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Article

Analysis of Sub μg/kg Lincomycin in Honey, Muscle, Milk, and Eggs Using Fast Liquid Chromatography–Tandem Mass Spectrometry

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A fast liquid chromatography–tandem mass spectrometry (LC–MS–MS) method is developed to determine lincomycin (LM) in honey, muscle, milk, and egg. Samples are cleaned-up at pH 4.7 using Strata-X-C mixed-mode polymeric strong cation exchange solid-phase extraction (SPE) cartridges, which could selectively adsorb the lincomycin from matrices under the acidic condition. LM is separated on the recently introduced Kinetex XB core-shell type HPLC column using isocratic elution mode with a mobile phase containing 0.1% formic acid in water/acetonitrile (93/7, v/v, pH 2.6) at a flow rate of 0.7 mL/min. The subsequent MS/MS detection has decreased ion effect, which allows the limit of detection (LOD) of LM for honey to be 0.05 μg/kg for honey and 0.5 μg/kg for muscle, milk, and egg. These LODs are much lower than those reported previously. The other main advantage of the developed method is the analysis time of only 3.5 min, which is about three times shorter than other reported LC–MS–MS methods. Recoveries varies between 94.2% and 125.2% and in-house reproducibility ranges from 3.7% to 28.7%. The developed method is validated according to European Union (EU) Commission Decision 2002/657/EC using a matrix-comprehensive validation strategy. All studied analytical parameters fulfills the EU guidelines.

Introduction

Lincomycin (LM) derived from Streptomyces lincolnensis, belongs to lincosamides group and is one of the most widely used antibiotics administered to animals and humans against gram positive bacteria. In the protein synthesis, LM acts as an RNA-dependent inhibitor (1). The risk of LM present in food products such as honey and milk may cause bacterial resistance and allergic reactions (2). The frequency and content of LM in biological samples suggest the contamination of foodstuffs (3). Therefore, the European Union (EU) has established maximum residual levels (MRLs) in the different foods of animal origin (4). MRLs of LM were set as 50, 100, 150, 500, and 1500 μg/kg for eggs, muscle, bovine milk, liver, and kidney, respectively (4). Although, no MRL have been established for antibiotics in honey, but some countries including Belgium, Hungary, Switzerland, and the United Kingdom have set limits, which vary from 10—50 μg/kg (5). It is therefore imperative to develop fast and efficient analytical procedure to detect LM in food samples.

A number of methods have been used to quantify LM, which include microbiological and chemical methods as well as chromatographic techniques (1, 2, 6–8). A use of high-performance liquid chromatography (HPLC) with UV and mass spectrometry detectors is common practice for analysis of LM [9]. LM has weak UV absorbance in the low wavelength range ($\varepsilon_{194nm} = 2.00 \times 10^4$ dm^2 mol^{-1} and $\varepsilon_{210nm} = 0.50 \times 10^4$ dm^2 mol^{-1}) (9), hence, LC with UV detection is not sensitive enough to determine LM in biological matrices. The application of LC–UV method was able to determine LM in fermentation broths at concentrations ranged from 600 to 50,000 μg/L (9). More powerful detection techniques such as LC–MS, LC–MS–MS or LC–TOFMS (time-of-flight) are used to detect low levels of residues in biological samples (10, 11). In previous works, limit of detections (LODs) in biological sample were achieved in the range from 0.75 to 8.8 μg/kg in ≥ 10 min analysis time for LM using HPLC method (12–17). The present paper demonstrates for the first time analysis of much lower LOD of LM in a shorter analysis time using Kinetex XB C-18 HPLC column for LC separation and subsequent MS/MS detection. However, the effectiveness of Kinetex C-18 column in antibiotic separations has already been published (18, 19), the HPLC column used in this study (Kinetex XB C-18) is the latest generation of Kinetex family and has not been used for LM determination yet. Kinetex XB has been developed especially for basic molecules such as LM.

In recent years, the focus of our research is to develop validated methods by applying pH control in sample preparations and cleaning procedures for LC separations, followed by MS–MS detection, to analyze low levels of corticosteroids and sulfonamides in environmental and biological samples (11, 20–23). The objectives of the current paper are to demonstrate: (i) fast separation of LM by using the latest core-shell type column (Kinetex XB), (ii) enhanced sensitivity by decreasing ion suppression effects from the matrix components in honey, muscle, milk, and eggs in the MS/MS analysis, and (iii) validation of an analytical procedure for determining LM in accordance with the European Union Commission Decision 2002/657/EC [24] by using a matrix-comprehensive method (16, 25, 26).

Experimental

Reagents and instruments

Lincomycin standard was purchased from Sigma-Aldrich Ltd. (Budapest, Hungary). HPLC grade acetonitrile, methanol,

ethyl acetate, and acetone were obtained from Promochem (Wesel, Germany). Suprapur acetic acid (100%) and formic acid (98–100%) were purchased from Merck Ltd. (Budapest, Hungary). Ammonia solution (25%) was obtained from Scharlau (Barcelona, Spain). Stock solution of LM was prepared by dissolving 10 mg standard in 10 mL of methanol to achieve 1 mg/mL concentration of and was stored at −20°C. This stock solution was stored for a week. A working standard solution (1 μg/mL) was prepared daily by diluting 50 μL of the stock solution with distilled water. Amoxicillin, penicillin G, ceftiofur, sulfadimethoxine, and sulfamethoxazole standards were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary). Acetic acid solution (0.01% (v/v), pH 4.7) was prepared by diluting 100 μL concentrated acetic acid to 1 L with distilled water. The blank and monitoring samples for LM analysis originated from a Hungarian residue control monitoring program from January 2010 to January 2011 and were stored at −20°C until analysis.

Strata-X-C (6 mL, 500 mg) SPE cartridges were purchased from Gen-lab Ltd. (Budapest, Hungary). Oasis HLB (6 mL, 200 mg) SPE columns were obtained from Waters Ltd. (Budapest, Hungary). In the LC separations, an Agilent 1200 HPLC system (G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat) was used (Agilent Technologies, Palo Alto, CA, USA). HPLC was connected to an Agilent 6410A Triple Quad mass spectrometer equipped with an Agilent multimode ion source (G1978B) (Agilent Technologies, Palo Alto, CA, USA). Data acquisition, quality, and quantitative evaluation were performed using the Agilent Mass Hunter B.01.04 Data Acquisition, Qualitative and Quantitative software.

Preparation of samples

Honey

Five gram samples were weighed into 50 mL PP centrifuge tubes and were dissolved in 15 mL diluted acetic acid solution by vortex-mixing for 30 s, followed by shaking at 700 min^{-1} for 40 min. Samples were cleaned-up and concentrated on SPE cartridges.

Milk

One gram samples were weighed into 50 mL PP centrifuge tubes and diluted with 5 mL acetonitrile before vortex-mixing for 30 s. Mixed samples were centrifuged at 4000 rpm speed with a Sigma 3-18K centrifuge (Osterode am Harz, Germany) at 25°C for 10 min. The upper layers (∼ 5.5 mL) were evaporated to 50-200 μL (not to dryness) in a TurboVap LV (Hopkinton, MA) under a gentle nitrogen stream at 45 °C. Next, samples were diluted by 5 mL 0.01% (v/v) acetic acid and subjected to vortex-mixing for 30 s before cleaning with SPE cartridges.

Muscle and egg

In case of egg, ten pieces whole eggs were homogenized. One gram samples were weighed into 50 mL PP centrifuge tubes and 2.5–2.5 mL acetonitrile was added to each sample. Samples were then vortex-mixed for 60 s, followed by shaking on a Janke & Kunkel IKA KS125 shaker (Staufen, Germany) at 700 min^{-1} for 20 min. Samples were subsequently centrifuged at 25 °C for 10 min at 4,000 rpm and the upper layers were collected in glass tubes. This extraction was repeated one more time and the supernatants were combined together. Extracts were evaporated to 50–200 μL (not to dryness) under a gentle nitrogen stream at 45°C and then diluted with 5 mL 0.01% (v/v) acetic acid solution (pH 4.7) before vortex-mixing for 30 s. Samples were cleaned-up and concentrated on SPE cartridges.

Solid-phase extraction clean-up

All samples were cleaned-up on Strata-X-C (6 mL, 500 mg) SPE cartridges, which were conditioned by passing 6 mL methanol, 6 mL water, and 6 mL 0.01% (v/v) acetic acid solution (pH 4.7) through the cartridges. Samples were passed through the cartridges dropwise (∼ 0.3 mL/min). Then SPE columns were rinsed with 6 mL 0.01% (v/v) acetic acid solution (pH 4.7) and then with 6 mL methanol. Cartridges were then dried under vacuum for 5 min and samples were eluted with 6 mL methanol-25% ammonia solution (95/5, v/v). The eluted samples were evaporated under a gentle nitrogen stream at 45 °C to 50-100 μL. In the case of honey, the samples were re-dissolved in acetonitrile–0.1% formic acid in water (7/93, v/v, pH 2.6) solution and adjusted to a final volume to 500 μL. This allowed concentrating honey samples by ten times. For muscle, milk and egg, the samples were re-dissolved in acetonitrile–0.1% formic acid in water (7/93, v/v, pH 2.6) solution and adjusted to a final volume to 1000 μL. Samples were filtered using a 0.45 μm Phenex nylon membrane filter (Gen-lab Ltd., Budapest, Hungary) and transferred into HPLC autosampler vials.

SPE clean-up procedure was also tested on Oasis HLB (6 mL, 200 mg). Cartridges were conditioned with 6 mL methanol, 6 mL water, and 6 mL 0.01% (v/v) acetic acid solution (pH 4.7). Prepared samples were passed through drop wise. Cartridges were rinsed with 6 mL 0.01% (v/v) acetic acid solution (pH 4.7) and dried with vacuum for 5 min. Samples were eluted with 6 mL methanol and evaporated until 50-100 μL under a gentle nitrogen stream at 45 °C. Samples were re-dissolved the same way as written in Strata-X-C clean-up.

LC–MS–MS analysis

LM was separated on Phenomenex Kinetex XB (100 mm x 3 mm, 2.6 μm) C-18 column (Gen-lab Ltd., Budapest, Hungary) using an isocratic elution. The mobile phase was a mixture of acetonitrile-0.1% formic acid in water (7/93, v/v, pH 2.6). The flow rate was 0.7 mL/min and the injection volume was 10 μL. The separation time was 3.5 min and the column thermostat was set at 30°C.

The QqQ mass spectrometer was used in the MRM (multiple reaction monitoring) mode and LM was detected by applying two ion traces (quantify and qualify). The quantify ion transition was 407.2 >> 126.2 (fragmentor voltage: 70 V, collision energy: 30 V) and qualify ion transition was 407.2 >> 359.3 (fragmentor voltage: 70 V, collision energy: 20 V). Dwell time of 200 ms and 150 V delta electron multiplier voltage were used for both ion traces. The multimode ion source (MMI) was set in the positive ESI mode. The ion source settings

were: drying gas temperature: 350°C, gas flow: 5 L/min, vaporizer: 250°C, nebulizer pressure: 413.7 kPa, capillary voltage: 2500 V, charging voltage: 2000 V. Drying and collision gas were nitrogen. The collision gas pressure was 1.07 Pa.

Quantification

Nine-point (including zero) matrix-matched curves were prepared for quantification. Results were evaluated by external standard method using a linear regression between absolute areas and concentrations. The calibration curve was weighted with $1/y^2$, because the calibration range was wide, and therefore, the accuracy of the lowest valuable point (5 μg/kg) was not acceptable. Using this way to calibration, the low values were also acceptable, while the accuracy of higher points have not changed.

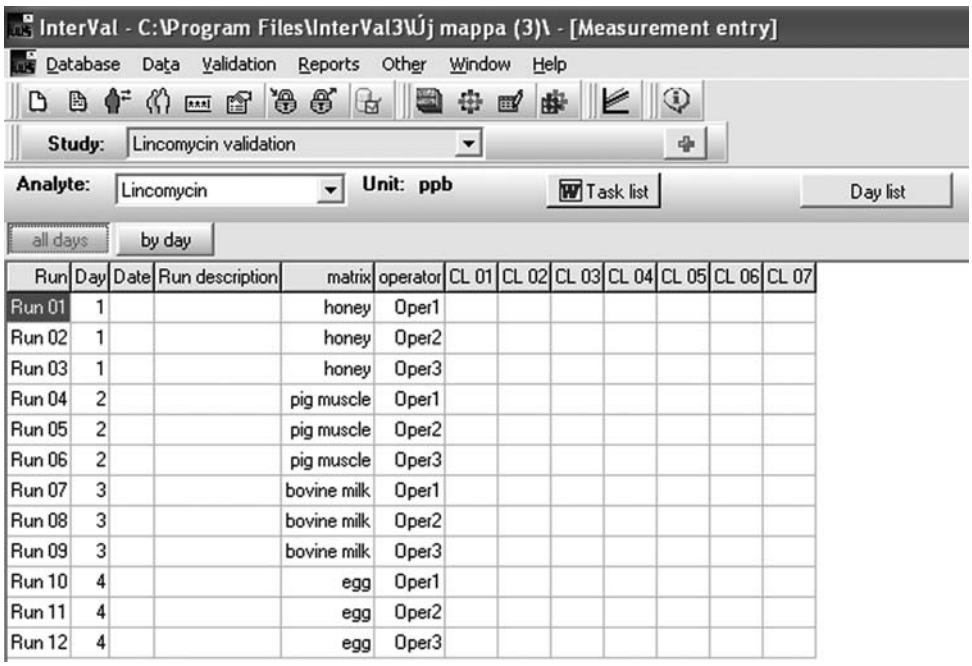
Validation method

In the validation, spiking levels were set to 1 mrpl (50 μg/kg), 1.5 mrpl (75 μg/kg), and 2 mrpl (100 μg/kg) for honey and 0.5 MRL, MRL, and 1.5 MRL for muscle, milk and egg that have permitted limits. A low level of 5 μg/kg was also tested for all matrices. The mrpl is either the lowest concentration of the analyte expected to be detected (screening method) or the lowest level at which its identity can be unequivocally confirmed (confirmatory methods). The detection capability (CCβ) of the screening method equals to mrpl concentration. Measurements were repeated on four different days under different conditions in order to determine the repeatability and reproducibility of the method. The comprehensive validation of the method according to the EU directive was matrix-comprehensive in-house method (25). The software of the method was InterVal 3 (version 3.1.2), developed for veterinary residues by the Federal Office of Consumer Protection and Food Safety (BVL, European Reference Laboratory for Residues, Berlin, Germany). This software is based on factorial design (27, 28), which allowed the simultaneous validation of matrices and required reduced number of samples. In the validation, two factors (matrix and operator) were defined in which matrix was the leading factor (Fig. 1). The leading factor had four levels: honey (level 0), pig muscle (level 1), bovine milk (level 2) and egg (level 3). The other factor, operator (different persons), had three levels: Oper1 (level 0), Oper2 (level 1) and Oper3 (level 2). Studied analytical parameters were: selectivity, identification, linearity, recovery, repeatability, in-house reproducibility, decision limit (CCα), detection capability (CCβ), limit of detection (LOD), and limit of quantification (LOQ).

Results and Discussion

General conditions for liquid chromatography

The LC was performed using the new XB (100 mm x 3 mm, 2.6 μm) C-18 column, a shell type stationary phase. This phase has been developed by using sol–gel processing techniques in which homogeneous porous shell is grown on the solid silica core. Some recent studies have demonstrated advantages of new core–shell particles over sub-2 μm fully porous particles (18, 19, 29–32]. The sufficient retention on the column was determined by carrying out two isocratic basic model runs. DryLab software was used to predict the optimal solvent ratio. The mobile phase was a mixture of acetonitrile-0.1% formic acid in water. The organic modifier content was set as 5% and 10% for the two basic runs. The column thermostat was set at 30°C. The calculated optimal mobile phase composition was



Run	Day	Date	Run description	matrix	operator	CL 01	CL 02	CL 03	CL 04	CL 05	CL 06	CL 07
Run 01	1			honey	Oper1							
Run 02	1			honey	Oper2							
Run 03	1			honey	Oper3							
Run 04	2			pig muscle	Oper1							
Run 05	2			pig muscle	Oper2							
Run 06	2			pig muscle	Oper3							
Run 07	3			bovine milk	Oper1							
Run 08	3			bovine milk	Oper2							
Run 09	3			bovine milk	Oper3							
Run 10	4			egg	Oper1							
Run 11	4			egg	Oper2							
Run 12	4			egg	Oper3							

Figure 1. Validation plan of InterVal (print screen). Matrix was the leading factor with four levels, other factor was the operators with three levels. Fortification levels were: 5 μg/kg (CL 01), 25 μg/kg (CL 02), 50 μg/kg (CL 03), 75 μg/kg (CL 04), 100 μg/kg (CL 05), 150 μg/kg (CL 06) and 225 μg/kg (CL 07) for all matrices.

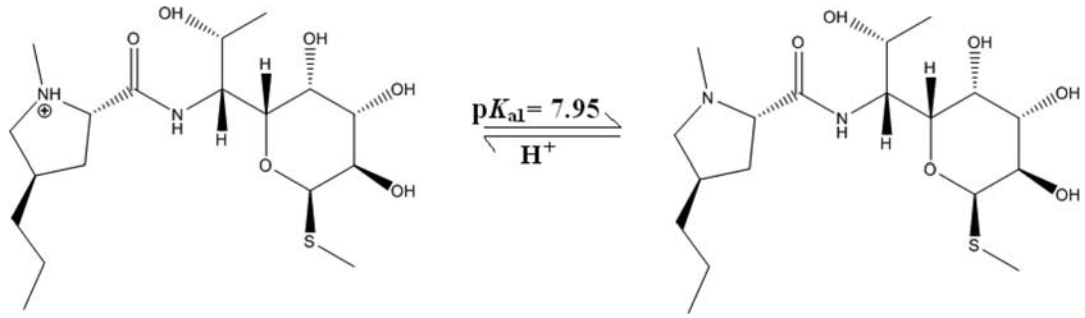


Figure 2. Structure of lincomycin.

7/93 (v/v) (acetonitrile/0.1% formic acid in water). This mobile phase composition provided a sufficient retention factor for LM having $R_t < 4$ min and 0.1 min peak shape.

Optimization of MS–MS parameters

During the optimization step, the ion transitions (precursor ion >> product ion) were set to maximum intensity. Mass spectra of LM (MW 406 dalton) were recorded in both positive and negative mode. Flow injection analysis (FIA) was carried out by connecting the injector outlet of the HPLC system directly to the nebulizer of the mass spectrometer. Methanol/0.1 % formic acid in water (70/30, v/v) mobile phase was used at flow rate of 0.3 mL/min. Firstly, MS2 scan mode was used to find the precursor ion. In positive mode, $[M + H]^+$ precursor ion was determined while $[M\text{-}H]^-$ ion was the detected species during negative ionization. In MMI, $[M + H]^+$ molecular ion was more intensive compared to the opposite polarity ion. MMI can perform both APCI and ESI ionization. LM is a polar species and ESI ionization has better detector response than that of APCI ionization.

Selected precursor ion was optimized employing different fragmentor voltage between 60 and 110 V. A voltage of 70 V had the highest response. After selecting the most intense fragmentor voltage for the precursor ion, the collision energies (CEs) of the ion traces were optimized between 0–30 V using the product ion scan mode. After choosing the two product ions with their optimal CEs, the detector was set to the MRM mode. The more intensive ion transition was used for quantification, and other one was used for qualification. The MRM ion transitions were further enhanced using different vaporizer temperature between 150–250°C, but there was not significant difference in detector signals between the tested vaporizer temperatures. Vaporization temperature of 250°C was used since the mobile phase contained low level of organic phase.

Optimization of SPE clean-up

LM has a pK_{a1} of 7.95 that suggests the presence of the cationic species of LM at pH 4.7 (Figure 2). A mixed-mode cation exchange SPE cartridge was therefore used in the present study for cleaning procedure. Strata-X-C sorbent contains both high capacity polymeric reversed-phase surface and strong cation exchange sulfonic acid groups. At pH 4.7, LM adsorbed selectively on the cation exchange phase while neutral and acidic matrices components would concentrate on the reversed-phase surface of the cartridge.

Table I
Recovery on 100 μg/kg Level Using Strata-X-C and Oasis HLB Cartridges

Sample	Recoveries (%)	
	Strata-X-C	Oasis HLB
Honey	91	162
Muscle	102	164
Milk	89	112
Egg	88	92

During the method development, different washing and elution conditions were tested to elute separately LM and matrices from the cartridges. Blank samples were fortified to 100 μg/kg according to procedure explained in previous sections. In the first condition, cartridges were rinsed first with 6 mL 0.01% (v/v) acetic acid solution (pH 4.7) and then with 6 mL ethyl acetate solution, followed by elution with 6 mL ethyl acetate–25% ammonia solution (95/5, v/v). During the second condition, cartridges were washed first with 6 mL 0.01% (v/v) acetic acid solution (pH 4.7) and then with 6 mL acetone. Samples were eluted with 6 mL acetone–25% ammonia solution (95/5, v/v). In the third condition, 6 mL 0.01% (v/v) acetic acid solution (pH 4.7) and subsequent 6 mL methanol were used for washing solutions, and elution was performed by using 6 mL methanol–25% ammonia solution (95/5, v/v). Acetic acid solution under different conditions removed polar proteins in samples, but kept the acidic phase on the cartridge. All organic solvents (ethyl acetate, acetone, and methanol) could elute both polar and non-polar matrices from the reversed-phase of cartridges. However, LM interacted with sulfonic acid groups of Strata-X-C and hence neutral organic solvents could not elute it from the cartridges. Elution of LM was possible only under basic conditions. Under first condition, LM was not detected in samples. During the second condition, the absolute recoveries were not higher than 12% for all samples. Under methanol condition (third method), absolute recoveries were between 71-76% and are in the range of satisfaction.

Optimization using Oasis HLB and Strata-X-C cartridges were compared. Before the SPE clean-up, samples were spiked to 100 μg/kg and were loaded to cartridges. Table I shows the results of absolute recoveries. The recoveries of milk and egg on HLB cartridges were 92% and 112%, respectively. However, the recoveries of honey and muscle (162% and 164%) were out

of satisfaction range (70–125%). In case of honey and muscle samples, ion enhancement was observed, which were not in the acceptable range (Table I). Comparatively, all matrices had good recovery (88-102%) using Strata-X-C. In HLB cartridges, matrices concentrated and eluted along with LM under methanol elution. The advantage of Strata-X-C is the cation exchange selectivity that enabled the selective sorption of LM under acidic condition.

Experiment on matrix effects

Absolute and relative matrix effects (ME%) were determined for all matrices at their set limits using an earlier published method (10–11). Five blank samples, which originated from different sources, for each matrix were cleaned-up and spiked after the sample preparation. Blank samples were spiked at 5, 100, 150, and 50 μg/kg for honey, muscle, milk, and egg samples, respectively. Standard solutions, which contained LM at the same concentrations as spiked ones, were also prepared in a HPLC clean solvent without matrix. Samples were injected into LC–MS–MS and areas were integrated. Absolute ME% was determined as 100 x [(area of LM in spiked sample/area of LM in standard solution)-1] for all spiked samples and matrices. Relative ME% was determined for all matrices as the relative standard deviation (RSD%) of areas of the five spiked samples.

This experiment assumes that there was no matrix effect in a clear standard solution, prepared in solvent without any matrices. If there was no ion effect in a matrix spiked sample (area of LM in matrix solution and area of LM in clear standard solution are equal), the absolute ME% was considered zero. Since the fortification was done after the clean-up, the reduction or enhancement of detector responses in matrix spiked solutions showed ion suppression or enhancement in the ion source, respectively. Generally, the matrix effects cannot be fully eliminated, however, a good sample preparation leads to similar ion effects between samples in a matrix. If the clean-up was satisfied, the absolute ME% between samples in a matrix would be nearly equal, and consequently, the RSD% of the areas of spiked samples (relative ME%) would also result in low. This relative ME% can strongly influence the reproducibility of the LC–MS–MS method (11).

Results are summarized in Table II. The RSD% of areas varied between 1.9 and 4.3% in different matrices; consequently, there was not significant difference in matrix effects between samples in a matrix. Since relative ME% could be minimized, it did not influence the reproducibility of analysis during the validation. In the case of honey and muscle samples, absolute ME% were eliminated using Strata-X-C clean-up (Table II). Results showed ~20% ion suppression when milk samples were analyzed, however, these suppressions were similar between milk samples. In egg samples, ~10% ion enhancement was observed in all samples (Table II). Absolute ME% was compensated using the matrix-matched curve for calibration.

Table II
Experiment on Matrix Effect*

	Honey Area (cps)	Muscle Area (cps)	Milk Area (cps)	Egg Area (cps)
Sample 1	187631	434883	461401	209224
Sample 2	185969	415912	481547	208575
Sample 3	176720	414571	490287	201600
Sample 4	169956	422895	487311	200386
Sample 5	173519	418145	469272	205007
Relative matrix effect (RSD%)	4.3	2.0	2.6	1.9
Standard solution	183561	426795	591522	184763
Absolute matrix effect%				
ME% (1)	+2	+2	−22	+13
ME% (2)	+1	−3	−19	+13
ME% (3)	−4	−3	−17	+9
ME% (4)	−7	−1	−18	+8
ME% (5)	−5	−2	−21	+11

* Five blank samples for each matrix were cleaned-up on Strata-X-C cartridges. Samples from the same matrix originated from different sources. Samples were spiked after the sample preparation. Fortification levels were: 5 μg/kg for honey, 100 μg/kg for muscle, 150 μg/kg for milk and 50 μg/kg for egg, respectively. Relative matrix effect was calculated as the RSD% of areas of spiked samples. Absolute matrix effect was determined as 100 x [(area of LM in spiked sample/area of LM in standard solution)-1].

Validation

Selectivity

Three samples for all matrices were spiked with beta-lactam (amoxicillin, penicillin G, ceftiofur) and sulfonamide (sulfadimethoxine, sulfamethoxazole) type antibiotics and were analyzed by the described method. These types of antibiotics have similar properties to LM (basic ones). In the blank chromatograms (Figure 3), there were no interfering species observed where LM was eluted (Figure 3), consequently, the condition of selectivity was satisfied.

Identification

The qualifier/quantifier ratio (ion ratio) of a compound was determined in the identification. The ion ratio is the intensity ratio of the qualifier and quantifier transitions. Ion ratio of LM was determined in a standard solution (8.2%), and also in spiked samples (7.0–8.3%). The developed method thus fulfilled the requirements of EU guideline.

Linearity

Calibration was performed on 0, 5, 25, 50, 75, 100, 150, 225, and 250 μg/kg levels for each matrix. Blank samples were fortified before the sample preparation with different volume of working standard solution and were prepared for analysis. The correlation coefficients (r^2) in different matrices were > 0.9912.

Recovery, repeatability, and in-house reproducibility

The fortification levels were 5 μg/kg (CL 01), 25 μg/kg (CL 02), 50 μg/kg (CL 03), 75 μg/kg (CL 04), 100 μg/kg (CL 05), 150 μg/kg (CL 06) and 225 μg/kg (CL 07) for all matrices (Figure 1). The levels included the mrpl, 1.5 mrpl, 2 mrpl levels for honey, and also included 0.5 MRL, MRL, 1.5 MRL levels for MRL compounds. These fortification levels fulfilled the criteria of 2002/657/EC Decision. Samples were analyzed on four days. One day, one kind of matrix was measured with three operators. All operators prepared one spiked sample on each level (Figure 1), therefore 21 samples were analyzed in one day. During the four days, 84 samples were analyzed with different operators and matrices. This is agreement with the minimum

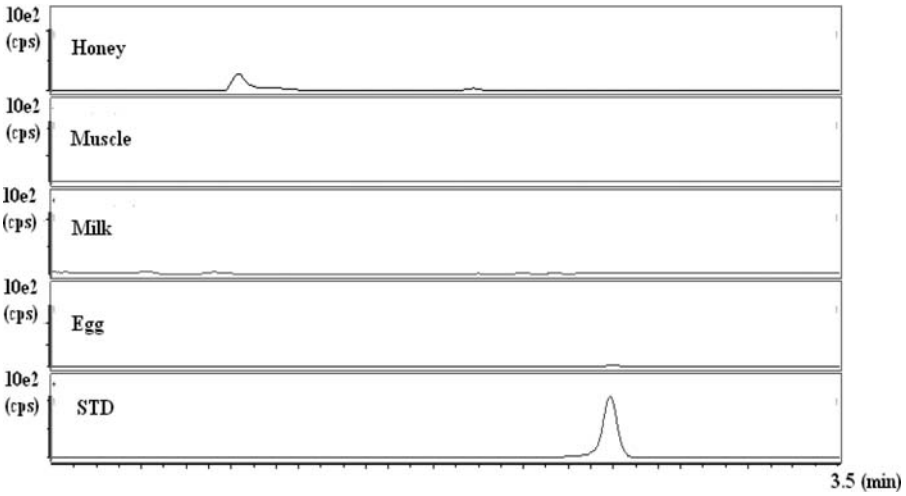


Figure 3. Total ion chromatograms (TICs) of blank samples (honey, muscle, milk, and egg) and TIC of a 5 μg/kg standard solution.

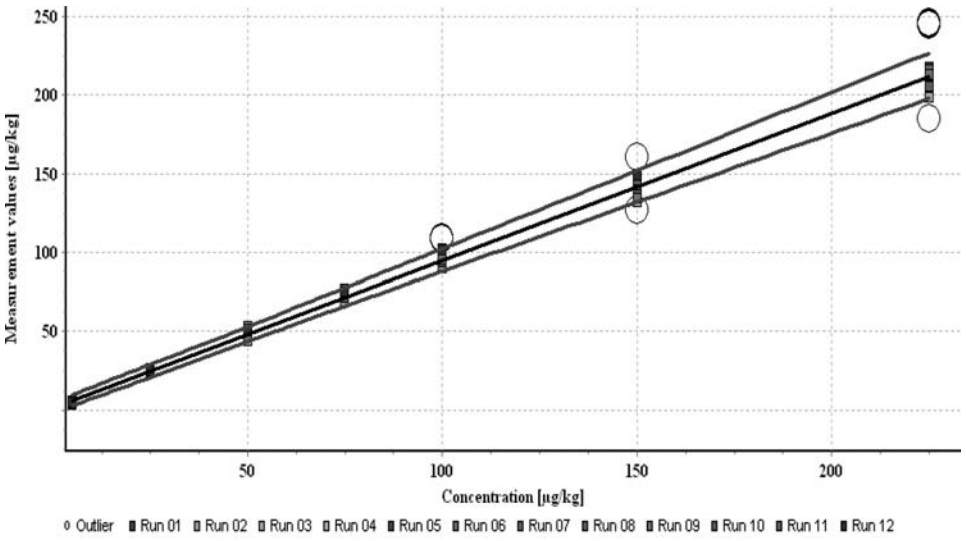


Figure 4. Overall calibration curve (middle line), prediction interval (top and bottom lines) and outliers (rings).

requirement of 80 analyses in a fractional factorial experimental plan (33).

During the evaluation of validation, the results (not corrected for recovery) were linearly regressed versus the fortification concentration (run calibrations). Overall calibration curve and predicted interval are represented in Figure 4. Significantly, factorial effects were not detected during the statistical evaluation. Using Grubbs test, outliers were detected. Seven outliers were found: in run 1, 10, 11 and 12 at 225 μg/kg, in run 2 and 4 at 150 μg/kg, and in run 5 at 100 μg/kg (Figure 4). Several causes are responsible for such outliers and are described in detail (25). In this study, outliers were detected only at high concentration levels. The sensitivity of the developed LC–MS–MS method is very high, so higher concentrations may decrease the accuracy of MS–MS detection. In daily routine practice, if high concentrations had to be measured, diluting the samples prior to analysis is recommended. The analytical parameters were determined after the outlier eliminations.

Recoveries were similar between 25 and 225 μg/kg levels (94.2–99.8%), but recovery increased to 125.2% at 5 μg/kg level (Table III). The measurement uncertainties on different levels are summarized in Table III. The repeatability and in-house reproducibility (relative uncertainty) slightly decreased from 225 μg/kg to 50 μg/kg. Under 50 μg/kg, the corresponding uncertainties sharply decreased. It should be pointed out that InterVal does not use the conventional relative standard deviation (RSD%) determination (100 x S/average of detected concentrations, where S means the standard deviation). It calculates RSD% as $100 \times S^2$/fortification level concentration. The repeatability and in-house reproducibility varied between 1.4–28.1% and 3.7–28.7%, respectively (Table III).

Analytical limits

Decision limit (CCα) means the limit at and above which it can be concluded with an error probability of α that a sample is non-compliant (24). In case of honey, where no permitted limit had been established (α= 1%), CCα was determined as three times of signal-to-noise ratio (SNR). Generally, 20 different blank samples need to be analyzed to determine the noise levels in the time window, where LM is expected. However, the noise levels were zero on both ion transitions, so SNR was determined at the lowest fortification level (5 μg/kg) by Mass Hunter Quantitative software and was interpolated to three. CCα was determined as 0.05 μg/kg for honey. In case of muscle, milk and egg matrices (MRL compounds), CCα (α= 5%) was determined by InterVal that calculates the analytical limits from the run calibrations according to [30] and was found from 55.0 to 160.8 μg/kg (Table IV).

In case of a mrpl compound, detection capability (CCβ) is the lowest concentration at which a method is able to detect truly contaminated samples with a statistical certainty of 1−β. CCβ (β = 5%) was calculated for honey as CCα + 1.64 x $S_{CC\alpha}$ and was determined as 0.07 μg/kg. In case of MRL substances, CCβ is the concentration at which the method is able to detect permitted limit concentrations with a statistical certainty of 1−β [24]. CCβ was also calculated by InterVal. It can be determined from the power functions (Figure 5), which allow the calculation of the probability of false negative results over the validated concentration range. The probability of 95% (β = 5%) adds the detection capabilities (63.2–179.1 μg/kg) (25).

LODs were calculated for all matrices as three times of SNR and were found 0.05 μg/kg for honey and 0.5 μg/kg for muscle, milk and egg, respectively. LODs were confirmed by analyzing five samples for each matrix, which were spiked to individually calculated values prior to sample preparation. LOD was accepted when both ion transitions appeared and the ion ratio was in acceptable range. LOQ was established as 3.33 × LOD (Table IV).

Table III
Recovery, Repeatability and In-House Reproducibility Results on Validation Levels

Concentration (μg/kg)	S_r^2 (μg/kg)	Repeatability RSD%	S_{wR}^2 (μg/kg)	in-house reproducibility RSD%	Recovery%
5	1.4	28.1	1.4	28.7	125.2
25	2.2	8.9	2.3	9.0	99.8
50	2.5	5.1	2.6	5.3	96.6
75	2.7	3.6	3.4	4.5	95.6
100	2.9	2.9	4.1	4.1	95.1
150	3.1	2.0	5.7	3.8	94.5
225	3.3	1.4	8.3	3.7	94.2

S_r repeatability standard deviation.
S_{wR} in-house reproducibility standard deviation.

Table IV
Decision Limit (CCα), Detection Capability (CCβ), Limit of Detection (LOD), and Limit of Quantification (LOQ)

Matrix	CCα (μg/kg)	CCβ (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)
Honey	0.05	0.07	0.05	0.17
Muscle	55.0	63.2	0.5	1.7
Milk	107.7	120.7	0.5	1.7
Egg	160.8	179.1	0.5	1.7

Analysis of Real Sample

This LC–MS–MS method was developed to confirm LM residues in food samples. LM is generally screened in laboratories using microbiological method. In our laboratory, a four plates agar gel microbiological test is applied for antibiotics. LM adds a specific inhibition zone on the third plate (*M. Luteus*, pH 8, Takácsy-type agar gel). On other plates LM does not give an inhibition zone. In national monitoring program, we analyzed two milk samples by LC-MS/MS since January 2010, which added inhibition (2-3 mm) only on third plate, but no LM was detected in samples.

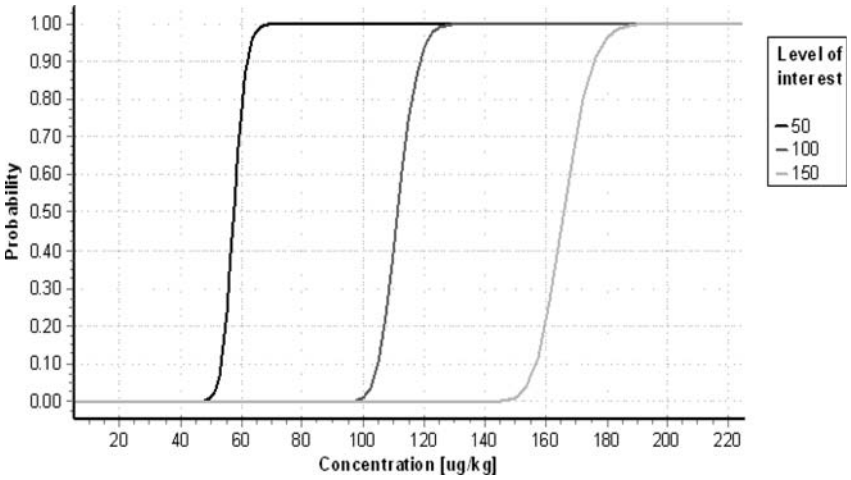


Figure 5. Corresponding power functions.

Conclusions

A fast LC–MS–MS method for analyzing LM in food matrices was developed by applying the acidic pH control and cleaning-up using Strata-X-C SPE cartridges. The separation on the Kinetex XB core-shell type HPLC column using an isocratic solvent minimized the ion effects for MS–MS detection to analyze LM in honey, muscle, milk and egg in 3.5 minutes. The obtained LOD of 0.05 μg/kg for honey is at least ten times lower than previous reported LC-MS/MS methods (14, 15, 17). In samples of muscle, milk, and egg, LOD achieved were 0.5 μg/kg, which are at least three times lower than other authors using LC-MS/MS techniques (12, 13). Recoveries in the method varied from 94.2% to 125.2% and in-house reproducibility varied between 3.7% and 28.7%. Significantly, the validation of the method using InterVal software was successful in meeting guidelines of the EU 2002/657/EC Decision.

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