Sample Preparation of Eggs From Laying Hens Using QuEChERS Dispersive Extraction for the Simultaneous Determination of Melamine and Cyromazine Residues by HPLC-DAD

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ABSTRACT: A quick, easy, cheap, effective, rugged, and safe (QuEChERS) dispersive extraction method is proposed herein for the isolation and cleanup of melamine and cyromazine from chicken egg yolk. Analytes are determined by high-performance liquid chromatography using photodiode array detector after separation on a LiChroCART® (250 × 4 mm)—LiChrospher® RP-8e, 5 µm analytical column using a mobile phase of 0.1% trifluoracetic acid and methanol (80:20 v/v) delivered isocratically at a flow rate of 1 mL/minute. Extraction of isolated compounds was achieved by methanol and acetonitrile mixture (1:1 v/v). Recovery rates ranged between 74.5% and 115.8%. The method was validated in terms of 657/2002/EC decision. The within-laboratory reproducibility, expressed as a relative standard deviation, was <11%. Decision limits (CCalfa) were 2.56 mg/kg for melamine and 0.22 mg/kg^{-1} for cyromazine, and the corresponding results for detection capability (CCbeta) were 2.8 mg/kg for melamine and 0.24 mg/kg for cyromazine. Ruggedness was estimated according to the Youden approach studying egg yolk mass, sorbent mass, centrifugation time, organic solvents volume, evaporation temperature, and vortex time.

KEYWORDS: cyromazine, egg, HPLC, melamine, QuEChERS

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Introduction

Melamine (2,4,6-triamino-1,3,5-triazine) is an organic base, found usually in the form of white crystals, with a high nitrogen content, ~66% by mass.[1] It is used for the manufacturing of several laminates, plastics, dishware, and kitchenware, as well as in the synthesis of melamine formaldehyde resins. It can migrate from these household items into food under acidic conditions or at higher temperatures.[2] It has also been illegally added as a nitrogen source in animal feeds and human foods, such as milk and milk products, to increase the protein concentration because it increases the nitrogen content, which is actually measured by the Kjeldahl method.[3] It can also be found in animal products due to the degradation of cyromazine present in animal feed, since melamine is one of the primary metabolites of cyromazine. By forming an insoluble salt, it may precipitate in kidneys and lead to renal function failure.[4] European Commission has agreed a maximum acceptable limit of 2.5 mg/kg for melamine in imported foods and 1 mg/kg in infant formula.

Cyromazine (*N*-cyclopropyl-1.3.5-triazine-2.4.6-triamine) is a triazine derivative used as pesticide and insect growth regulator. It is effective against blowfly larvae on sheep and lambs. It is also effective against other Diptera such as houseflies and mosquitoes.[3] It is used as a chitin synthesis inhibitor to control fly in cattle manure, crops, vegetables, and fruits.[4] Additionally, cyromazine is administered in the feed of laying hens to prevent flies from hatching in their manure.[3] After exposure to cyromazine, some residues of cyromazine and melamine remain in animal tissues.[4]

Many analytical methods have been developed to quantitate the level of melamine in food samples and a few of them have been developed to determine the level of cyromazine, using various techniques such as gas chromatography/mass spectrum (GC-MS),[5] liquid chromatography/mass spectrometry LC-MS/MS,[3] high-performance liquid chromatography (HPLC) and LC-MS/MS,[4] micellar electrokinetic capillary chromatography with amperometric detection (MECC-AD),[6] Raman spectroscopy,[7] HPLC with fluorescence detection (HPLC-Fluo),[8] biosensors, and enzyme linked immunosorbent assay (ELISA).[1] For the determination of melamine and cyromazine in chicken eggs, only one method used QuEChERS and LC-MS/MS[3] and another publication has reported the determination of melamine in chicken eggs with UPLC-MS/MS.[5]

QuEChERS is the acronym for quick, easy, cheap, effective, rugged, and safe. It is based on the work introduced

by Anastassiades et al,[9] who has developed an extraction method for pesticides in fruits and vegetables, coupled with a cleanup method that removes sugars, lipids, organic acids, sterols, proteins, pigments, and excess water. It is applied in two steps: in the first step, a homogenized sample is extracted and partitioned using acetonitrile (ACN) and a salt solution, and then, the supernatant is cleaned using a dispersive solid-phase extraction technique. QuEChERS was developed to be a simple, effective, and inexpensive way to extract and clean pesticide residues from a wide range of sample matrices. It is a generic sample preparation that has been efficient for various complex matrices.[10]

The aim of this study was to develop a simple and quick HPLC-DAD method for the determination of melamine and cyromazine in egg's yolk using QuEChERS dispersive extraction. The method was validated according to the European Union Decision 2002/657/EC with regard to selectivity, linearity, accuracy, precision, stability, and ruggedness by applying the Youden approach and sensitivity.[11]

Experimental

Reagents and materials. Melamine (99%) was purchased from Alfa Aesar GmbH & Co KG, and Cyromazine (99.8%) from Sigma-Aldrich. HPLC grade methanol Lichrosolv® (MeOH, 99.8%) was purchased from Merck, ACN (99.99%) from Fisher Scientific and trifluoracetic acid (TFA: CF_3COOH, 99%) from ACROS Organics. Ultrapure water was used throughout the study, provided by a Milli-Q® purification system (Millipore). QuEChERS for fatty samples were purchased from Agilent Technologies and consist of 150 mg magnesium sulfate, 50 mg primary and secondary amines, and 50 mg C_{18EC}. Syringe nylon (nylon 66) filters (13 mm diameter, 0.22 µm membrane) were purchased from BGB Analytik and were used for sample filtration prior to HPLC analysis. A LiChroCART® (250 × 4 mm)—LiChrospher® RP-8e, 5 µm analytical column from Merck (Darmstadt, Germany) was used for the chromatographic separation. Solid-phase extraction (SPE) cartridges Strata-X (polymeric reversed phase) were supplied from Phenomenex and Merck-Lichrolut RP-18 (200 mg/3 mL) from Merck. Eggs were purchased from the local market and kept at +4°C.

Instrumentation. Mobile phase was delivered to the analytical column by a Shimadzu LC-10ADVP pump equipped with a Shimadzu SCL-10ALVP System Controller. The solvent lines were mixed in an FCV-10ALVP mixer. For the degassing of the mobile phase, helium sparging was performed in the solvent reservoirs by a DGU-10B degassing unit. Samples were injected manually using a Rheodyne 7725i injection valve (Rheodyne) with a 20 µL loop. Analytes were monitored using a photodiode array detector SPD-M10AVP, with data acquisition software Lab Solutions–LC solutions by Shimadzu.

For the filtration of the aquatic solutions, a glass vacuum-filtration apparatus purchased from Alltech Associates using Cellulose Nitrate 0.2 µm membrane filters from Sartorius Stedim Biotech GmbH was used. A Glasscol small vortexer (Terre Haute) was employed for the pretreatment of egg's yolk samples.

Sonication was performed by an ultrasonic bath Transsonic 460/H (35 kHz, 170 W; Elma) and centrifugations were carried out using a Hermle centrifuge, model Z-230 (B. Hermle). A Visiprep™ SPE Vacuum Manifold by Supelco and a nine-port Reacti-Vap™ (model 18780) by Pierce were used for sample preparation.

Chromatographic conditions. A LiChroCART® (250 × 4 mm)—LiChrospher® RP-8e, (5 µm) analytical column, at room temperature, was used for the separation. The analytes were monitored at 240 nm. The elution was isocratic and the mobile phase consisted of 0.1% TFA (CF_3COOH) and methanol (CH_3OH; 80:20 v/v) delivered isocratically at a flow rate of 1 mL/minute. Inlet pressure was between 180 and 190 bar. The injection volume was 20 µL.

Preparation of standards. Stock solutions of melamine and cyromazine at 100 ng/µL were dissolved in ultrapure water and ACN (50:50 v/v) and stored refrigerated at +4°C. They were found to be stable for at least two months. Working aqueous standards were prepared by the appropriate dilution at a range of 0.2–30 ng/µL.

Sample preparation. Two SPE sorbents (Strata-X and Lichrolut RP-18) were investigated for their efficiency for the isolation of the examined analytes from standard solutions, using different organic solvents. The sorbent providing the higher recovery was selected for further study with egg yolk. An aliquot of 0.5 g of the egg yolk was extracted by 1 mL of ACN and 1 mL of 10% TFA. After centrifugation at 1900 *g* for 10 minutes, the supernatant was cleaned up by SPE. Elution was performed by 1 mL methanol and 1 mL of ACN. However, the results were not encouraging. Therefore, a dispersive approach was selected for sample preparation and optimized as shown in Table 1.

Table 1. Comparison of different extraction methods.

EXTRACTION MEDIUM	ELUENT	RECOVERIES (%)	
		MELAMINE	CYROMAZINE
Standard solutions			
Strata-X (SPE)	1 mL MeOH 1 mL ACN	47.5	58.7
Lichrolut RP-18 (SPE)	1 mL MeOH 1 mL ACN	<5%	
Egg yolk			
Strata-X (MSPD)	1 mL MeOH 1 mL ACN	34.1	12.9
QuEChERS	2 mL ACN	8.7	52.7
QuEChERS	2 mL MeOH	67.8	40.8
QuEChERS	1 mL MeOH 1 mL ACN	74.5	75.3

Egg's yolk was separated from the white. The content of 10 eggs was homogenized and held at room temperature until used, and then, it was stored at +4°C. An aliquot of 125 mg of QuEChERS material was placed in a falcon tube with 1 mL methanol, 1 mL ACN, and 0.5 g yolk, which was spiked with 500 µL of a standard solution containing melamine and cyromazine. Then, the sample was vortexed for 30 seconds and centrifuged at 1900 *g* for 10 minutes. The supernatant was evaporated to dryness in water bath at 40°C under a light stream of nitrogen, and the dry residue was dissolved in 500 µL in ultrapure water and was filtered prior to the injection into the HPLC system.

Method validation. Validation of the developed method was done according to the European Union Decision 2002/657/EC[11] using spiked samples since no validated reference material was available. Selectivity, linearity, precision (repeatability and between-day precision), decision limit (CCalfa), detection capability (CCbeta), stability, and ruggedness were studied.

Linearity was studied using working standards at concentration levels between 0.2 and 30 ng/µL. In egg yolk, linearity was examined using spiked samples covering the range between 0.2 mg/kg and 30 mg/kg and calibration curves were calculated. Limit of detection (LOD) was calculated based on the signal/noise ratio of 3.3, and limit of quantitation (LOQ) was three times the LOD. The selectivity of this method was expressed as lack of interference of endogenous compounds examined by the analysis of blank samples of egg's yolk. Precision and accuracy were calculated for melamine by analyzing spiked samples of yolk at the concentration levels of 10 mg/kg, 15 mg/kg, and 20 mg/kg and for cyromazine at 2 mg/kg, 5 mg/kg, and 10 mg/kg. Within-day repeatability was examined by five measurements at the above concentration levels. Between-day precision was studied applying the same procedure in a period of five days. The recovery was calculated using the formula of the percentage of the ratio of the analyte mass that was found in the spiked sample, to the spiked mass. Calculation of the decision limit CCalfa was done by the mean concentration found at the LOQ of each analyte plus 1.64 times the SD of duplicate measurements of 20 samples at LOQ, while calculation of the detection capability CCbeta was based on CCalfa plus 1.64 times the SD of duplicate measurements of 20 samples spiked at levels of CCalfa. Stability for the spiked samples of yolk was studied as short-term stability which was evaluated after 2 and 24 hours of storage in room temperature and long-term stability, which was assessed after one, three, and seven days of storage at 4°C. The ruggedness of the method was assessed according to the Youden's approach.[12] The concept is that several variations are introduced at once, instead of studying one alteration at a time. Eight different experiments are carried out with seven small changes of the operating parameters (variables). The changes involved: QuEChERS mass, evaporation temperature, egg yolk mass, volume of elution solvents, vortex, and centrifugation time. Egg's yolk samples were spiked at 10 mg/kg and recovery of target analytes was estimated. Standard deviation of the differences *Di* (*SDi*) was calculated according to the equation:

$$SDi = \sqrt{2x\sum\left(\frac{Di^2}{7}\right)}$$

when *SDi* is significantly larger than the standard deviation of the method carried out under intermediate precision conditions, it is a predetermined conclusion that all factors collectively have an effect on the result, even if every single factor does not show a significant influence, and that the method is not sufficiently robust against the modifications that are chosen.[12] The investigated factors and their levels of variation are reported in Table 2.

Table 2. Youden's ruggedness test by applying seven small but deliberate changes in the operating parameters.

PARAMETER	UNITS	OPTIMAL CONDITIONS	HIGH LEVEL CAPITAL CASE VALUES	LOW LEVEL CAPITAL VALUES	EXPERIMENT NUMBER							
					1	2	3	4	5	6	7	8
A = egg yolk mass	g	0.5	0.7	0.5	A	A	A	A	a	a	a	a
B = QuEChERS mass	mg	125	125	62.5	B	B	b	b	B	B	b	b
C = centrifugation time	min	10	10	5	C	c	C	c	C	c	C	c
D = MeOH volume	mL	1	1.5	1	D	D	d	d	d	d	D	D
E = ACN volume	mL	1	1.5	1	E	e	E	e	e	E	e	E
F = evaporation to dryness temperature	°C	40	40	25	F	f	f	F	F	f	f	F
G = Vortex time	s	30	30	0	G	g	g	G	g	G	G	g
Observed results					**s**	**t**	**u**	**v**	**w**	**x**	**y**	**z**

COMPOUND	SD (METHOD)	SD	Da	Db	Dc	Dd	De	Df	Dg
Melamine	44	5941.8	3300	1936.2	3491.6	2394.7	3463.1	3392	–8216.2
Cyromazine	221.8	2133.0	–306.9	–2483.9	15	–1851.7	1278	–1108.1	–1835.8

Application to real samples. The developed method for the determination of melamine and cyromazine was applied to the verification of the occurrence of the examined analytes in eggs from local markets. Twenty eggs were collected and analyzed using the developed method. Neither melamine nor cyromazine was detected in the examined samples.

Results and Discussion

Chromatography. The mobile phase consisted of 0.1% TFA and methanol (80:20 v/v), which was delivered isocratically. The separation of melamine and cyromazine was achieved within seven minutes. Retentions times of the analytes were approximately at 3.196 minutes for melamine and 6.721 minutes for cyromazine.

Sample preparation. In order to optimize the extraction procedure, different SPE and QuEChERS protocols were examined. Initially, SPE extraction was used in standard solutions in order to choose the sorbent that provides the highest recovery rates. The best recoveries were obtained using Strata-X cartridges with ACN and MeOH (1:1 v/v) as eluent, while with Lichrolut RP-18, extraction was insufficient. For melamine, recovery was 47.5%, and for cyromazine, it was 58.7%. However, when these sorbents were applied for cleanup after solid–liquid extraction from egg's yolk, recoveries were very low, which were 34.1% for melamine and 12.9% for cyromazine. Therefore, QuEChERS was applied using various eluents. At first, 125 mg QuEChERS, 0.5 g yolk, and 2 mL ACN were used, and the recovery for melamine was 8.7% and for cyromazine was 52.7%. Then, instead of 2 mL ACN, 2 mL of MeOH was used and the recoveries were 67.8% for melamine and 40.8% for cyromazine. Finally, a combination of 1 mL ACN and 1 mL MeOH was used and the recovery was 74.5% for melamine and 75.3% for cyromazine. Concerning the elution solvents, best recoveries were observed after elution with methanol and ACN (1:1 v/v). Typical chromatograms of blank and spiked egg yolk samples at 10 mg/kg for melamine and cyromazine after QuEChERS procedure are shown in Figure 1.

Method validation results.

Linearity and sensitivity. The calibration curves of both standard solutions and spiked egg's yolk samples were all linear with coefficient of determination values ranging between 0.9931 and 0.9998. The LOQ of the method was found at 0.2 mg/kg for cyromazine, while linearity extended at least up to 30 mg/kg and for melamine LOQ was 2.5 mg/kg while linearity was extended at least up to 30 mg/kg.

Calibration curves for working standards were $y = 47.3x - 3529.4$ ($R^2 = 0.9997$) for melamine and $y = 54.5x + 22747.5$ ($R^2 = 0.9996$) for cyromazine. The respective calibration curves in spiked yolk matrix were $y = 37.0x + 77131.2$ ($R^2 = 0.9998$) for melamine and $y = 28.1x - 10170.6$ ($R^2 = 0.9931$) for cyromazine, where x = mg/kg and y = peak area of analytes.

Selectivity. Selectivity was investigated by analyzing blank samples of egg's yolk by the same procedure. No endogenous compounds that can interfere with the examined analytes were found.

Precision and accuracy. The precision of the method was based on within-day repeatability and between-day reproducibility. The former was assessed by replicate ($n = 5$) measurements from three spiked samples of egg's yolk at 10, 15, and 20 mg/kg for melamine and 2, 5, and 10 mg/kg for cyromazine. The recoveries of spiked samples were calculated at three different concentrations by comparison of the peak area ratios for extracted compounds toward the values derived from calibration curves. Between-day reproducibility was determined using the same concentrations. A triplicate determination of each concentration was performed for a period of five days. RSD values were <10.1% for all analytes. Recovery rates for

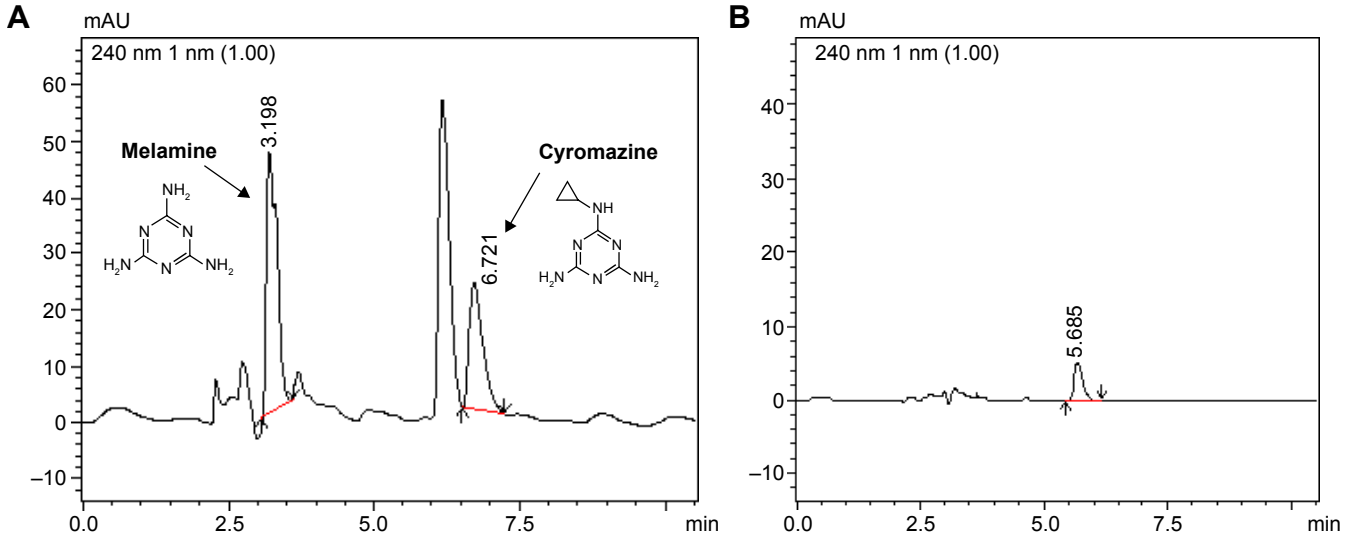

Figure 1. HPLC chromatogram of melamine and cyromazine in egg's yolk, monitored at 240 nm, using the chromatographic conditions described in text. **A:** Spiked egg yolk sample at 10 mg/kg. **B:** Blank egg yolk sample.

Table 3. Validation parameters of the developed method for the determination of melamine and cyromazine in egg yolk.

VALIDATION PARAMETERS	MELAMINE	CYROMAZINE
Linear range mg/kg	2.5–30	2–30
Linearity R^2	0.9998	0.9931
Linear equation (y = peak area, x = mg/kg)	y = 37.0x + 771312	y = 28.1x – 101706
LOQ S/N = 10 (mg/kg)	2.5	2
Intra-day precision and accuracy% RSD% n = 3 levels × 3 samples	75–110% RSD < 4%	75–79% RSD < 7%
Inter-day precision and accuracy% RSD n = 3 levels × 3 samples × 5 days	82–108% RSD < 3.5%	76–116% RSD < 10%
Recovery%	74%	75%
CCalfa (mg/kg)	2.6	2.0
CCbeta (mg/kg)	2.8	2.2

each compound ranged as follows: for melamine: 74.5%–110% and for cyromazine: 75.3%–115.8%.

The analytical performance parameters are summarized in Table 3.

Decision limit and detection capability. In compliance with the European Commission Regulation,[11] the CCalfa and CCbeta were calculated after spiking 20 egg's yolk samples with melamine and cyromazine at the LOQ level. Table 3 shows the CCalfa and CCbeta values for egg's yolk.

Stability of the solutions. In order to evaluate the stability of melamine and cyromazine, samples of egg's yolk were used, spiked at 10 mg/kg. Short-term stability was assessed after 1, 2, and 24 hours of storage at room temperature. Long-term stability was evaluated after one, three, and seven days of storage at +4°C. Spiked samples were found stable for at least three days at 4°C and melamine was found stable for seven days also. When stored at room temperature, cyromazine was found to be stable for 24 hours, according to the degradation criterion of –15%.[13]

Ruggedness. A Youden and Steiner test was applied to estimate ruggedness. Seven factors were chosen in the total analytical process because of their potential critical influence. These were centrifugation time, QuEChERS material quantity, evaporation temperature, yolk weight, volume of MeOH, volume of ACN, and the presence or absence of vortex. Blank yolk samples spiked at 10 mg/kg were used for these eight experiments. The basic idea of this test is the simultaneous application of several variations, instead of one alteration at a time. A set of A–G indicate the nominal values for the seven selected factors that could potentially influence the results, if their nominal values are changed to some extent. Their alternative values are represented by the corresponding lower case letters a–g. Eight determinations are performed using a combination of the chosen factors (A–G). Letters "s–z" express the observed results as analyte's amount from each Youden experiment as shown in Table 2. The results from the eight relevant robustness experiments are reported in the same table. For melamine, vortex and centrifugation time had the greater effect, in particular, centrifugation time had a positive impact and vortex had a negative impact, while for cyromazine, the quantity of QuEChERS had a negative effect and volume of ACN had a positive effect.

Application to real samples. Twenty egg samples from the local market were collected and analyzed using the developed method. Neither melamine nor cyromazine was detected in the examined samples.

Concluding Remarks

The method described earlier is a simple, validated assay for the simultaneous determination of melamine and cyromazine in egg's yolk by HPLC-DAD using QuEChERS dispersive extraction. It was validated according to European Union Decision 2002/657/EC in terms of selectivity, linearity, accuracy, precision, stability, sensitivity, and ruggedness. RSD values were <10.1% and recoveries ranged between 74.5% and 115.8%. Compared to another study by Wang et al,[3] which uses also QuEChERS dispersive extraction for the determination of cyromazine and melamine in eggs, the elution of the analytes was achieved in <7 minutes, instead of 17 minutes. Moreover, the extraction of the analytes using the method by Wang et al required up to 5 mL ACN, five times higher than the volume required in the proposed method.

By suitable preconcentration, the proposed method can reach the maximum residue limit set by the European Commission for cyromazine with no sophisticated equipment to be necessary.[14,15]

To the best of our knowledge, HPLC-DAD was herein applied for the first time for the simultaneous determination of melamine and cyromazine in egg's yolk using QuEChERS dispersive extraction.

The described method is quick, easy, and friendly to the environment. The required instrumentation can be easily found in any laboratory.

Author Contributions

Conceived and designed the experiments: VS, NT. Analyzed the data: VS, NT. Wrote the first draft of the manuscript: VS. Contributed to the writing of the manuscript: VS, NT. Agree with manuscript results and conclusions: VS, NT. Jointly developed the structure and arguments for the paper: VS, NT. Made critical revisions and approved final version: VS, NT. All authors reviewed and approved of the final manuscript.

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