Journal of Chromatography B, 879 (2011) 652–656

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Low-level quantification of melamine and cyanuric acid in limited samples of rat serum by UPLC–electrospray tandem mass spectrometry

Cristina C. Jacob, Gonçalo Gamboa da Costa*

Division of Biochemical Toxicology, US FDA National Center for Toxicological Research, Jefferson, AR, United States

ARTICLE INFO

Article history:
Received 2 November 2010
Accepted 30 January 2011
Available online 21 February 2011

Keywords:
Melamine
Cyanuric acid
Rat serum
UPLC–ESI-MS/MS

ABSTRACT

This paper reports the development and validation of a methodology for the low-level quantification of melamine and cyanuric acid in limited samples of rat serum. The methodology, based upon ion-exchange solid phase extraction (SPE) and ultra-performance liquid chromatography (UPLC) coupled with electrospray tandem mass spectrometry (MS/MS) in multiple reaction monitoring (MRM) mode, relies on the use of stable isotope-labeled internal standards and requires only 15 μL samples of serum. The method provides a recovery of 80–110% of melamine with a signal suppression of *ca.* 55%, and a recovery of 50–90% of cyanuric acid with a signal suppression *ca.* 40–60%, affording lower limits of quantification (LLOQ) for melamine or cyanuric acid of, respectively, 5 ppb (mean accuracy 109%; CV = 4.9%) and 10 ppb (mean accuracy 96%; CV = 8.6%). The small sample requirements, excellent sensitivity, accuracy and precision, and high-throughput (5 min of instrument run time) make this methodology optimal for toxicokinetic or exposure assessments studies.

Published by Elsevier B.V.

1. Introduction

In 2007 the intentional adulteration of pet food with melamine and derivatives, including cyanuric acid, was implicated in the kidney failure and death of a large number of cats and dogs in the USA [1]. While individually these compounds present low toxicity, co-exposure can lead to the formation of melamine cyanurate crystals in the nephrons and eventual kidney failure [2,3]. In the subsequent year, in China, the adulteration of baby formula with melamine resulted in the illness of approximately 300,000 infants, among which 50,000 underwent hospitalization and six deaths were reported [4]. These events precipitated a worldwide effort to elucidate the toxicology of melamine and derivatives, with particular emphasis to the combined exposures to melamine and cyanuric acid, a scenario deemed to present the highest nephrotoxic potential. A key point to consider under this scenario is the detailed determination of the toxicokinetic profiles of melamine, cyanuric acid, and their combinations. A number of methodologies has since been reported for the quantification of melamine in serum or plasma samples of animals [5–11]; however, the availability of methodologies for the quantification of cyanuric acid remains very limited [8,12], in particular when considering the limited sample availability, high sensitivity, accuracy and precision required for toxicokinetic studies in small laboratory animals like rats. We report here the development and validation of an isotopic dilution mass spectrometry methodology specifically designed for the low-level quantification of melamine and cyanuric acid in limited samples of rat serum.

2. Experimental

2.1. Chemicals and reagents

Unlabeled melamine (CAS 108-78-1) and cyanuric acid (108-80-5), and all reagents used in the sample preparation were purchased from Sigma–Aldrich, St. Louis, MO. $^{15}N_3$-labeled melamine and $^{13}C_3$-labeled cyanuric acid, used as internal standards, were purchased from Toronto Research Chemicals Inc., North York, Ontario, Canada. All solvents used in the sample preparation, standards preparation, and chromatography were Optima LC/MS grade and were acquired from Thermo-Fischer Inc., Waltham, MA. OASIS MCX 96-well plates (30 μm; 30 mg) and OASIS MAX 96-well plates (30 μm; 30 mg) were purchased from Waters Corporation, Milford, MA.

2.2. Preparation of standard solutions and fortified serum samples

Stock solutions of unlabeled melamine or cyanuric acid were prepared by carefully weighing the compounds in class A volumet-

* Corresponding author at: Division of Biochemical Toxicology, US FDA National Center for Toxicological Research, HFT-233, 3900 NCTR Road, Jefferson, AR 72079, United States. Fax: +1 870 543 7136.
E-mail address: goncalo.gamboa@fda.hhs.gov (G. Gamboa da Costa).

1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2011.01.035

ric vials and adding the appropriate amount of 50% methanol in water. Working solutions at 100 μg/mL were prepared by diluting the stock solutions with 50% methanol in water. Stock solutions of isotopically labeled melamine or cyanuric acid were prepared in a similar manner. The concentrations of the labeled internal standards were determined by HPLC with diode array detector, through comparison of their peak areas with those of identical injections of the unlabeled solutions. The solutions were analyzed in a Thermo Surveyor Plus HPLC system (Thermo-Fischer Inc., Waltham, MA) using a Synergi Hydro-RP 2 mm × 250 mm, 4 μm particle size column (Phenomenex, Torrance, CA) eluted isocratically with pH 7.0, 10 mM ammonium phosphate buffer containing 5% of acetonitrile at 400 μL/min. The eluate was monitored at 235 nm for melamine and 214 nm for cyanuric acid.

F344 rat serum was obtained from animals in the breeding colony of the National Center for Toxicological Research. The blood collection was done according to the guidelines and under approval of the Institutional Animal Care and Use Committee. Upon collection, the blood was allowed to clot on ice, and serum was obtained by centrifugation. Solutions of labeled and unlabeled melamine or cyanuric acid in rat serum were obtained by diluting appropriate amounts of the solutions prepared as described above in rat serum. All solutions were kept in tightly sealed Teflon-lined vials at −21 °C.

2.3. Sample preparation

2.3.1. Melamine

15 μL Samples of rat serum were diluted with 315 μL of water and 75 μL of a 100 ppb solution of $^{15}N_3$-labeled melamine. The samples were mixed in a 96-well plate agitator for 30 min at room temperature. An OASIS MCX (cationic exchange) 30 mg 96-well plate was conditioned by sequentially washing each well with 1 mL of 0.1 M NaOH in methanol, 1 mL of methanol, 1 mL of 0.01 M HCl in methanol, and 1 mL of methanol. After equilibration of the wells with 1 mL of acetonitrile followed by 1 mL 4% formic acid in water, 200 μL of 4% formic acid in water were loaded into each well, followed by the serum samples. The samples were washed with 1 mL of 4% formic acid in water, followed by 1 mL of acetonitrile, 1 mL of 0.2% diethylamine in acetonitrile and 1 mL of acetonitrile and recovered by elution with 2 × 250 μL of 2% diethylamine in acetonitrile. The eluates were taken to dryness under a stream of nitrogen at 75 °C and finally reconstituted in 75 μL of acetonitrile containing 1% water.

2.3.2. Cyanuric acid

15 μL Samples of serum were diluted with 315 μL of water and 75 μL of a 100 ppb solution of $^{13}C_3$-labeled cyanuric acid. The samples were mixed in a 96-well plate agitator for 30 min at room temperature. An OASIS MAX (anionic exchange) 30 mg 96-well plate was conditioned by sequentially washing each well with 1 mL of 0.01 M HCl in methanol, 1 mL of methanol, 1 mL of 0.1 M NaOH in methanol, and 1 mL of methanol. After equilibration of the wells with 1 mL of acetonitrile followed by 1 mL of 5% ammonium hydroxide in water, 200 μL of 5% ammonium hydroxide in water were loaded into each well, followed by the serum samples. The samples were washed with 1 mL of 5% ammonium hydroxide in water, followed by 1 mL of methanol and 1 mL of acetonitrile and recovered by elution with 2 × 250 μL of 2% formic acid in acetonitrile. The combined eluates were taken to dryness under a stream of nitrogen at 75 °C and finally reconstituted in 75 μL of acetonitrile containing 1% water.

2.4. UPLC–MS/MS system

The UPLC–MS/MS system comprised an Acquity UPLC system (Waters Corporation) interfaced with a Waters Quattro Premier XE tandem mass spectrometer equipped with an electrospray ionization probe. Chromatographic separation was performed on a Waters BEH Amide, 1.7 μm, 2.1 mm × 100 mm column maintained at 35 °C, using a 600 μL/min gradient of acetonitrile (eluent A) and 10 mM ammonium acetate in water (eluent B) as follows: 2 min isocratic elution with 95% A; 1 min linear gradient to 30% A; 0.8 min isocratic elution with 30% A; 0.2 min linear gradient to 95% A; 1 min equilibration at 95% A. An autosampler containing the samples was thermostated at 8 °C and an injection volume of 10 μL (partial loop with needle overfill) was used. During the first 2.5 min, the ESI source was used in the negative ionization mode and the optimized MS parameters were as follows: capillary voltage at 3.0 kV; cone voltage at 25 V; source temperature at 120 °C; desolvation temperature at 400 °C; cone gas flow at 50 L/h; desolvation gas flow at 900 L/h; the collision gas flow was kept at 0.3 mL/min, enabling a collision cell pressure of approximately 7.9 μbar. After 2.5 min and until the end of the run, the ESI source was used in the positive ionization mode and the optimized MS parameters were as follows: capillary voltage at 0.3 kV; cone voltage at 30 V; The remaining parameters were unchanged from the negative mode acquisition. The following MRM transitions (*m/z*) were monitored in negative mode (0–2.5 min): $^{13}C_3$-cyanuric acid 130.7 → 43.3 (internal standard transition; collision energy 12 eV); cyanuric acid 127.8 → 42.3 (quantification transition; collision energy 12 eV); cyanuric acid 127.8 → 85.1 (confirmatory transition; collision energy 9 eV). The transitions monitored in positive mode (2.5–5 min) were as follows: $^{15}N_3$-melamine 129.9 → 87.1 (internal standard transition; collision energy 16 eV); melamine 126.9 → 85.0 (quantification transition; collision energy 16 eV); melamine 126.9 → 68.2 (confirmatory transition; collision energy 23 eV). The dwell time for the negative mode transitions was 250 ms, and for the positive mode transitions was 80 ms. In all cases an inter-scan delay of 20 ms and an inter-channel delay of 5 ms was used.

2.5. Method validation

2.5.1. Calibration curves

Calibration solutions of unlabeled melamine or cyanuric acid were prepared individually in acetonitrile containing 1% water. The plot of response ratios for labeled versus unlabeled melamine and cyanuric acid was linear over the concentration range of 5–1000 ppb of unlabeled compound plus 100 ppb of the respective labeled internal standard and afforded a slope of 1.29 with a correlation coefficient (r^2) of 0.9993 for melamine and a slope of 1.33 with an r^2 of 0.9997 for cyanuric acid. Eight different concentration ratios, analyzed in quadruplicate, were used for each plot and the respective residuals were within 10% of the nominal values.

2.5.2. Accuracy, precision, and lower limit of quantification

The intra- and inter-assay accuracy of the method was evaluated by comparing the mean experimental and nominal concentrations of five independent blank rat serum samples spiked with 50, 500, or 1000 ppb of melamine or cyanuric acid, for a total of 15 independent samples per compound per day, on two consecutive days. The precision of the method was estimated by the intra- and inter-day coefficient of variation of the determinations. The lower limit of quantification (LLOQ) was estimated by analyzing blank rat serum samples spiked with decreasing concentrations of melamine or cyanuric acid (25, 10, 5, and 2.5 ppb; $n = 5$ for each concentration) and determining the lowest concentration at which the deviation from the nominal concentration and the imprecision (CV) did not exceed 10% [13]. Given a pervasive and stable low-level background con-

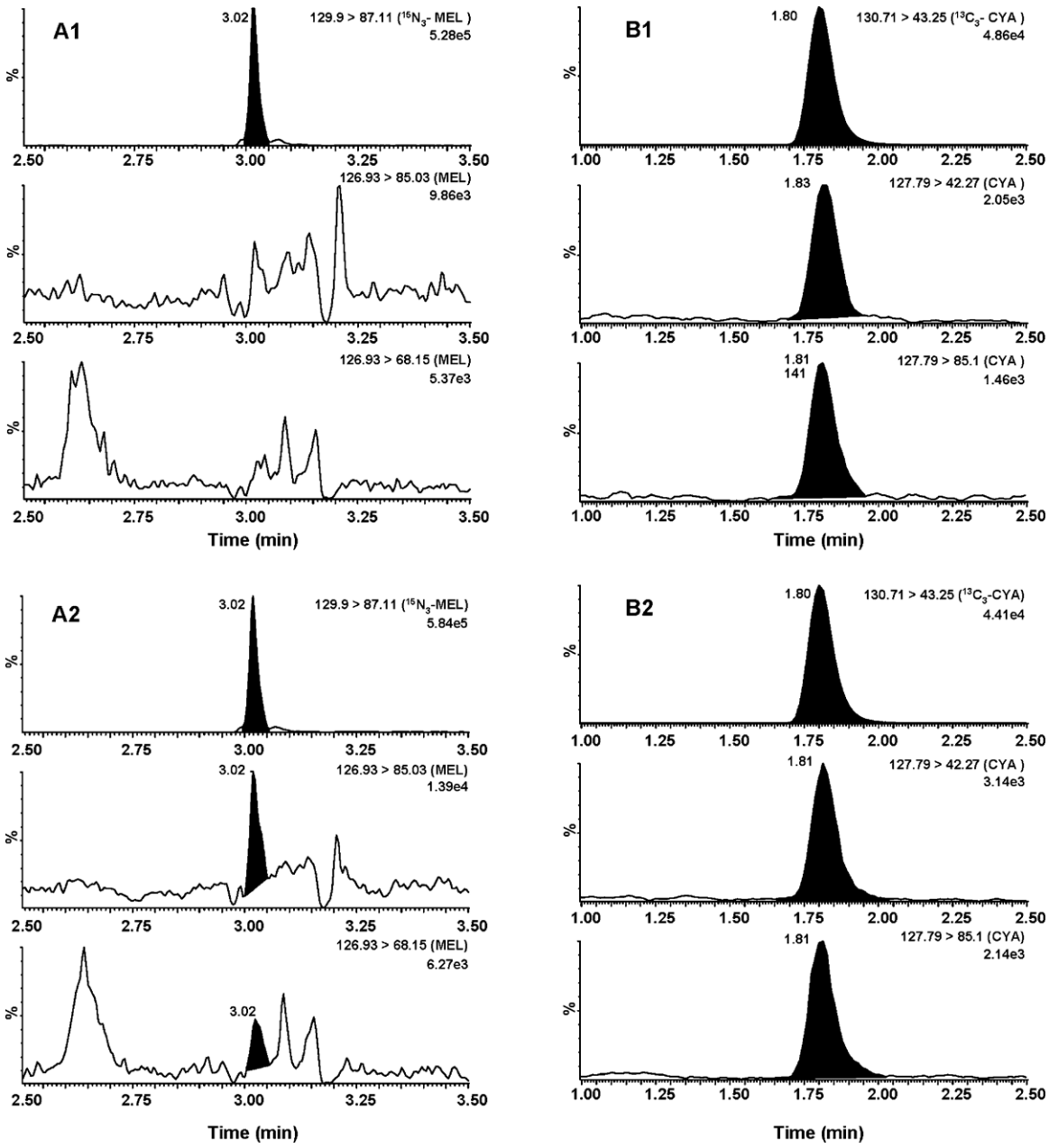

Fig. 1. Representative chromatograms obtained in the analysis of melamine in blank rat serum (panel A1) and rat serum spiked with 5 ppb of melamine (panel A2). The transitions, monitored in positive electrospray mode, were as follows: $^{15}N_3$-melamine 129.9 → 87.1 (internal standard transition; collision energy 16 eV); melamine 126.9 → 85.0 (quantification transition; collision energy 16 eV); melamine 126.9 → 68.2 (confirmatory transition; collision energy 23 eV). Representative chromatograms obtained in the analysis of cyanuric acid in blank rat serum (panel B1) and rat serum spiked with 10 ppb of cyanuric acid (panel B2). The transitions, monitored in negative electrospray mode, were as follows: $^{13}C_3$-cyanuric acid 130.7 → 43.3 (internal standard transition; collision energy 12 eV); cyanuric acid 127.8 → 42.3 (quantification transition; collision energy 12 eV); cyanuric acid 127.8 → 85.1 (confirmatory transition; collision energy 9 eV).

tamination of cyanuric acid, the mean of the method blank values was subtracted from the integration of each sample.

2.5.3. Recovery and matrix effect

A quantitative assessment of the matrix effect was performed at the 100 ppb level, and was estimated as the ratio of the peak area provided by a blank rat serum extract fortified with 100 ppb of melamine or cyanuric acid, and that of a quality control standard at the same concentration. The extraction recovery was calculated as the ratio of the peak areas of a 100 ppb processed sample and that of the fortified serum extracts described above. These analyses were performed in triplicate.

3. Results and discussion

The method reported herein was designed and optimized to provide lower limits of quantification in the low ppb range for both melamine and cyanuric acid, while requiring the use of only limited amounts of rat serum (15 μL for each compound). Initial attempts to prepare samples by simple protein precipitation with organic solvents were unsuccessful due to low recoveries and/or very high signal suppression (data not shown). Considering the expected advantages of incorporating a pre-purification procedure orthogonal to the chromatography, we modified and optimized a solid phase extraction protocol for ionic exchange media suggested for the analysis of melamine (Oasis MCX cationic exchange

Table 1
Statistical analysis of the validation of the UPLC–MS/MS methodology. The intra- and inter-assay accuracy of the method was evaluated by comparing the mean experimental and nominal concentrations of five independent blank rat serum samples spiked with 50, 500, or 1000 ppb of melamine or cyanuric acid, for a total of 15 independent samples per compound per day, on two consecutive days. The precision of the method was estimated by the intra- and inter-day coefficient of variation of these determinations.

Compound	Nominal concentration in serum (ppb)	Measured concentration in serum (ppb)	SD	Precision (CV, %)	Accuracy (%)
Intra-assay validation ($n = 5$)					
Melamine	50	50.6	2.1	4.1	101.3
	500	497.8	13.0	2.6	99.6
	1000	969.1	28.8	3.0	96.9
Cyanuric acid	50	50.2	2.1	4.1	100.4
	500	476.1	11.2	2.4	95.2
	1000	928.5	13.4	1.4	92.9
Inter-assay validation ($n = 5$)					
Melamine	50	48.8	1.3	2.6	97.7
	500	503.3	18.0	3.6	100.7
	1000	1001.0	33.3	3.3	100.1
Cyanuric acid	50	50.8	2.7	5.4	101.5
	500	489.6	9.7	2.0	97.9
	1000	931.4	20.4	2.2	93.1

resin) and cyanuric acid (Oasis MAX anionic exchange resin) in infant formula and other food matrices. The protocol was adapted to 96-well format SPE media, and modifications and optimizations, focused on the conditioning and recovery steps of the SPE process resulted in reduced signal suppression and improved recovery of both compounds. In order to maximize the LLOQ, while assuring short instrumental run times, the methodology was developed using a UPLC with 1.7 μm particle size columns. This configuration initially proved to be problematic given that UPLC columns referenced by the vendors as adequate for the chromatography of melamine and cyanuric acid in feed matrices did not provide adequate separation from early eluting serum interferences that significantly suppressed the signal of cyanuric acid. Upon comparing a number of columns from different vendors it was determined that the Waters UPLC BEH Amide HILIC column allowed for a good separation of melamine and cyanuric acid, washing, and regeneration in a total of 5 min, with cyanuric acid eluting at 1.80 min (Fig. 1, panel A), and melamine at 3.02 min (Fig. 1, panel B). It should be noted that good baseline separation of the two compounds was crucial to maximize the mass spectral response by avoiding the need to conduct polarity cycling between negative ESI mode for cyanuric acid and positive ESI mode for melamine. The relevant mass spectral parameters were optimized by infusion of standard solutions of melamine or cyanuric acid in the mass spectrometer. Interestingly, the melamine signal consistently optimized at very low capillary voltages (*ca.* 300 V). The selectivity provided by the use of the MRM mode assured low background signal and thus signal to noise maximization. The method was validated with regard to its accuracy and precision by analyzing rat serum samples spiked with 50, 500, or 1000 ppb of melamine or cyanuric acid, revealing good statistical performance, with intra- and inter-day accuracies of *ca.* 93–102% and imprecision under 5% (Table 1). As optimized, the method provided a recovery of 80–110% of melamine with a signal suppression of *ca.* 55%, and a recovery of 50–90% of cyanuric acid with a signal suppression *ca.* 40–60%. Although a considerable effort was made to minimize the signal suppression by adjusting the chromatographic and SPE conditions, the relatively poor retention of cyanuric acid and melamine in the chromatographic and SPE media precluded further improvements. The LLOQ of the methodology, corresponding to the lowest experimental concentration at which the deviation from the nominal concentration and the imprecision (CV) did not exceed 10% was 5 ppb for melamine (mean accuracy 109%; CV = 4.9%) and 10 ppb for cyanuric acid (mean accuracy 96%; CV = 8.6%). These LLOQ values, determined according to US National Toxicology Program guidelines [13], are comparable with those estimated by the equation $LOQ = X + 10\sigma$, where X is the mean value of the blank serum response ($n = 10$), and σ the respective standard deviation [14], corresponding, after correction for the dilution factor in the sample preparation procedure, to *ca.* 6 ppb for melamine, and 25 ppb for cyanuric acid in the serum samples. Discrepancies on LOQ values well in excess of the one observed here for cyanuric acid have been reported in mass spectral methods following the use of different internationally accepted guidelines for LOQ determination [15]. The estimated limits of detection (LOD), calculated as $LOD = X + 3\sigma$ [14] were *ca.* 1.9 ppb for melamine and 8 ppb for cyanuric acid. These values compare favorably with those reported for other methodologies in the literature for melamine [5–12] and cyanuric acid [8,12] in serum or plasma, in particular considering that the sample requirements reported herein are at least 3-fold lower than those previously reported. It should be noted that the higher LLOQ obtained for cyanuric acid was due to a pervasive low-level contamination of cyanuric acid, the origin of which could not be conclusively traced, and that affected the statistical performance of the method at these very low concentrations. Similar problems have been reported by other authors in the LC–MS analysis of melamine and cyanuric acid [12].

4. Conclusions

A methodology has been developed for the low-level analysis of melamine and cyanuric acid in limited volumes of rat serum. The methodology, based upon sample preparation by ionic exchange SPE and UPLC-isotopic dilution tandem mass spectrometry allows the quantification of melamine or cyanuric acid with good accuracy and precision using 15 μL samples of serum containing concentrations as low as 5 ppb of melamine or 10 ppb of cyanuric acid. The sensitivity of the method, the multiplex nature of the SPE sample preparation, and short run times (5 min) make it optimal for toxicokinetic and exposure monitoring studies in small animals. Although this methodology was developed and validated using rat serum, given the use of isotopically labeled internal standards that can inherently account for reasonable differences in the sample matrix, it is envisioned that the methodology should be applicable to human serum samples with only minor modifications. Given the very low sample requirements and sensitivity of the method, the availability of such a methodology could prove extremely useful in the analysis of pediatric samples in the event of a suspected exposure to melamine or cyanuric acid.

Acknowledgements

This study was supported through an interagency agreement between the FDA and the National Toxicology Program at NIEHS (FDA IAG:224-07-0007; NIH Y1ES1027).

The opinions expressed in this paper do not necessarily represent those of the U.S. Food and Drug Administration.

References

[1] University of Guelph Laboratoy Services Division Urgent Bulletins – Pet Food Recall. http://www.labservices.uoguelph.ca/urgent.cfm, 2007 (accessed 20.10.10).
[2] R.L.M. Dobson, S. Motlagh, M. Quijano, R.T. Cambron, T.R. Baker, A.M. Pullen, B.T. Regg, A.S. Bigalow-Kern, T. Vennard, A. Fix, R. Reimschuessel, G. Overmann, Y. Shan, G.P. Daston, Toxicol. Sci. 106 (2008) 251.
[3] B. Puschner, R.H. Poppenga, L.J. Lowenstine, M.S. Filigenzi, P.A. Pesavento, J. Vet. Diagn. Invest. 19 (2007) 616.
[4] World Health Organization. http://whqlibdoc.who.int/publications/2009/9789241597951_eng.pdf, 2009 (accessed 20.10.10).

[5] R.E. Baynes, B. Barlow, S.E. Mason, J.E. Riviere, Food Chem. Toxicol. 48 (2010) 2542.
[6] R.E. Baynes, G. Smith, S.E. Mason, E. Barrett, B.M. Barlow, J.E. Riviere, Food Chem. Toxicol. 46 (2008) 1196.
[7] W. Jingbin, M. Ndong, H. Kai, K. Matsuno, F. Kayama, Food Chem. Toxicol. 48 (2010) 1791.
[8] X.W. Lv, J. Wang, L. Wu, J. Qiu, J.G. Li, Z.L. Wu, Y.C. Qin, J. Agric. Food Chem. 58 (2010) 943.
[9] Y.T. Wu, C.M. Huang, C.C. Lin, W.A. Ho, L.C. Lin, T.F. Chiu, D.C. Tarng, C.H. Lin, T.H. Tsai, J. Chromatogr. A 1216 (2009) 7595.
[10] Y.T. Wu, C.M. Huang, C.C. Lin, W.A. Ho, L.C. Lin, T.F. Chiu, D.C. Tarng, C.H. Lin, T.H. Tsai, J. Agric. Food Chem. 58 (2010) 108.
[11] F. Yang, Y. Mao, X.D. Zhang, Z.Q. Ma, X.R. Zhang, J. Sep. Sci. 32 (2009) 2974.
[12] G.Y. Liu, S.J. Li, J.Y. Jia, C.Y. Yu, J. He, C. Yu, J.M. Zhu, Regul. Toxicol. Pharmacol. 56 (2010) 193.
[13] US National Toxicology Program, Specifications for the Conduct of Studies to Evaluate the Toxic and Carcinogenic Potential of Chemical, Biological and Physical Agents in Laboratory Animals for the National Toxicology Program (NTP), Available from http://ntp.niehs.nih.gov/files/Specifications_2006Oct1.pdf, 2006 (accessed 14.01.11).
[14] EURACHEM, The Fitness for Purpose of Analytical Methods. A LABORATORY GUIDE to Method Validation and Related Topics, LGC, Teddington, Middlesex, United Kingdom, 1998.
[15] M.M. Sanagi, S.L. Ling, Z. Nasir, D. Hermawan, W.A. Ibrahim, A. Abu Naim, J. AOAC Int. 92 (2009) 1833.