMS that has been commercialized and widely applied for the detection of various small molecules.[16-19] Samples could be analyzed in various states including gases, liquids and solids. For instance, liquid analysis could be conducted by dipping a glass rod into the fluid followed by directly placing the glass rod in front of the DART ionizing beam. As AMS is capable to provide real-time information, it has been widely applied for high throughput analysis.

In this work, we proposed DART-MS as a new method for the determination of four Sudan dyes. Simple liquid extraction was combined with DART-MS direct analysis, and the method was supposed to be easy, fast and highly efficient.

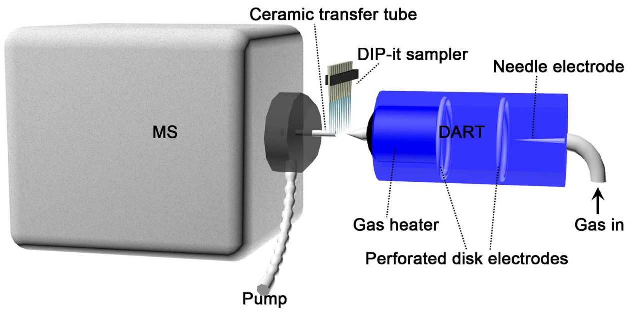
Results and discussion

Optimization of DART-MS parameters

In order to realize higher ionization efficiency and better detection performance, DART-MS parameters including working gas types, gas heater temperature (50-450 °C, in 50 °C steps), the distance between DART orifice and MS inlet and sampling speed were all investigated with diluted mixed stock solution (5 ppm). The working gas types and gas temperature were found to be the most important factors influencing the signal intensity. As is shown in Fig. 1 (a), only Sudan I and II could be detected when nitrogen was used as working gas (gas heater temperature 300 °C). The excited state of helium (2^3S, electronic excited state, 19.8 eV) has higher energy than that of nitrogen, thus more kinds of analytes could be ionized.[16] Therefore, helium was chosen as working gas for the following experiments.

Working gas temperature would affect the analytes desorption efficiency, then the signal intensity. Fig. 1 (b) showed that for low molecular weight targets like Sudan I and

II, the highest peak intensity was observed at 150 °C. Along with the raise of gas temperature, the desorption efficiency of higher boiling point Sudan III and IV started to increase. But the signal intensity of Sudan I and II decreased due to insufficient ionization efficiency, which was caused by ion competition ionization. 250 °C would be the best for Sudan III and IV. Taking all four target analytes into consideration, 300 °C was selected as gas heater temperature eventually. Other DART parameters were further evaluated with mixed standard working solutions (5 ppm). A distance of 2.2 cm from the DART orifice to ceramic transfer tube orifice and a rate of 0.2 mm/s for DIP-it sampler were finally chosen for later analysis. The analysis diagram was showed in Scheme 1.



Scheme 1 the schematic diagram of DART-MS.

Under all the optimized conditions, mixed standard solutions were detected as demonstrated. Fig. 2 showed that all the four target analytes were observed in their protonated molecular ions (Sudan I ($[M + H]^+$, *m/z* 249.21), Sudan II ($[M + H]^+$, *m/z* 277.25), Sudan III ($[M + H]^+$, *m/z* 353.27), Sudan IV ($[M + H]^+$, *m/z* 381.30)). That is because proton transfer is the dominant mechanism in positive mode.

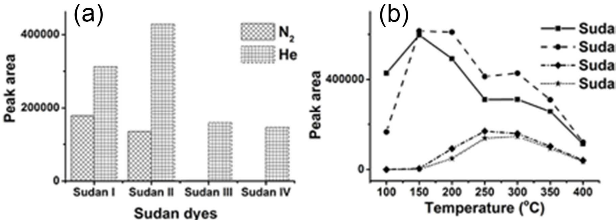


Fig. 1 The optimization of DART parameters affecting the detection performance of four Sudan dyes: (a) working gas types; (b) gas heater temperature.

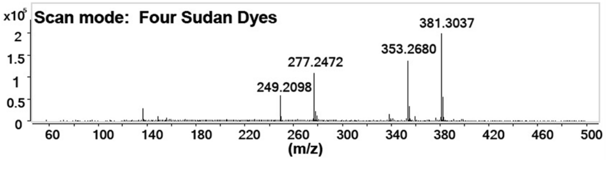


Fig. 2 Scan spectra of Sudan molecules under optimized conditions
(Sudan I ($[M + H]^+$, *m/z* 249.21), Sudan II ($[M + H]^+$, *m/z* 277.25), Sudan III ($[M + H]^+$, *m/z* 353.27), Sudan IV ($[M + H]^+$, *m/z* 381.30)).

Matrix effect, Limit of detection (LOD), linearity and reproducibility

The high resolution feature of QTOF-MS was useful for identification and confirmation of target analytes. However, when considering the unknown complex components, only protonated molecular ions were not sufficient to confirm the presence of target molecules. To make the results more reliable, MS/MS detection were used, and a precursor ion and at least one daughter ion were used to confirm the existence of target analytes. To evaluate the effect of chili powder matrix on the detection, LODs of the four Sudan dyes in methanol and in matrix solutions were measured. Collision energies were optimized to obtain the best MS/MS performance, and the finally selected values were listed in Table 1. MS/MS spectra and proposed fragmentation pathways were showed in Fig. 3. Under optimized conditions, the LODs measured in methanol and in matrix solutions were listed in Table 2. Sudan I and II could be detected at a concentration as low as 100 ng/mL, while Sudan III and IV could be detected at 80 ng/mL in methanol. Owing to the matrix interferences especially in higher molecular ranges, detection sensitivities in matrix solutions were worse than those in pure organic solution. Sudan III and IV couldn't be observed until 0.5 μg/mL, while Sudan I and II were detected at 0.4 μg/mL and 0.3 μg/mL, respectively. LODs obtained in this method was better than other reported ambient MS approach[20]. Due to method reliability, liquid extraction without further clean-up were adopted for sample preparation. Along with the further increase of analytes concentration, clear protonated molecular ions could be apparently observed even in matrix solutions. Therefore, MS scan method was adopted and the extracted protonated ion chromatograms were integrated for the following quantitative analysis for method simplicity. Each analysis was measured according to the average peak areas of 6 repeated samples.

The calibration curve was built by sampling five matrix-diluted standard solutions at 1, 2, 5, 10 and 20 ppm, respectively. Higher concentrations (50, 100 ppm) couldn't be obtained in good linearity due to ion competition and suppression effect. Four calibration curves with correlation coefficients (R^2) higher than 0.99 were obtained as showed in Fig. 4. The reproducibilities were measured at all concentrations covering the calibration curve and all achieved satisfactory RSDs (*n*=6) lower than 15%. To further increase the reproducibility, isotope-internal standard may be needed.

Table 1 Collision energy selected for four Sudan dyes

Compound	Precursor ion (*m/z*)	Daughter ions (*m/z*)	Collision energy (eV)
Sudan I	249.21	156.13[a],232.19	13
Sudan II	277.25	121.16[a],156.13	15
Sudan III	353.27	120.13,198.19[a]	16
Sudan IV	381.30	225.23[a], 277.23	17

([a] Fragment ions used for qualitative analysis)

Method application

Three kinds of chili powders were purchased from the local market and all detected as Sudan dyes adult-free samples. Therefore, the recovery of the proposed liquid-extraction DART-MS approach was conducted by spike experiments. Method reproducibility and recovery were all listed in Table 3. Good recoveries for all four Sudan dyes at three concentrations were obtained, ranging from 88% to 116% with satisfactory repeatability under 26% (RSD, *n*=3).

Table 2 LODs of four Sudan dyes in methanol and in chili powder matrix solutions.

Compound	Methanol (μg/mL)	Matrix (μg/mL)
Sudan I	0.1	0.4
Sudan II	0.1	0.3
Sudan III	0.08	0.5
Sudan IV	0.08	0.5

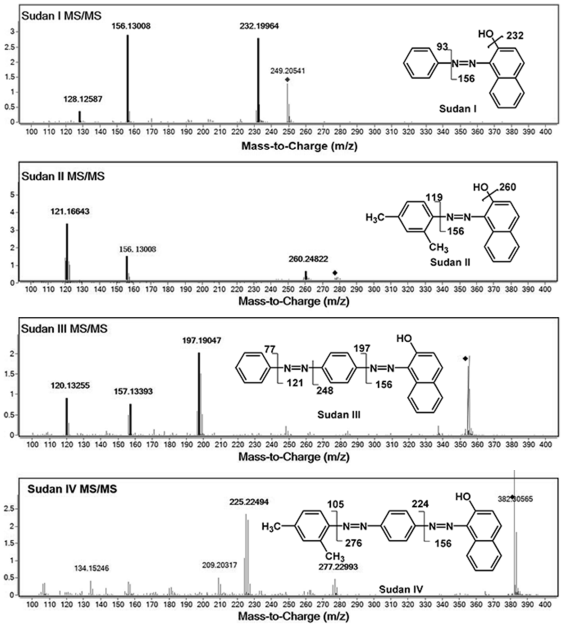


Fig. 3 MS/MS spectra of the four Sudan dyes together with the proposed fragmentation pathways.

Table 3 Accuracy and precision of spiked samples.

Compound	Spiked samples (ppm)	Calculated concentration (ppm)	RSD (*n*=3)	Recovery (%)
Sudan I	0	ND [b]	ND	ND
	10	11.6	0.19	116
	20	18.9	0.10	95
	40	36.8	0.19	92
Sudan II	0	ND	ND	ND
	10	10.1	0.25	101
	20	17.6	0.05	88
	40	36.5	0.21	91
Sudan III	0	ND	ND	ND
	10	11.2	0.26	112
	20	21.5	0.22	107
	40	38.5	0.07	96
Sudan IV	0	ND	ND	ND
	10	9.6	0.06	96
	20	19.2	0.15	96
	40	37.5	0.11	94

ND [b] not detected

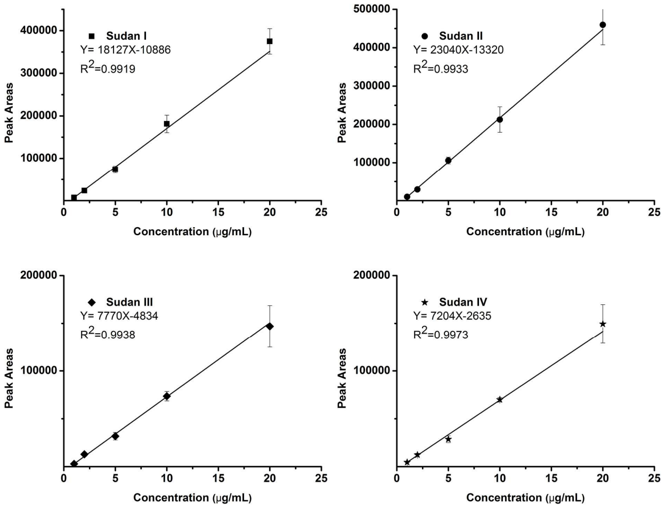


Fig. 4 Equation and correlation coefficient of four calibration curves (*n*=6).

Experimental

Reagents and Standards

Four standard Sudan dyes (Sudan I, 1-[(2,4-dimethylphenyl)azo]-2-naphthalenol); Sudan II, 1-(phenylazo)-2-naphthol; Sudan III, 1-(4phenylazophenylazo)-2-naphthol; Sudan IV, o-tolyazo-o-tolylazo-betanaphthol) were obtained from Sigma Aldrich (St Louis, MO). Methanol and hexane of HPLC-grade were supplied by Dikma Technologies Inc. (Richmond, VA, USA). Four standard stock solutions were individually prepared in methanol at the concentration of 0.5 mg/mL. Mixed matrix-free working solution (5 ppm) used for method optimization was prepared by diluting the stock solutions with hexane. All solutions were stored at 4 °C in darkness.

Pepper powder was purchased from the local market.

Helium gas (purity 99.999%) was purchased from Beijing helium gas industry Co. Ltd. (Beijing, China), nitrogen gas (purity 99.999%) was purchased from Haike Yuanchang applied gas Co. Ltd. (Beijing, China).

Sample preparation

Preliminary experiment showed that the chili powder used were adult-free. Then 0.5 g chili powder was weighted into 10 mL centrifugal tube, and 5 mL hexane was added for liquid extraction. Tubes were manually shaken for a few seconds, followed by 5 minutes' sonication. At last, the samples were centrifuged for 3 minutes at 3000 r/min. Supernatant layer liquid were obtained as matrix solution.

Matrix-diluted solutions were provided to measure the LODs and calibration curves. Stock solutions were diluted with matrix solution to obtain a series of working standards ranging from 1 to 100 ppm. For recovery experiments, corresponding standard stock solutions were directly spiked into 0.5 g chili powder and aired dried. Further sample preparations were done in the same way as above. A fused glass rod which called DIP-it economically was dipped into the extraction hexane layer for a few seconds, then placed into DART working gas stream for DART-MS analysis.

Apparatus and parameters

All analyses were performed on an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight mass spectrometer (Agilent Technologies, CA, USA), equipped with a DART®-SVP source (IonSense, Saugus, MA, USA) after removing the Agilent Jet Stream ESI source. A ceramic transfer tube (3.75 mm i.d., 83 mm length) and vacuum pump was equipped outside and aligned in line with the QTOF-MS orifice for ion transfer (see Scheme 1), according to existing publications[21]. Both DART and QTOF-MS were operated in positive mode. An automatic 12-sample DIP-it sampler (IonSense, Saugus, MA, USA) was equipped for sampling. It allowed multiple sample analysis automatically at one time, thus greatly improved approach efficiency. The MS operating conditions were set as commonly used parameters, which have been optimized earlier in our lab: capillary entrance voltage 3500 V, fragmentor 175 V, and skimmer 65 V. Both the MS and MS/MS data were acquired at a rate of 1.02 spectra per second in the mass range of 50-500 (*m*/z) by MassHunter Data Acquisition B.02.00 (Agilent Technologies, CA, USA). Data analysis were conducted with MassHunter Qualitative Analysis B.02.00 (Agilent Technologies, CA, USA). The voltage of discharge needle and the grid electrode of DART were set to 6 kV and 100 V, respectively, according to optimization experiments before. The working gas types and gas heater temperature were optimized to obtain the best sensitivity.

Conclusions

A simple method for the fast determination of four Sudan dyes (I-IV) was developed in this work. Simple liquid extraction with DIP-it sampling and DART-MS detection were combined together. DART parameters were systematically optimized, and finally helium gas and gas heater 300°C were selected for the analysis. Matrix-diluted solutions were provided to measure the LODs and calibration curves. Reliable results were confirmed by MS/MS detections and satisfactory sensitivity (0.5 ppm) was obtained. The detection method was proved to have good linearity (1-20 ppm, $R^2>0.99$) and repeatability (RSD<15%). The whole analytical process could be completed within 15 minutes with good recoveries (88-116%) and satisfactory repeatability (<26%, *n*=3).

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant No. 21275012).

Notes and references

Beijing National Laboratory for Molecular Sciences, Key Laboratory of Bioorganic Chemistry and Molecular Engineering of Ministry of Education, College of Chemistry and Molecular Engineering, Peking University, Beijing 100871, China. E-mail: hwliu@pku.edu.cn; Fax: +86-10-62751708; Tel: +86-10-62754976

1. F. Calbiani, M. Careri, L. Elviri, A. Mangia and I. Zagnoni, *J. Chromatogr. A*, 2004, **1058**, 127-135.
2. F. Calbiani, M. Careri, L. Elviri, A. Mangia, L. Pistarà and I. Zagnoni, *J. Chromatogr. A*, 2004, **1042**, 123-130.
3. M. Stiborová, V. Martínek, H. Rýdlová, P. Hodek and E. Frei, *Cancer Research*, 2002, **62**, 5678-5684.
4. C. Schummer, J. Sassel, P. Bonenberger and G. Moris, *J. Agric. Food Chem.*, 2013, **61**, 2284-2289.
5. V. Cornet, Y. Govaert, G. Moens, J. Van Loco and J.M. Degroodt, *J.Agric.Food Chem.,* 2006, **54**, 639-644.
6. E. Ertas, H. özer and C. Alasalvar, *Food Chemistry*, 2007, **105**, 756-760.
7. L.M. He, Y.J. Su, B.H. Fang, X.G. Shen, Z.L. Zeng and Y.H. Liu, *Analytica Chimica Acta*, 2007, **594**, 139-146.
8. E. Mejia, Y.S. Ding, M. F. Mora and C. D. Garcia, *Food Chemistry*, 2007, **102**, 1027-1033.
9. M. R. V. S. Murty, N. Sridhara Chary, S. Prabhakar, N. Prasada Raju and M. Vairamani, *Food Chemistry*, 2009, **115**, 1556-1562.
10. T. S. Fukuji, M. Castro-Puyana, M. F. Tavares and A. Cifuentes, *J. Agri. Food Chem.*, 2011, **59**, 11903-11909.
11. C.M. Ju, Y. Tang, H.Y. Fan and J.D. Chen, *Analytica Chimica Acta*, 2008, **621**, 200-206.
12. F. J. López-Jiménez, S. Rubio and D. Pérez-Bendito, *Food Chemistry*, 2010, **121**, 763-769.
13. H.Y. Yan, J.D. Qiao, H. Wang, G.L. Yang and K. H. Row, *Analyst*, 2011, **136**, 2629-2634.
14. M.M. Zheng, J.H. Wu, Y.Q. Feng and F.H. Huang, *Anal. Methods*, 2011, **3**, 1851.
15. Z. Takats, J. M. Wiseman, B. Gologan and R. G. Cooks, *Science*, 2004, **306**, 471-473.
16. R. B. Cody, J. A. Laramée and H. D. Durst, *Anal. Chem.*, 2005, **77**, 2297-2302.
17. J. H. Gross, *Anal. Bioanal. Chem.*, 2014, **406**, 63-80.
18. L. P. Li, B. S. Feng, J. W. Yang, C. L. Chang, Y. Bai and H. W. Liu, *Analyst*, 2013, **138**, 3097-3103.
19. B.S. Feng, Y. Bai and H. W. Liu, *SCIENTIA SINICA Chimica*, 2014, **44**, 784-788.
20. D. Taverna, L. Di Donna, F. Mazzotti, B. Policicchio and G. Sindona, *J. Mass Spectrom.*, 2013, **48**, 544-547.
21. S. Yu, E. Crawford, J. Tice, B. Musselman and J.T. Wu, *Anal. Chem.*, 2008, **81**, 193-202.