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Article *in* Analytica chimica acta · July 2010

DOI: 10.1016/j.aca.2010.03.056 · Source: PubMed

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Confirmatory method for the determination of streptomycin in apples by LC–MS/MS

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ARTICLE INFO

Article history:
Received 4 January 2010
Received in revised form 16 March 2010
Accepted 26 March 2010
Available online 24 April 2010

Keywords:
Streptomycin
Apples
LC–MS/MS
Validation
SANCO/3131/2007
Limit of quantification

ABSTRACT

The method was specifically developed for the determination and confirmation of streptomycin in apple samples using the whole mellow apple. The method is simple, rapid, sensitive and was validated for streptomycin in accordance with SANCO/3131/2007. After extraction with phosphate buffer and a pH change, the clean-up was performed by the way of SPE with polymeric phase. The LC–MS/MS analysis was carried out using a HILIC column for the separation of the analytes and a triple quadrupole mass spectrometer in positive ESI mode to measure the transitions of the substances in MRM mode. For the quantification of streptomycin a matrix calibration curve in the linear range of 1.0–20 $\mu g\,kg^{-1}$ and the internal standard dihydrostreptomycin (10 $\mu g\,kg^{-1}$) were used. The calculated validation parameters like the recovery (101–105%) for 2, 5, 10 and 20 $\mu g\,kg^{-1}$ and the relative standard deviation (RSD, 4.1–11.4%) of the 6 replicates fulfil the requirements of SANCO/3131/2007. The LOQ was determined as 2 $\mu g\,kg^{-1}$.

1. Introduction

Streptomycin (STR) is an aminoglycoside antibiotic that is particularly active against aerobic gram-negative bacteria. As a pesticide this substance can be used for the treatment of fire blight (agent: the bacterium *erwinia amylovora*), which is amongst common diseases of apples. The toxicology of streptomycin is well-studied and several cases of allergic reactions have been reported. At this moment there is no efficient alternative to substitute the antibiotic substance streptomycin. In September 2008 the maximum residue limit (MRL) of streptomycin was set at 10 $\mu g\,kg^{-1}$ following Regulation (EC) No 396/2005 [1]. For this reason it was necessary to develop a method with a low limit of quantification (LOQ) for apples [2,3]. More methods were developed in the last few years for the determination of streptomycin in food of animal origin than for plant products [4–11]. Residues of streptomycin may be found in food products such as apples and honey [12,13], because bees transfer streptomycin from the apple tree to the beehive.

2. Experimental

2.1. Chemicals

Streptomycin (STR) and dihydrostreptomycin (DSTR) were purchased from Ehrenstorfer (Augsburg, Germany). LC-grade acetonitrile (ACN) and methanol (MeOH) were obtained from LCS (Amsterdam, The Netherlands). LC-grade water was bought from Biosolve (Valkensward, The Netherlands). Hydrochloric acid (HCl), sodium hydroxide (NaOH), potassium dihydrogen phosphate (KH_2PO_4), acetic acid, formic acid, trichloracetic acid (TCA) and titriplex III ($Na_2EDTA{\cdot}2H_2O$) were purchased form Merck (Darmstadt, Germany). Solid-phase extraction (SPE) was performed by means of Oasis HLB cartridges (200 mg, 6 mL) from Waters (Milford, MA, USA).

2.2. Materials

A vortex shaker from Heidolph (Schwabach, Germany), an analytical balance from Mettler Toledo (Greifensee, Switzerland), a high-volume centrifuge from Heraeus (Hanau, Germany), an evaporator from Zymark (Idstein, Germany), a pH meter from WTW (Weilheim, Germany) and a vacuum station from Supelco (Deisenhofen, Germany) were used for the sample preparation and the extraction process.

2.3. Standard solutions

Stock solutions (S0) at concentrations of 1 $mg\,mL^{-1}$ were prepared by dissolving 10 mg of streptomycin and dihydrostreptomycin respectively in 10 mL of water. To prepare the working solutions, the stock solutions were diluted with 0.1% acetic acid, resulting in concentrations of 100 $\mu g\,mL^{-1}$ (S1) and 1 $\mu g\,mL^{-1}$ (S3). The working solution S3 was used for preparing the samples for matrix calibration as well as the spike samples.

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doi:10.1016/j.aca.2010.03.056

Table 1
LC–MS/MS parameter.

Analyte	RT (min)	Transition (*m/z*)	DP (V)	FP (V)	CE (V)	CXP (V)
Streptomycin	8.3	582 → 263	101	230	45	18
		582 → 246	101	230	54	18
Dihydrostreptomycin (Internal Standard)	8.3	584 → 263	110	240	43	18

RT, retention time; DP, declustering potential; FP, focusing potential; CE, collision energy; CXP, cell exit potential.

2.4. Solutions for sample preparation and mobile phases

For the phosphate buffer solution, 1.36 g of KH_2PO_4 was dissolved in approximately 950 mL of H_2O. The pH value was adjusted to 4 with 1N HCl using a pH meter. Then 0.15 g of $Na_2EDTA{\cdot}2H_2O$ and 20 g of TCA were added. The solution was filled up to 1000 mL with H_2O. The solutions of 3% formic acid in MeOH, 0.1% acetic acid in water, 5N NaOH in water, 0.05% formic acid in water (mobile phase component A) and 0.05% formic acid in ACN (mobile phase component B) were prepared by mixing the equivalent volumes. The reconstitution solution was prepared by mixing the components of mobile phases A and B with 30/70 (v/v).

2.5. Preparation of apple samples

The laboratory sample of blank material of the whole mellow apple was divided into sub-samples (e.g. of approximately 100 g) in order to allow a sufficient number of parallel analyses in the 4 test series. The sub-samples were stored in a freezer at approximately −20 °C to exclude modifications in the apple texture and to simulate the storage of real samples. After the thawing step the samples were homogenised and 5 g of sample material was transferred into a centrifugation tube. Afterwards the samples for the matrix calibration and for the recovery were spiked with different volumes (see Sections 2.8 and 2.9) of the S3 working solution of streptomycin (1 μg mL^{-1}). Then 50 μL of the S3 working solution of the internal standard of dihydrostreptomycin (1 μg mL^{-1}) was added to the test sample. Before the next step, a waiting time of 10 min had to be respected.

2.6. Extraction and clean-up procedure

20 mL of phosphate buffer was added to the sample of 5 g of apple. Then the sample was vortexed for approximately 1 min, shaken for 10 min und treated in an ultrasonic bath for 5 min. After centrifugation at 4000 rpm for 10 min at 5 °C, the supernatant was decanted and filtered through filter paper into a 50 mL centrifuge tube. Then the extract was adjusted with 5N NaOH to pH 7.5 and was controlled with a pH meter. The extract was centrifuged and decanted a second time. The SPE cartridge was conditioned with 6 mL of methanol and 6 mL of water. Then the entire extract solution was applied directly onto the cartridge. After the rinsing step with 3 mL of water, the cartridge was air-dried for 10 min. The analyte was eluted with 6 mL of a mix solution of 3% formic acid in methanol. Then the eluate was concentrated to dryness in a TurboVap evaporator (operation mode "sensor and time"; water bath at 50 °C, nitrogen stream at 0.4 bar). The dry residue was reconstituted in 500 μL of a mobile phase mix (component A/B = 30/70, v/v) using a vortex mixer. The solution was transferred into vials and centrifuged for 10 min at 4000 rpm. The clear solution was filtered through a microfilter, carefully transferred into dark glass vials with inserts and analysed by LC–MS/MS.

2.7. LC–MS/MS analysis

The LC–MS/MS system comprised the LC instrument 1100 from Agilent Technologies (Waldbronn, Germany) with quaternary pump, auto sampler, degasser, column oven and system controller. The LC was coupled to a triple mass spectrometer API 3000 from Applied Biosystems (Darmstadt, Germany). The analytical column for the separation of the analytes was a HILIC "Atlantis" (150 mm × 2.1 mm, 3 μm particle size) with adequate guard. The separation of the analytes was performed by applying a gradient of components A (water with 0.05% formic acid) and B (ACN with 0.05% formic acid) at an oven temperature of 30 °C and a flow rate of 0.3 mL min^{-1} without splitting. The injection volume was 20 μL. The samples were kept in the auto sampler at a temperature of 10 °C. The gradient started with 70% of component B for 1 min and then decreased to 10% within 3 min. This composition was kept for 4 min, then increased to 70% of component B within 1 min. With the following equilibration time of 11 min, the resulting total run time was 20 min. The instrument parameters for the mass spectrometry measurement by means of the software Analyst, version 1.4.1, were as follows: ESI+; scan type: MRM; dwell-time: 100 ms; resolution Q1 and Q3 = unit; gas = nitrogen; nebuliser gas = 12 psi; curtain gas = 12 psi; collision gas = 8 psi; ion spray voltage = 5500 V; source temp. = 400 °C.

The mass spectrometry parameters applied for the substances streptomycin and dihydrostreptomycin with regard to the transitions from precursor to product ions are shown in Table 1.

2.8. Validation of the method

The validation was performed in accordance with SANCO/3131/2007 [14]. This document declares: "A minimum of 5 replicates is required (to check the precision) at both the reporting level (to check the sensitivity) and at least another higher level, perhaps an action level, for example the MRL. The (method) LOQ is defined as the lowest validated spike level meeting the method performance acceptability criteria (mean recoveries in the range of 70–120% with an RSD of ≤20%)." We applied an extended validation approach with 4 concentration levels and 6 replicates each. The 6 samples for each concentration to check the accuracy were spiked with 10, 25, 50 and 100 μL of the working solution of 1 μg mL^{-1} to obtain concentration levels of 2, 5, 10 and 20 μg kg^{-1} based on 5 g of sample and a final volume of 0.5 mL. The chosen

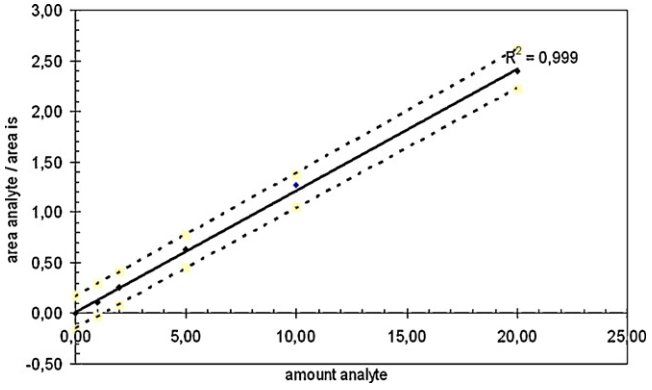

Fig. 1. Matrix calibration curve with 1.0–20 μg kg^{-1} streptomycin and 10 μg kg^{-1} internal standard dihydrostreptomycin.

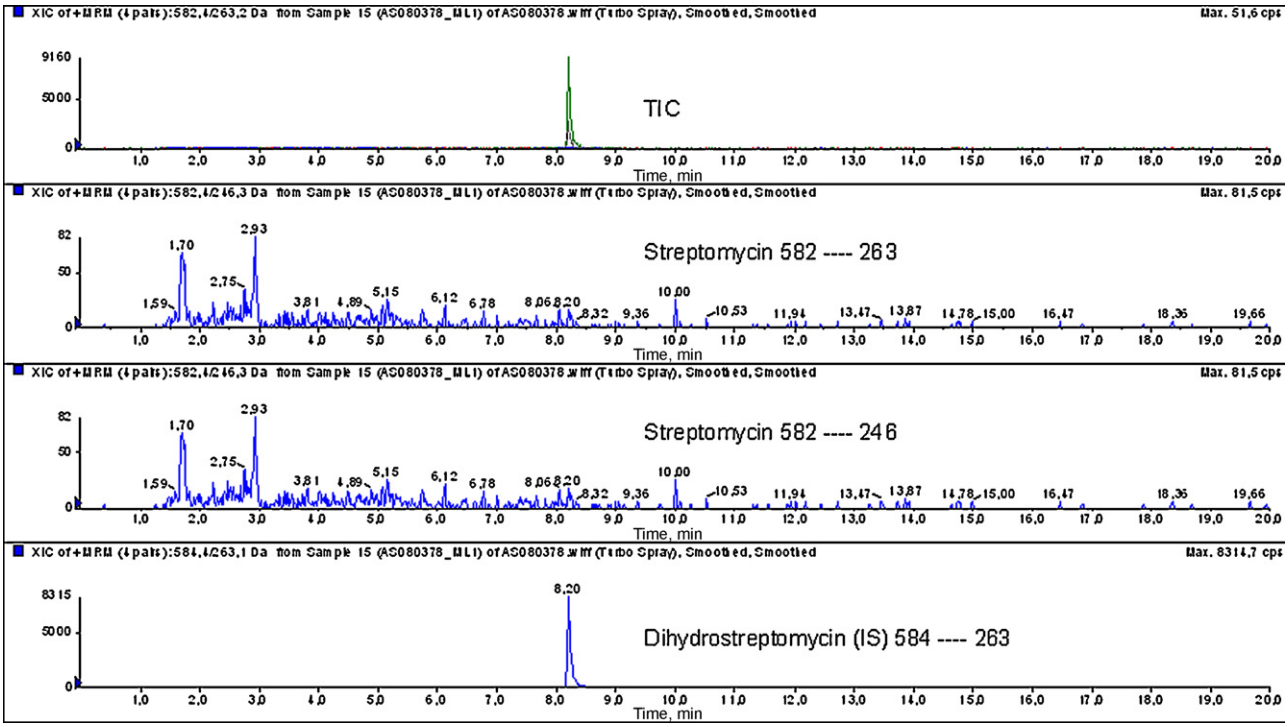

Fig. 2. Total ion and product ion chromatograms of blank apple sample spiked with 10 μg kg^{-1} internal standard dihydrostreptomycin.

concentration levels fulfil the demands of SANCO/3131/2007 [14] with regard to the above definition. A spike volume of 50 μL of the internal standard dihydrostreptomycin results in a concentration of 10 μg kg^{-1} each sample.

2.9. Identification and quantification

The confirmation of the substance streptomycin was performed on the basis of the ratio of the intensities of the two most abundant product ions obtained from the precursor ion with a signal-to-noise ratio of ≥3:1 (see Table 1). Furthermore the substance was confirmed with the help of the relative retention times of streptomycin and the internal standard dihydrostreptomycin. The ratio of the areas of the most intensive product ions of streptomycin (*m*/*z* 263) and the internal standard dihydrostreptomycin (*m*/*z* 263) were used for the quantification (see Fig. 1). The calculation of the concentration of the spike samples for the determination of the recovery was carried out with the help of matrix calibra-

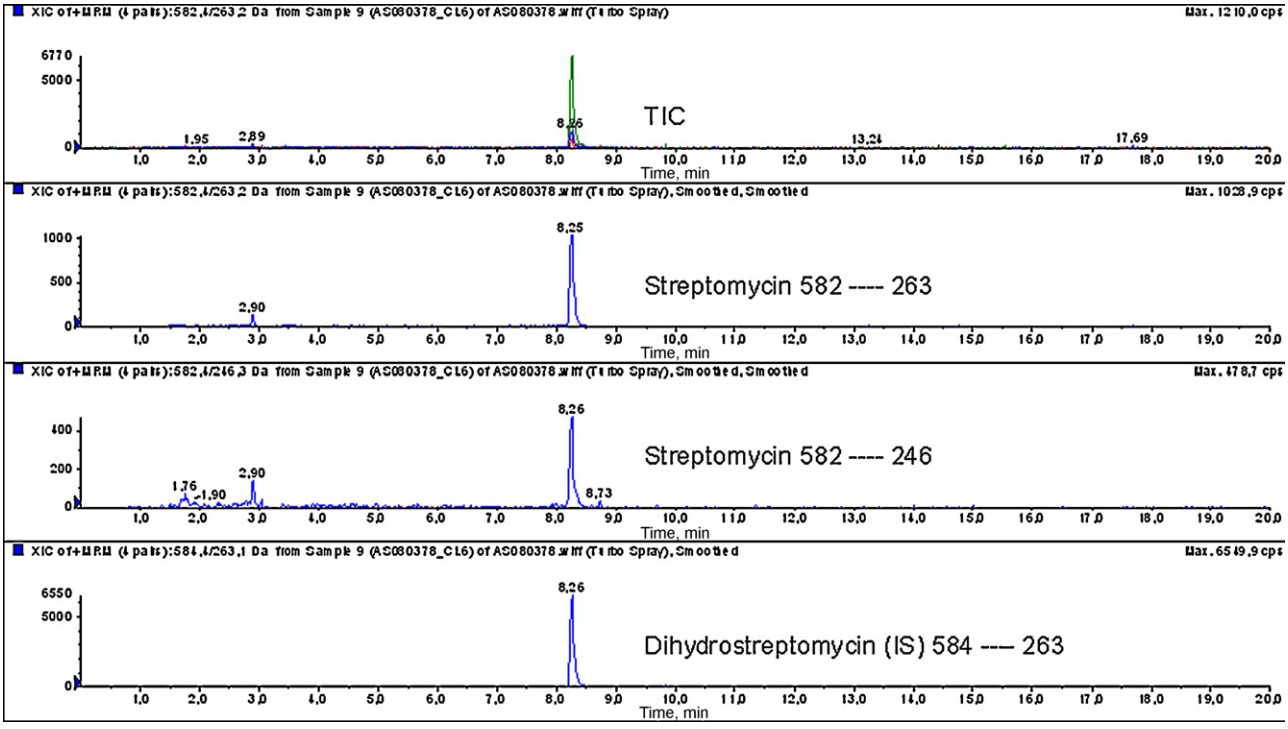

Fig. 3. Total ion and product ion chromatograms of apple sample spiked with 5 μg kg^{-1} streptomycin and 10 μg kg^{-1} internal standard dihydrostreptomycin.

Table 2
Validation parameter.

Day	Spike sample ($\mu g\,kg^{-1}$)	Replicates (n)	Recovery (%)	RSD (%)	Intra-day RSD (%)	Inter-day RSD (%)
1	2	6	101	4.1	4.5	
	10(MRL)	6	105	4.3		7.3
2	5	6	101	8.4	9.6	
	20	6	102	11.4		

tion curves of 6 concentration levels. These matrix calibration samples were spiked with the working solution of $1\,\mu g\,mL^{-1}$. In the case of streptomycin they were spiked with 0, 5, 10, 25, 50 and 100 μL, and in the case of the internal standard dihydrostreptomycin, with 50 μL. The samples were prepared in the same manner as the recovery samples. Due to the use of 5 g of matrix sample and a final volume of 0.5 mL, concentration levels of 0, 1, 2, 5, 10 and $20\,\mu g\,kg^{-1}$ for streptomycin and of $10\,\mu g\,kg^{-1}$ for the internal standard dihydrostreptomycin were achieved. The linearity of the matrix calibration curve for streptomycin was given for the overall concentration range $1–20\,\mu g\,kg^{-1}$ (see Fig. 1).

3. Results and discussion

The selectivity of the discussed LC–MS/MS method was researched by analysing blank matrix samples spiked with the internal standard dihydrostreptomycin. The extracted ion chromatograms in Fig. 2 do not show any signals for neither streptomycin nor any interfering compounds at the corresponding retention time. The chromatogram of a spiked sample with $5\,\mu g\,kg^{-1}$ streptomycin shows a satisfactory peak shape for both transitions of streptomycin (see Fig. 3) at the MRL. In Table 2 the results of the validation according to document SANCO/3131/2007 [14] are described. Assuming that there are no significant differences between the recovery values in the range of $2–20\,\mu g\,kg^{-1}$, the intra-day and inter-day precisions were calculated.

All results for recovery and the relative standard deviation (RSD) fulfil the requirements of the above document (mean recoveries in the range of 70–120% and RSD $\leq$ 20%). The LOQ is defined as the lowest validated spike level meeting the performance acceptability criteria and was determined with the value of $2\,\mu g\,kg^{-1}$. The method was checked in an international laboratory comparison with real apple samples contaminated with streptomycin. The laboratory of the authors attained z-scores values of well below ±2 for the analysis of five investigated samples.

4. Conclusions

The method can be applied for the identification and quantification of streptomycin in apples with an LOQ of $2.0\,\mu g\,kg^{-1}$. The use of the matrix calibration curve and of the internal standard dihydrostreptomycin is necessary and suited for the quantification of streptomycin. The linearity was proven by matrix calibration in the range of $1.0–20\,\mu g\,kg^{-1}$. The calculated recovery and RSD are in compliance with the required acceptable values according to SANCO/3131/2007 [14].

Acknowledgements

Special thanks to Ms. M. Jüsgen, Ms. S. Mönch and Mr. A. Petruck for technical assistance and the analytical investigations.

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