Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1058 (2004) 137–142
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Determination of streptomycin and dihydrostreptomycin in milk and honey by liquid chromatography with tandem mass spectrometry

Michel van Bruijnsvoort*, Stef J.M. Ottink, Klaas M. Jonker, Enne de Boer

Dutch Food and Non-Food Authority/Inspectorate for Health Protection and Veterinary Public Health, P.O. Box 202, 7200 AE Zutphen, The Netherlands

Available online 11 September 2004

Abstract

Two liquid chromatography–tandem mass spectrometry (LC–MS/MS) methods were developed for the determination of streptomycin (STR) and its derivative dihydrostreptomycin (DHSTR) in milk and honey. These aminoglycoside antibiotics are used as veterinary drugs. In the EU, the presence of dihydro- and streptomycin residues in honey is forbidden, the maximum residue level (MRL) in milk is 200 μg/kg. The methods were optimised with regard to sensitivity and chromatographic efficiency, and validated by a procedure consistent with EU directive 2002/657. Average recoveries and accompanying standard deviations were satisfactory. The limit of quantification of STR was 2 μg/kg in honey and 10 μg/kg in milk, of DHSTR it was a factor two lower. The precision of the milk analysis was improved by using STR as the internal standard for DHSTR and vice versa. In a survey of 186 honeys available on the Dutch market, 26% of the honeys of foreign origin were positive for (DH)STR. This occurence rate was consistent with previous surveys, but lower concentrations were found.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Dihydrostreptomycin; Streptomycin; Milk; Honey

1. Introduction

Streptomycin (STR) and its derivative dihydrostreptomycin (DHSTR) are aminoglycoside antibiotics that are particularly active against aerobic gram-negative bacteria. They are used as veterinary drugs or crop-protection agents, often combined with a penicillin or tetracycline in broad-spectrum anti-infection formulations. (DH)STR offers a potent treatment of American or European foulbrood, which are amongst the most common diseases of honeybees [1]. The toxicology of these classic antibiotics is well studied, and several cases of allergic reactions have been reported [2]. Residues of the treatment with streptomycin antibiotics may be found in food products such as meat, milk and honey. In the EU, streptomycins are allowed to be used as a veterinary drug for cattle; the maximum residue limit (MRL) in milk is 200 μg/kg [3]. On the other hand, to protect the image of honey as a healthy natural product, these bactericides are banned from honey.

A variety of analytical and immunochemical methods for the determination of aminoglycoside antibiotics in food matrices have been developed [4]. Since STR and DHSTR do not possess a strong UV-absorbing chromophore group, the state-of-the-art analytical method for their determination at trace levels is liquid chromatography (LC) with post-column derivatization and fluorescence detection [5]. The complexity of food samples makes sample clean-up by solid-phase extraction (SPE) prior to LC-fluorescence analysis necessary. A cation-exchange SPE clean-up has been used for the determination of (DH)STR in animal tissue, milk and honey [6–9]. With a suitable counter-ion such as heptanesulfonic acid, streptomycins can be retained on a C_{18} SPE column [10,11]. Edder et al. have combined two SPE procedures to reach a reliable and sensitive method, that was validated in the matrices of honey, milk, meat, liver and kidney [12]. The limit of quantification (LOQ) was 10 μg/kg in honey and 50 μg/kg in milk.

There are several enzyme immunoassays (EIA) for the screening of streptomycins in milk and honey [13–16]. With EIAs, incidence rates of ca. 20% were found in milk and honey [12,17]. However, due to cross-reactions with food matrices, EIA tests are generally susceptible to generating

* Corresponding author.
E-mail address: michel.van.bruijnsvoort@vwa.nl (M. van Bruijnsvoort).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.07.101

false-positives. Surprisingly, with LC-fluorescence analysis an even higher incidence of 42% in honey was found [12]. There is a clear need for a reliable, sensitive confirmatory analytical method for streptomycin antibiotics, to monitor and control their use in the food industry.

Tandem mass spectrometry (MS/MS) is a powerful alternative to fluorescence detection. It has the advantage that the ratio between different product ions provides additional confirmation of the identity, reducing the risk of false-positive results. In 1993, McLaughlin and coworkers reported an LC–MS/MS method for the determination of aminoglycoside antibiotics, including STR and DHSTR, in bovine kidney [18,19]. These authors reached detection limits (LODs) of about 0.1 mg/kg. In recent years, the sensitivity and robustness of MS equipment have improved dramatically; Kaufmann et al. presented an LC–MS/MS analysis of streptomycin in honey with a detection limit of 1 μg/kg [20]. We have developed LC–MS/MS methods for the determination of STR and DHSTR in milk and honey. Vital aspects of the method development, regarding the mobile phase composition and sample preparation, are discussed. The emphasis in this paper is on the application to honey. With a simplified sample preparation, the method was made suitable for the analysis of milk. The performance of both methods was evaluated by a validation procedure that is in accordance with EU Directive 2002/657 [21]. A survey was conducted to investigate the presence of streptomycin antibiotics in honeys on the Dutch market.

2. Experimental

2.1. Reagents and chemicals

All reagents were obtained from standard suppliers and, with the exception of pentafluoropropionic acid (PFPA) 98+% (Fluka, Buchs, Switzerland), of at least p.a. quality. Vetranal® streptomycin sesquisulfate (Riedel-Haën, Seelze, Germany) and dihydrostreptomycin sesquisulfate >99% (Fluka) served as analytes. Distilled water, purified by a MilliQ system (Millipore, Bedford, MA, USA), was used. Stock solutions of STR and DHSTR were prepared in water and stored at 4 °C. Working standards were prepared in a 0.1 mol/L sodium heptanesulfonic acid (HSA) solution.

2.2. Apparatus

An Alliance 2695 LC system (Waters, Watford, Hertfordshire, UK) was used for chromatography. It was equipped with a 150 × 2.1 mm 5 μm Alltima C_{18} column (Alltech, Deerfield, IL, USA) that was thermostatted at 30 °C. The flow rate was 200 μL/min and the injection volume 25 μL. During the first 5.5 min of the experiment, the flow was directed to waste. Detection was performed by a Quattro Ultima (Micromass, Altrincham, Chesire, UK) triple quadrupole mass spectrometer with an electrospray interface (ESI). Positive ions were detected in the multiple reaction monitoring (MRM) mode with a dwell time of 0.3 s. The collision gas was argon at a pressure of 2.5 mbar, while nitrogen was the nebulizer and cone gas, flowing at 800 and 50 L/h, respectively. The desolvation temperature was set at 350 °C, the source temperature at 120 °C, the capillary voltage at 3.5 kV, the cone voltage at 50 V and RF lens 1 at 70 V. Data were processed by QuanLynx 4.0 software (Micromass).

2.3. Sample preparation

2.3.1. Honey

Unless noted otherwise, all preparations were carried out at room temperature. 1.5 g of honey were dissolved in 15 mL of extraction solvent, i.e., 50 mmol/L HSA in a 25 mmol/L trisodium phosphate buffer, adjusted to pH 2 with *ortho*-phosphoric acid. The solution was centrifuged for 10 min at 4000 rpm. A 3 mL SPE cartridge containing 200 mg of octadecyl packing (catalogue number 7020-02, Malinckrodt Baker, Philipsburg, NJ, USA), was activated with 3 mL of methanol (MeOH), 3 mL of water, followed by 2 mL of extraction solvent. The honey solution was loaded on the SPE cartridge in 10–15 min. The column was washed with 3 mL of water, dried for 10 s (underpressure ca. 20 kPa), then washed with 3 mL of *tert*-methylbutylketone and dried for another 10 s. Samples were eluted with 4.0 mL of MeOH into a preweighed tube. The MeOH was evaporated till almost dry, and the extract was reconstituted to 2.0 g with water.

2.3.2. Milk

Milk was diluted with four volumes of water, or reconstituted from milk powder by dissolving 1.0 g in 50 mL of water. Five milliliter was transferred to a centrifugation tube and 0.5 mL of a 5% 5-sulfosalicyl acid dihydrate solution was added. The sample was centrifuged for 10 min at 5 °C (4000 rpm). To 1.0 mL of supernatant, 3.0 mL of a 0.1 mol/L HSA solution was added and the mixture was allowed to stand for at least 15 min. The resulting opalescent solution was filtered through a 0.45 μm regenerated cellulose membrane filter.

3. Results and discussion

3.1. Method development

The settings of the mass spectrometer were optimized while infusing a 0.2 mg/L aqueous solution of the analytes, acidified with 0.1% (v/v) formic acid, into the mass spectrometer. The mass spectrum recorded of the product ions of STR with the collision energy set at 30 eV, is shown in Fig. 1. DHSTR, differing from STR by the substitution on the middle saccharide ring, generates a virtually similar mass spectrum. The fragments *m/z* 263, 246, 221, 176 and 407 were also found in earlier MS experiments [22,23]. The most abundant transitions of the respective protonated molecular ions (*m/z*

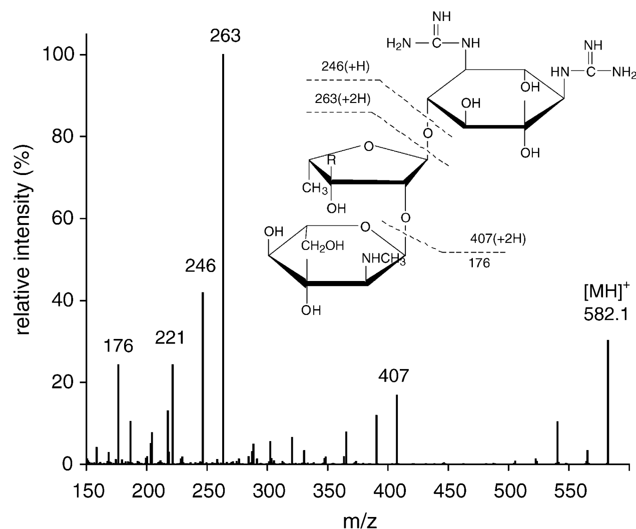

Fig. 1. ESI + mass spectrum of streptomycin. The insert shows the chemical structure of the streptomycins, where R is CHO for streptomycin and R is CH_2OH for dihydrostreptomycin.

582.1 for STR and *m/z* 584.2 for DHSTR) to *m/z* 263 were used for screening and quantification, while the ratios with the product ion *m/z* 246 were used for confirmation of the identity.

For the determination of (DH)STR by LC–MS/MS, a reversed-phase LC system with an aqueous mobile phase containing acetonitrile (ACN) and the counter-ion pentafluoropropionic acid (PFPA) was used, following McLaughlin and Henion [18]. As a starting point for method development, a mobile phase with 15% ACN and 1.9 mmol/L PFPA was chosen; a retention factor of ca. 6 was obtained. Higher concentrations of PFPA led to a decreased detector response, while with a lower concentration a poor peak shape was obtained. The analytes were dissolved in an aqueous solution of 0.1 mol/L HSA. Without the counter-ion in the injected solution, the analytes were unretained on the column. It appeared that addition of ammonium formate to the mobile phase could improve the peak shape and signal-to-noise-ratio. In the absence of ammonium formate, peaks were comparatively broad and fronting. Addition of 3.2 mmol/L ammonium formate led to a more symmetrical peak shape and the separation efficiency could be further improved with higher salt concentrations. The signal-to-noise ratio appeared to be optimal with 3.2 mmol/L of ammonium formate and these conditions were chosen for the analysis of honey samples. Further addition of electrolyte to the mobile phase led to a decrease of the detector response, which is a common trend in electrospray MS detection [24].

Streptomycins are very hydrophilic compounds. Hence, prior to the LC–MS/MS analysis an SPE clean-up is necessary to separate the analytes from the sugars that are abundantly present in the honey matrix. A C_{18} solid-phase is preferable to a cation-exchange material. Compounds are extracted from a C_{18} phase with methanol, which can be easily evaporated to concentrate the analytes. The extraction of streptomycins from a C_{18} solid-phase depends strongly on the brand [10]. Satisfactory results were obtained using a C_{18} cartridge, containing 200 mg of sorbent (Baker).

With the optimized method, the analytes could be detected in the low μg/kg range. For this method, we set our minimum required performance level (MRPL) at 10 μg/kg for STR and 5 μg/kg for DHSTR. When the method is run in our laboratory, it is a prerequisite that the signals of the confirmatory ions in a honey sample, spiked at the MRPL level, exceed the LOQ. Fig. 2 shows the LC–MS/MS chromatograms of a mixed-flower honey sample, spiked with 10 μg/kg of STR and 5 μg/kg of DHSTR. All peak shapes in the LC–MS/MS chromatograms were satisfactory. The STR chromatogram of a blank honey sample displayed an interference of matrix components that were eluted close to the analyte, but this did not hamper the quantification or confirmation of the identity. The LOQ (S/N = 6) was 2 μg/kg for STR and 1 μg/kg for DHSTR. Surprisingly for such similar components, the response of DHSTR was approximately twice that of STR. The method was more sensitive than current LC methods with fluorescence detection while a less extensive sample preparation was required.

The MRL of (DH)STR in milk is 200 μg/kg, more than one order of magnitude higher than the MRPL that was established in honey. The surplus of detector signal at the MRL level suggested that a time-consuming sample clean-up by SPE would not be necessary, but that mere dilution of the sample would suffice. In initial experiments, milk samples were diluted 20-fold in 0.1 mol/L HSA and injected after centrifugation and filtration. With this direct approach, recoveries were less than 60% due to suppression of the ionisation of the analytes by matrix components. Suppression could be eliminated completely by precipitation of the milk proteins by 5-sulfosalicylic acid. However, when the mobile phase composition for honey analysis was used, double peaks with severe peak tailing were observed. Apparently, the separation was affected by the milk matrix. By increasing the concentration of ammonium formate in the mobile phase to 6.4 mmol/L, i.e., exchanging sensitivity for chromatographic efficiency, peak shapes could be improved significantly. The LC–MS/MS chromatograms from a spiked milk sample were similar to the chromatograms generated from a spiked honey sample (Fig. 2). The chromatograms of blank milk samples displayed some disruptions of the baseline at the position of the analytes, but these were negligible compared to the signal at the MRL. The LOQ was 10 μg/kg for STR and 5 μg/kg for DHSTR, which is more than one order of magnitude below the MRL of 200 μg/kg.

3.2. Validation

3.2.1. Honey

Calibration plots, with concentrations of 0.5, 1, 2 and 4 times the MRPL, were set up in blank honey matrix. The regression coefficients for STR and DHSTR were 0.999 and

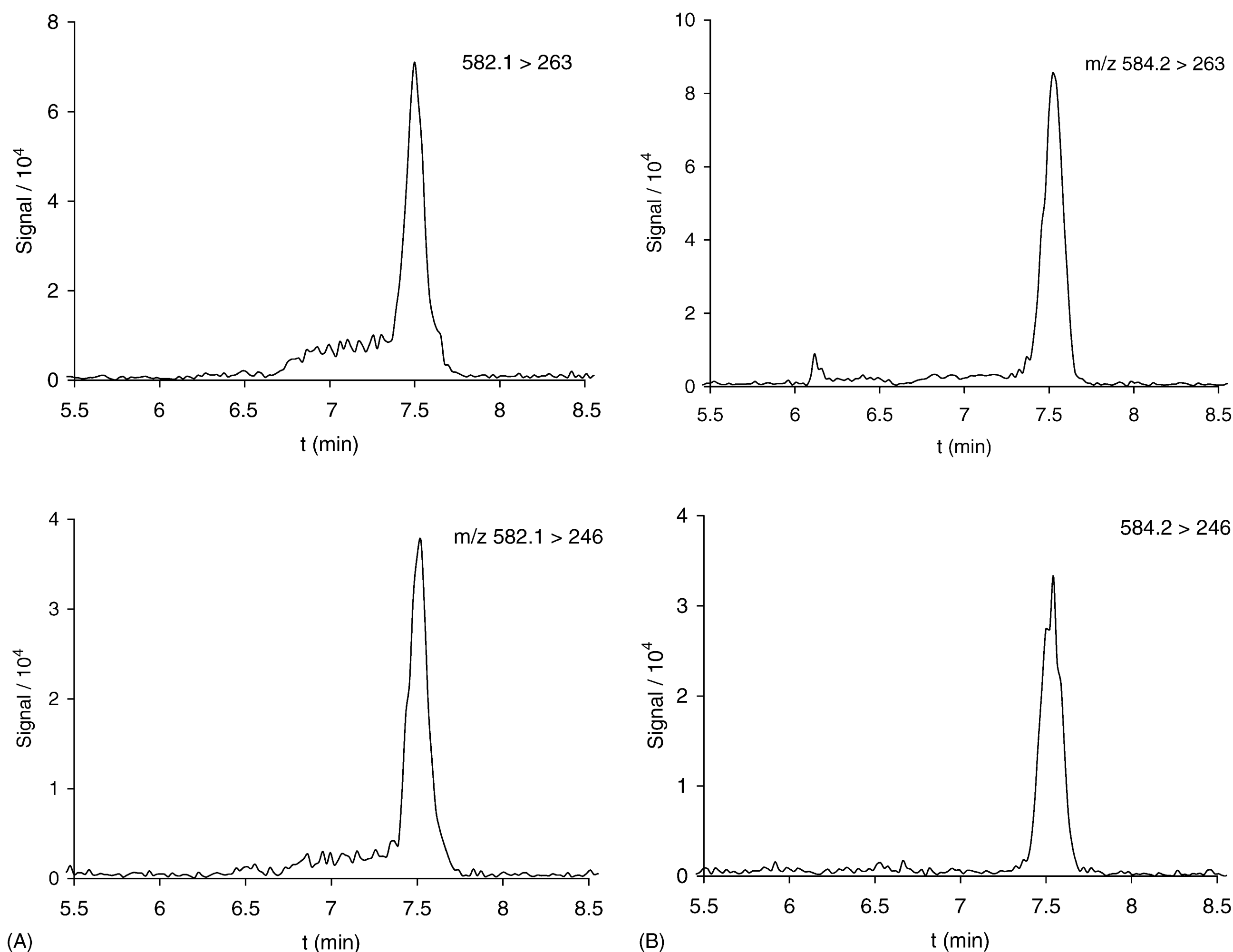

Fig. 2. LC–MS/MS chromatograms of a mixed-flower honey spiked with 10 μg/kg streptomycin (A) and 5 μg/kg dihydrostreptomycin (B). The upper trace shows the screening ion and the lower trace the confirmatory ion.

Table 1
Validation results of the methods developed for detecting (DH)STR in milk and honey

Matrix	Compound	Recovery (%)	R.S.D. (%)	LOQ (μg/kg)	CCα (μg/kg)[a]	CCβ (μg/kg)[b]
Honey	STR	81	12	2	–	LOQ[c]
	DHSTR	84	13	1	–	LOQ[c]
Milk (no correction for IS)	STR	92	15	10	0.23	0.28
	DHSTR	91	13	5	0.22	0.26
Milk (correction for IS)	STR	102	8	10	0.23	0.25
	DHSTR	99	8	5	0.22	0.25

For experimental conditions, see text.
[a] $\alpha = 5\%$.
[b] $\beta = 5\%$.
[c] Samples are declared non-compliant when the confirmatory ion exceeds the LOQ (S/N = 6).

0.992, respectively, and the intercepts with the y-axis did not deviate significantly from the origin. A blank mixed-flower honey was selected. It was spiked in 6-fold at the level of 1, 1.5 and 2 times the MRPL. This procedure was repeated by two analysts on different occasions. An ANOVA test revealed that all results could be pooled. The within-lab-reproducibility data from these 54 experiments are presented in Table 1. The average recoveries of more than 80%, with R.S.D.s of ca. 13%, are acceptable at the μg/kg level. During the three measurement series, retention times were stable (R.S.D. < 0.5%) and no deviation of more than 10% from the average ion ratio was observed. The decision limit (CCα) and detection capability (CCβ) [21] were not determined. Samples are declared positive when the confirmatory ion exceeds the LOQ (S/N = 6) and the ion ratio is within 20% of the reference value as determined from calibration standards. This tolerance in-

terval is determined by the value of the ion ratio, which was typically 0.6 for both analytes [21].

To investigate the effect of the matrix on the recovery, 12 different blank honeys were selected, spiked at the MRPL level and analysed. These honey samples included a wide variety of honey types, such as clover, heath, black forest and mixed-flower honey. Average recoveries were 84% for STR and 89% for DHSTR, with R.S.D.s of 19 and 15%, respectively. Although the precision was slightly reduced, the influence of the type of honey on the performance of the method appeared to be negligible. Importantly, the analytes could be recovered from all different honey samples, i.e., no false-negatives were observed. 13 additional honeys, collected from local beekeepers, were analysed. These honeys could be assumed to be free of streptomycins. The results for all honeys were negative, showing that no false-positive results were generated by the method.

A honey sample that contained STR was analysed repeatedly during a period of 5 months. No result deviated more than two times the standard deviation from the average, i.e., 6.3 ± 1.9 μg/kg (95% CI, 8 d.f.). This shows that STR is stable for at least 5 months in honey. Stock solutions of DHSTR and STR with a concentration of 0.32 g/L were stable for at least 9 months. However, stored at 5 °C the concentrations of a working solution containing 0.3 mg/L STR and 0.15 mg/L DHSTR were reduced to about 50% within a week. Arguably, this is caused by adsorption of streptomycins to the glassware. Hence, working solutions were freshly prepared from stock solutions before use.

The validation data show that the method is robust and is virtually insensitive to the type of honey that is analysed.

3.2.2. Milk

The method for the detection of (DH)STR in milk was validated by a procedure practically identical to the one used for validating the honey analysis. Calibration plots, in the range of between 50 and 800 μg/kg, were linear with a regression coefficient of 0.997, and intercepted the y-axis close to the origin. The within-lab-reproducibility in the range of between 100 and 300 μg/kg was determined by three analysts independently. There are two different approaches to treat the generated set of data. The recovery can be calculated directly, without correction for an internal standard (IS). Alternatively, the recovery can be calculated by designating DHSTR as the IS for STR and vice versa. The resulting within-lab-reproducibilities are shown in Table 1. Also, the determined decision limit CCα (α = 5%) and detection capability CCβ (β = 5%), calculated from the within-lab-reproducibility [21], are tabulated. Without IS, the R.S.D.s of the recovery (15% for STR and 13% for DHSTR) are rather large for the concentration level under investigation. Correction with an IS reduces the R.S.D.s to 8% and the apparent recoveries become close to complete. For a more precise result and lower CCβ, samples that are positive after initial screening can be spiked with IS and re-analysed. In 8 skimmed fat and 10 low-fat milk powders, no STR or DHSTR was detected. All samples originated from large batches, in which possible positive samples (from individual cows or farms) may have been diluted to concentrations below the detection limit. These validation data show that with a minimum of sample preparation, a satisfactory analysis of milk samples can be obtained.

3.3. Honey survey

The presence of streptomycin antibiotics in honeys on the Dutch market was surveyed, 186 honeys were collected and analysed. Fifteen percent of the tested samples were positive. While 3% of the Dutch honeys were positive, in 26% of the foreign honeys one of the analytes was detected. Foulbrood is not endemic in The Netherlands, which explains the low incidence of streptomycins in Dutch honeys. The high percentage of positive foreign honeys corresponds to data from previous surveys with EIA or LC-fluorescence analysis, where 20% or more non-complaint samples were found [10,12,16]. However, only 9% of the foreign honeys in our survey contained more than 10 μg/kg STR, which is the typical reporting level of EIA and LC-fluorescence methods. Assuming that no significant number of false-positive results were included in the previous surveys, these results suggest that the levels of streptomycins in honey have decreased in recent years. A proficiency study, comparing the available techniques, can be helpful to establish the value of the respective surveys.

It is clear from all surveys that the occurrence of streptomycins in honey is widespread and that it is necessary to monitor honey for these forbidden compounds on a regular basis. The high incidence has led to the suggestion that honey may become contaminated by alternative sources such as crop-protection agents [6]. Also, streptomycin-producing bacteria may be a source of contamination. The present analytical method can be a useful tool to further investigate these possibilities.

4. Conclusions

The two applications presented here are fine examples of the advantages LC–MS/MS can offer for trace analyses in food samples. The ion ratio of two product ions helps to confirm the identity of the analytes and reduces the risk of false-positive results. Owing to the high selectivity and sensitivity of LC–MS/MS, the sample preparation could be simplified. For milk samples, precipitation and dilution were the only sample preparations required and for honey samples, a single solid-phase extraction was sufficient. Detection limits were in the low μg/kg range. The validation data show that both methods perform well and can be used for routine analysis.

A minor survey of milk powder samples, stemming from large batches, revealed no positive results. A useful application can be the analysis of milk from individual bovines or farms, possibly in combination with a screening test such as

EIA. By contrast, a large proportion of the honeys of foreign origin tested in this work contained (dihydro)streptomycin. This result agrees with reports from surveys conducted with other, non-confirmatory methods. It is necessary to investigate the source of this widespread contamination, in order to reduce the occurrence of these forbidden substances in honey.

References

[1] L. Bailey, B.V. Ball (Eds.), Honey Bee Pathology, Academic Press, London, UK, 1991.

[2] R. Krieger (Ed.), Handbook of Pesticide Toxicology. Part 1. Principles, Academic Press, San Diego, CA, 2001.

[3] Commision Regulation (EEC) 2377/90, Off. J. Eur. Commun. L224 (1990) 1.

[4] D.A. Stead, J. Chromatogr. B 747 (2000) 69.

[5] H. Kubo, Y. Kobayashi, T. Kinoshita, Anal. Chem. 58 (1986) 2653.

[6] D. Klementz, W. Pestemer, Nachrichtenbl. Deut. Pflanzenschutz. 48 (1996) 280.

[7] G.C. Gerhardt, C.D.C. Salisbury, J.D. MacNeil, J. AOAC Int. 77 (1994) 334.

[8] H. Abbasi, K.E. Hellenäs, Analyst 123 (1998) 2725.

[9] V. Hormazábal, M. Yndestad, J. Liq. Chromatogr. 18 (1995) 2695.

[10] U. Kocher, Lebensmittelchemie 50 (1996) 112.

[11] G.C. Gerhardt, C. Salisbury, J.D. McNeil, J. AOAC Int. 77 (1994) 765.

[12] P. Edder, A. Cominoli, C. Corvi, J. Chromatogr. A 830 (1999) 345.

[13] P. Schnappinger, E. Usleber, Märtlbauer, G. Terplan, Food Agric. Immunol. 5 (1993) 67.

[14] W. Haasnoot, P. Stouten, H. Cazemier, A. Lommen, J.F.M. Nouws, H.J. Keukens, Analyst 124 (1999) 301.

[15] A. Strasser, R. Dietrich, E. Usleber, E. Märtlbauer, Anal. Chim. Acta 495 (2003) 11.

[16] W. Heering, E. Usleber, R. Dietrich, E. Märtlbauer, Analyst 123 (1998) 2759.

[17] P.M. Davidson, A.L. Branen (Eds.), Antimicrobials in Foods, Marcel Dekker, New York, 1993.

[18] L.G. McLaughlin, J.D. Henion, J. Chromatogr. 591 (1992) 195.

[19] L.G. McLaughlin, J.D. Henion, P.J. Kijak, Biol. Mass Spectrom. 23 (1994) 417.

[20] A. Kaufmann, P. Butcher, P. Kölbener, Rapid Commun. Mass Spectrom. 17 (2003) 2575.

[21] European Parliament Decision 2002/657/EC, Off. J. Eur. Commun. L221 (2002) 8.

[22] M. Suzuki, K.-I. Harada, Spectrosc. Int. J. 2 (1983) 267.

[23] H. Kambara, S. Hishida, H. Naganawa, J. Antibiotics 35 (1982) 67.

[24] M.G. Ikonomou, A.T. Blades, P. Kebarle, Anal. Chem. 62 (1990) 957.