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Thermal Degradation of Streptomycin Residues in Honey During Storage

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Summary

In Europe there is an increasing emphasis on the quality control of honey, especially on maximum limits of veterinary drug residues (particularly antibiotics) permitted in it. Streptomycin is an aminoglycoside antibiotic used in apiculture to protect bees against a variety of brood diseases. Romanian authorities have included it in the National Monitoring Program for honey manufacturers. In this study, an enzyme-linked immunosorbent assay (ELISA) screening test was validated as a detection method of streptomycin residues in honey. The ELISA experimental results were compared to those obtained by using an HPLC method. The values generated by the two methods were very close to each other. This fact certifies that ELISA method can be successfully used for quantitative detection of the amount of streptomycin in honey samples. Following validation, three types of honey (polyfloral, lime and acacia) were analyzed for streptomycin content after exposure to 4, 22, 30, 40 or 70 °C for 20 weeks. The results show that streptomycin mass fraction decreased with time and with the increase of temperature in all honey samples. The data collected were used to fit a second-order multiple linear regression model for predicting the degradation of streptomycin in honey samples as a function of temperature and storage period. Values of the calculated statistical indicators confirm a good predictive capability of mathematical and statistical models.

Key words: honey, streptomycin, ELISA, HPLC, degradation, statistical model

Introduction

Honey is an aliment, a unique blend of sugars that provides many nutritional benefits, which contains many minerals, vitamins, antioxidants, amino acids and numerous enzymes (*1*). Antibiotics are mainly used in apiculture for the treatment of bacterial brood diseases, *e.g.* American foulbrood (AFB) (*Paenibacillus larvae*) (*2*). They are effective in beekeeping only against the infestation of the hives with AFB. Some beekeepers still practice preventive treatments with various antibiotics (*3,4*).

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The presence of antibiotics in food products is seen by the European sanitary veterinary and food safety authorities as very harmful for the consumers. Streptomycin is a commonly used antibiotic in veterinary medicine (*5*). High concentrations of streptomycin may have ototoxic and nephrotoxic effects. At low concentrations, as found in foods, streptomycin may cause allergies, destroy intestinal flora and cause resistance to certain microorganisms.

According to the European Council Regulation No 470/2009 honey should be free of antibiotics (*6*). In Romania, the CRL Guidance Paper (*7*) is also used for the determination of residues in honey. It was developed by the Fougères laboratory, France, called the Community Reference Laboratory (CRL) for antimicrobial residues in food. According to this guidance, the recommended mass fraction of streptomycin is 40 μg/kg.

In order to determine the antibiotic content in honey, there are two main approaches: screening tests and confirmatory methods. Simple tests (optical surface plasmon resonance (SPR) biosensor, ELISA, Charm II, *etc.*) that allow identification of a single target analyte provide qualitative, semi-quantitative and quantitative results (*8*). Screening tests offer a low rate (<5 %) of false negative samples, ease of use, short time of analysis, good selectivity and a low price (*9*).

In his study about antibiotic residues in honey, Reybroeck (*10*) mentioned that confirmation of presumptive samples of streptomycin was performed with high-performance liquid chromatography coupled with fluorescence detection (HPLC-FLD). Over time various methods for determination of streptomycin residues in honey have been developed (*11-17*).

Regarding the stability of streptomycin, Regna *et al.* (*18*) found that at temperatures above 28 °C and pH greater than 7, the solutions of streptomycin were unstable. These conditions change when streptomycin is found in honey. Landerkin and Katznelson (*19*) studied the stability of streptomycin in honey at temperatures of 4 and 34 °C during 9 months. At the end of this period, 56 and 87.5 % of the initial quantity of streptomycin was degraded at 4 and 34 °C, respectively. In a study of The Food and Environment Research Agency (*20*) honey samples with a mean streptomycin mass fraction of 124 mg/kg were subjected to degradation during 332 days at room temperature. After the first 28 days, 94 % of streptomycin was degraded. After that, no significant changes in streptomycin residue were noticed. On the contrary, Pang *et al.* (*21*) concluded that streptomycin in honey is quite stable during a storage period of 4 months at room temperature.

The results of screening or confirmatory tests along with other parameters for honey samples can be correlated and interpreted. Thus, in many cases, the connection between two or more parameters that describe a certain process is sufficiently close so that the variation of one parameter can be controlled and expressed based on the variation of other parameters. Functional links of this type are called stochastic or probabilistic. The study of these kinds of relationships has brought about the development of multiple correlation theory (*22*).

The purpose of this paper is to validate the ELISA test as a detection method of streptomycin residues in honey and to develop a series of mathematical and statistical models for predicting the rate of degradation of streptomycin in different types of honey as a function of temperature and storage period.

Materials and Methods

Honey samples

In order to compare ELISA to HPLC methods, a commercial honey was used. It was analyzed prior to the testing and no antimicrobial substances were detected. The sample was divided into 16 replicates, then each replicate was fortified with streptomycin at a level of 20 μg/kg. In all cases, analyses for the presence of streptomycin residues were performed in duplicates using ELISA and HPLC methods.

For streptomycin degradation study, three types of commercial honey free of antibiotics (two monofloral: acacia and lime, and one polyfloral) were considered. The samples came from the same batch, and were kept under the same storage conditions ((21±1) °C, in the dark). A sample from each honey type was divided into 4 subsamples, to which streptomycin was added at a level of 200 μg/kg, and they were settled into glass pots with metal screws.

The subsamples fortified with 200 μg/kg of streptomycin were stored at five different temperatures (4, 22, 30, 40 and 70 °C), in the dark, for 20 weeks. Every 7 days, the samples were analyzed by ELISA method. All the determinations were performed in duplicates.

Antibacterial residue analyses and testing protocols

For the determination of streptomycin residues in honey, MaxSignal® Streptomycin ELISA Test Kit (Bioo Scientific Corporation, Austin, TX, USA), was used which has a detection limit of 10 μg/kg. ELISA is an immunoassay test used for the quantitative determination of antibiotics, so it was applied here for preliminary qualitative analysis. This test is based on the reaction between an antigen and an antibody. The wells of ELISA plate were coated with a protein conjugate of streptomycin. After the addition of the anti-streptomycin antibody, the free streptomycin and the linked one compete for streptomycin antibody (immunoassay competitive reaction). The unlinked antibodies are removed during washing. The sample preparation and their analysis with the ELISA test were performed according to the working protocol provided with the kit (*23*).

As a confirmatory test of ELISA screening, HPLC was used (*24*). The system consisted of a GBC series 8000 HPLC with a post-column derivatization system model 655A-13 (Merck/Hitachi, Sigma-Aldrich, St. Louis, MO, USA), a post-column reaction assembly (Merck, Darmstadt, Germany) used for separation, identification and quantification of streptomycin residues present in the samples, column Hypersil BDS C-18: 100×4.6 mm per 3 μm, precolumn ODS 20×2, 1 mm per 10 μm, working temperature 55 °C, injected volume 100 μL, isocratic gradient, reactive solution 0.2 M NaOH, reaction column:

10 m (volume 0.86 mL, i.d. 0.33 mm), excitation wavelength 263 nm, emission wavelength 435 nm.

For the extraction, (10±0.02) g of honey were weighed in a 100-mL Berzelius beaker. A volume of 25 mL of perchloric acid solution, pH=2, was added and stirred until it was dissolved. The elution was performed under low vacuum (by drops) with 6 mL (3×2 mL) of methanol in a conical tube. The eluate was evaporated in a rotary evaporator and brought to dryness at a temperature of 40 °C in nitrogen stream. If traces of water remained, the process was repeated with ethanol and it was evaporated to dryness. The residue was treated again with 1 mL of HPLC solution in a tube with a cap. Before measurement, the tube was centrifugated at 4000 rpm for 10 min.

Results and Discussion

Validation of ELISA for detection of streptomycin

In order to demonstrate the ELISA method reliability, the following parameters were investigated according to the European Commission Decision 2002/657/EC (*25*): repeatability, reproducibility, recovery, specificity and precision. The values of these parameters are presented in Table 1.

The 16 replicates of fortified honey were analyzed with ELISA as well as HPLC and the results of the two methods were compared (Fig. 1).

In Fig. 1 insignificant differences, minimum 0.030 μg/kg and maximum 2.313 μg/kg, between the amounts of streptomycin detected by ELISA and HPLC methods can be seen. All the samples analized with ELISA immunoassay test were confirmed by HPLC.

To compare the performance and precision of the two methods and the influence of some factors on the measured values, mathematical statistics was used. Using F-distribution (*26*), dispersions of the two series of experiments were compared. The values found were s_1^2= 2.3818 for ELISA screening method and s_2^2=1.8973 for HPLC method, respectively. By chosing the threshold of significance equal to 0.1, the value $\alpha/2$=0.05, with the degree of freedom $\nu_1=\nu_2$=15 was obtained.

Because 2.3818>1.8973, the following result is calculated:

$$F = \frac{s_1^2}{s_2^2} = \frac{2.3818}{1.8973} = 1.2553 \qquad /1/$$

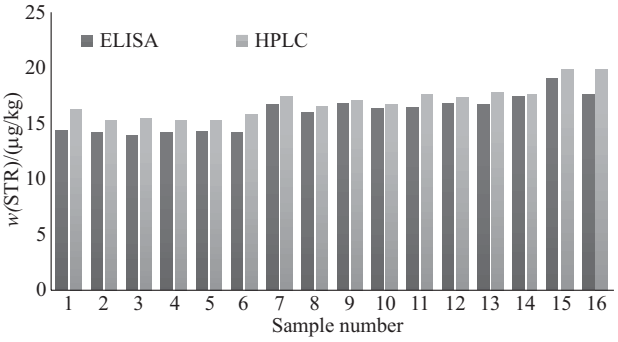

Fig. 1. Mass fraction of streptomycin in 16 replicates of honey samples doped with streptomycin (STR) 20 μg/kg, detected by ELISA and HPLC methods

According to critical values of F-distribution (*26*), $F_{0.05}$(15,15)=2.4034. Since this value is greater than F= 1.2553 found experimentally, it can be inferred that both sets of analyses are equally reproducible, regardless of the method. Thus, it was possible to conclude that ELISA method can be succesfully used to quantify the presence of streptomycin in honey.

Streptomycin degradation at different temperatures

The rate of degradation of streptomycin in the three types of honey during storage at four different temperatures is presented in Table 2.

The experimental results for the three types of honey stored at four different temperatures indicate a decrease in the overall content of streptomycin. Streptomycin mass fraction in honey samples decreased with the increase of storage temperature. In the case of honey samples preserved at 70 °C, it was noticed that streptomycin was completely degraded.

The data collected were used to fit a second-order multiple linear regression model, presented in Eq. 2, for predicting the degradation of streptomycin in honey samples as a function of temperature and storage period:

$$Y(T,t) = a_0 + a_1 \cdot T + a_2 \cdot t + a_3 \cdot T \cdot t + a_4 \cdot T^2 + a_5 \cdot t^2 \qquad /2/$$

where Y is mass fraction of streptomycin in honey samples in μg/kg, T is storage temperature in °C, t is time of storage in weeks, and a_0, a_1, a_2 are coefficients of the first-order multiple linear regression model.

Table 1. Parameters for method reliability

Parameters		*w*(STR)/(μg/kg)			*w*(N)/(μg/kg)
		20	40	60	40
Repeatability*/(μg/kg)		18.12±0.30	39.25±0.71	57.87±1.64	-
Recovery*/%		90.6	98.12	96.45	-
Reproducibility*/(μg/kg)	Analyst 1	18.12±0.30	39.25±0.71	57.87±1.64	-
	Analyst 2	18.83±0.25	38.90±0.66	56.95±1.95	-
Precision**/(μg/kg)		19.94±0.34	-	-	-
CV/ %		1.75	-	-	-
Specificity*/(μg/kg)		-	39.51±0.30	-	ND

*number of replicated samples *N*=6, **number of replicated samples *N*=15; ND=not detectable, STR=streptomycin, N=neomycin

Table 2. Streptomycin (STR) mass fraction in honey samples during storage at different temperatures

Time	Polyfloral honey				Lime honey				Acacia honey			
	t/°C											
week	4	22	30	40	4	22	30	40	4	22	30	40
	w(STR)/(μg/kg)				w(STR)/(μg/kg)				w(STR)/(μg/kg)			
0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0	200.0
1	199.0	197.9	195.8	194.9	199.0	196.8	194.7	194.1	197.9	196.8	195.8	193.8
2	198.8	196.6	195.6	194.3	197.7	195.6	193.5	192.4	197.7	196.6	194.5	191.5
3	198.2	196.1	195.1	193.9	197.2	195.1	193.1	192.0	197.2	196.1	194.1	193.4
4	197.9	195.8	194.8	193.5	196.8	194.8	192.9	191.3	195.8	194.8	193.8	192.5
5	197.1	194.2	193.2	192.7	195.2	193.2	191.3	190.4	194.2	192.2	191.3	190.6
6	194.9	192.9	191.9	192.3	193.9	190.9	189.0	188.5	192.9	190.0	188.0	186.9
7	192.5	191.5	190.4	190.1	191.5	188.3	187.3	186.9	190.4	189.4	185.3	183.8
8	191.4	190.3	189.3	188.7	190.3	187.2	186.1	184.8	188.2	186.1	184.1	182.7
9	190.4	188.0	186.9	185.4	189.2	185.7	183.4	182.2	186.9	184.5	181.2	180.7
10	188.4	186.3	184.2	183.4	188.4	183.2	180.1	179.3	182.1	181.1	180.1	178.8
11	186.0	184.8	180.6	179.7	184.8	182.7	179.5	177.9	180.6	178.5	177.5	173.9
12	184.0	181.4	177.6	177.3	182.7	180.1	175.2	174.7	176.4	175.2	172.9	170.6
13	183.0	178.1	175.8	174.5	179.3	175.8	171.3	170.4	174.7	171.3	169.2	167.8
14	181.1	173.3	172.5	170.1	175.9	174.2	169.3	167.9	173.3	167.7	164.7	164.1
15	179.0	169.9	167.0	165.3	174.3	173.6	164.2	162.8	172.1	163.5	162.9	161.8
16	174.9	164.4	163.1	162.9	172.2	170.8	162.5	160.3	168.9	160.7	159.5	159.3
17	170.0	162.4	159.5	159.0	168.4	164.6	160.9	159.8	163.1	158.1	154.1	153.7
18	168.7	160.8	156.7	155.5	164.3	163.6	156.1	155.7	158.7	156.1	152.2	150.1
19	161.9	159.4	155.3	154.8	160.2	158.6	153.0	151.9	156.9	155.3	150.7	148.3
20	159.4	154.1	151.2	150.3	154.1	151.9	150.5	148.9	152.6	150.5	149.1	146.2

Models were developed in MATLAB v. 7.9 (Math Works Inc., Natick, MA, USA). By processing the experimental data, a series of statistical models were obtained that indicate the cumulative influence of temperature and storage time on the streptomycin mass fraction in honey samples. Experimental data along with the regression planes corresponding to these models are presented in Fig. 2 for polyfloral honey.

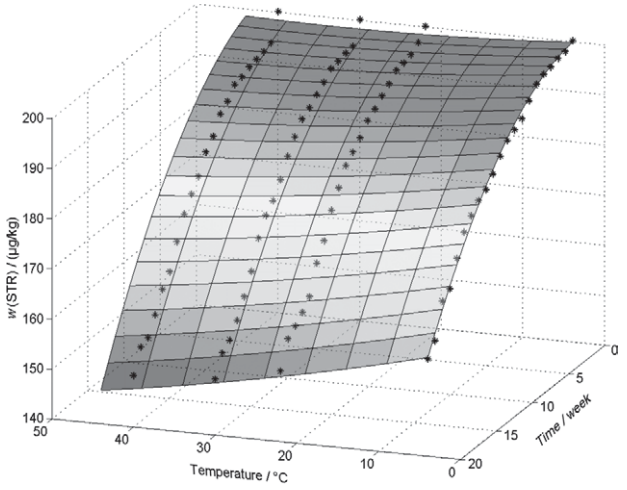

Fig. 2. Influence of time and temperature of storage on the variation of streptomycin (STR) mass fraction in polyfloral honey

Equations of second-order multiple linear regression models are presented in Table 3 and are valid for the studied range of values. Due to the fact that streptomycin is enterely degraded as soon as the temperature reaches 70 °C, the development of regression models for temperatures higher than 70 °C was not considered necessary.

Table 3. Equations of second-order multiple linear regression models

Honey type	Equations of statistical models
polyfloral honey	$Y=200.1822-0.1331\cdot T-0.0751\cdot t-0.0148\cdot T\cdot t+$ $+0.0021\cdot T^2-0.0933\cdot t^2$
lime honey	$Y=200.2249-0.0548\cdot T-0.5487\cdot t-0.0093\cdot T\cdot t-$ $-0.0012\cdot T^2-0.0762\cdot t^2$
acacia honey	$Y=201.1308-0.0576\cdot T-1.0948\cdot t-0.0094\cdot T\cdot t-$ $-0.00005\cdot T^2-0.0625\cdot t^2$

Correlation analysis showed that temperature of honey has a great influence on the degadation of streptomycin during storage. The study of both model equations and regression planes demonstrated that the degradation of streptomycin in relation to storage time and temperature is similar for all three types of studied honey.

In order to quantify how well the experimental data are modelled by the equations presented in Table 3, the following statistical indicators were calculated: standard deviation and coefficient of determination (Table 4).

Table 4. Statistical indicators used to assess the performance of statistical models

Honey type	σ/(μg/kg)	R^2
polyfloral honey	1.3540	0.9915
lime honey	1.4604	0.9899
acacia honey	1.3940	0.9925

σ=standard deviation, R^2=coefficient of determination

The values of statistical indicators confirm the fact that the equations of second-order multiple linear regression models describe the degradation of streptomycin with time as a function of storage temperature with sufficient accuracy.

Conclusions

Validation of the application of ELISA test for the determination of streptomycin residues in honey has been made. Good correlations between the results of ELISA and HPLC methods were observed. These results confirm that ELISA is a sensitive, accurate and low-cost method that can be a useful tool for screening residues of streptomycin in honey. Following validation, the mass fraction of streptomycin in honey was directly related to temperature and storage time. The results demonstrate that streptomycin mass fraction decreased with time and with temperature increase in all honey samples. Mathematical and statistical models can approximate the streptomycin mass fraction in honey with the variation in time.

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