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RESEARCH ARTICLE

Development of Chemometric Models Using Infrared Spectroscopy (MID-FTIR) for Detection of Sulfathiazole and Oxytetracycline Residues in Honey

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Abstract Chemometric models using mid-infrared (MID-FTIR) spectroscopy were developed for detection and quantification of oxytetracycline and sulfathiazole contamination in honey samples. Low standard error of calibration (SEC) and prediction (SEP) values were achieved using a partial least squares algorithm (SEC=1.02 and SEP=1.39 for oxytetracycline and SEC=1.24 and SEP=1.79 for sulfathiazole). Chemometric model-predicted concentrations of antibiotics were compared with ELISA results with coefficient of determination R^2=0.8577 for oxytetracycline and R^2=0.8216 for sulfathiazole. Classification of antibiotic contaminated honey samples and uncontaminated samples was carried out using Soft Independent Modeling Class Analogy analysis with a 100% correct classification rate with interclass distances in the range of 6.93-13.3. MID-FTIR chemometric models developed for detection and quantification of oxitetracycline and sulfathiazole in honey samples have been demonstrated.

Keywords: sulfathiazole, oxytetracycline, chemometric, honey, FTIR

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Introduction

Veterinary drugs are used during animal production to treat diseases, promote growth, and reduce production costs (1). Veterinary drug residues are small amounts of drugs that remain in animal products and eventually enter the food chain posing a risk to human health. A large amount of veterinary antibiotics administered are released daily into the environment through excretion or direct runoff (2). Although epidemiological studies investigating the magnitude of these effects are scarce, available information indicates that these residues are an important medium for dissemination of antibiotic resistant bacteria (3).

National governmental authorities control use of veterinary drugs based on adoption of rules and regulations for residues of dangerous or prohibited drugs in foods. Regulations establish maximum residue limits (MRL) that indicate the maximum concentration of a veterinary drug that is legally permitted or accepted in food. The presence of antimicrobial residues above MRL in food indicates that good veterinary practices are not being observed (4).

Use of antibiotics in apiculture has been known since 1940. Sulfonamides, tetracyclines, nitrofurans, and macrolides are used by farmers to prevent and combat diseases in cultivated plants and by beekeepers to prevent and combat diseases in honeybees (5). Beekeepers use antibiotics to treat clinical infections (bacterial brood diseases), such as European foulbrood (EFB) (*Melissococcus pluton*) and American foulbrood (AFB) (*Paenibacillus larvae*) in honeybees (6). Several techniques are available for detection of antibiotic residues in animal-based foods. Immunological methods consist primarily of commercially available enzyme-linked-immunosorbent assays (ELISA) and recent techniques are based on biosensors and chromatographic

methods, such as HPLC, coupled with different detection systems (7).

The utility of analytical methods for detection and quantification of antibiotics in honey has been demonstrated (8-11). However, these methods are costly, complex, and time-consuming, requiring a large investment in analytical time using trained personnel (7). Trends in analytical chemistry are moving toward simpler and less time-consuming analytical methods. Therefore, development of new methods that are simple, fast, and reliable has become necessary. Currently, infrared spectroscopy is being used in combination with chemometrics in different areas for food analysis (12) and these techniques are becoming more common for analysis of honey. Analytical methods applied to honey generally address the topics of determination of botanical or geographical origin, assessment of quality control according to current standards, and detection of adulteration (13). In all of these areas, except for residue analysis, infrared spectroscopy has recently been applied because it is a rapid, non-destructive, and promising approach.

Due to a need for low cost rapid analytical screening methods for samples (7), use of detection techniques, such as Fourier transform infrared (MID-FTIR) spectroscopy in combination with chemometrics has been useful for analysis of veterinary drug residues at μg/kg levels (14-16). This study describes models for detection of compounds using MID-FTIR in the mid-infrared region as an alternative to existing methods for determination of residues of 2 common antibiotics in honey samples.

Materials and Methods

Honey sample preparation Pure honey samples were acquired from the beekeeping area at the Centre for Environmental Education (CEA) under control of the Faculty of Medicine Veterinary and Zootechnics of the National Autonomous University of Mexico (UNAM) in México City. The facility ensures that the honey is produced without antibiotics. For each antibiotic, an initial working solution containing 0.4 mg/mL of oxytetracycline hydrochloride (O5750; Sigma-Aldrich, St. Louis, MO, USA) and sulfathiazole sodium (S0127; Sigma-Aldrich) was prepared and stored in amber vials in refrigeration at 4ºC. Intermediate standard solutions at different concentrations were prepared based on dilution of working solutions using distilled water. Honey samples were spiked with solutions containing different concentrations of corresponding antibiotics. For each 3.9 g honey sample, 0.1 mL of each solution at a different concentration of the corresponding antibiotic was added (15). Thirty-six samples of honey with oxytetracycline and sodium sulfathiazole in concentrations ranging from 2 to 1,000 μg/kg were prepared. Thirty samples were used for calibration of quantitative models while remaining samples were used for validation. Classification models was developed using Soft Independent Modeling Class Analogy (SIMCA) (17) using 27 samples of each class (80%) as a calibration set and the remaining 20% (3 samples of each class) were used as a validation group. The models were validated with a reliability of 95%; using a group of samples of honey with oxytetracycline (OTC) and sulfathiazole (SFZ) not included in the calibration set. Honey samples (3.9 g) added with 100 μL of antibiotic solution was prepared 24 h before spectroscopic analysis.

MID-FTIR Spectroscopy MID-FTIR spectra were obtained at ambient temperatures using a FTIR spectrophotometer (model Spectrum GX; Perkin Elmer, Norfolk, CT, USA) equipped with a deuteratedtriglycine sulfate detector. Spectra were obtained using an horizontal attenuated total reflectance (HATR) accessory (model 022-12xx; Pike Technologies, Madison, WI, USA) with a removable zinc selenide crystal (ZnSe crystal angle of 45º, surface with 10 internal reflections, and a 48×5 mm effective area).

The spectrum for each honey sample was scanned in triplicate from 4,000 to 650 1/cm at a 4 1/cm resolution over 64 scans, and results were recorded as the mean spectral value of replicates. Spectra were baseline-corrected and normalized using Spectrum® software (version 3.01.00; Perkin Elmer) and the background spectrum from the empty ZnSe cell (with ambient filters for CO_2 and H_2O) was subtracted from each of the spectra. After spectra for honey samples were acquired, the ZnSe crystal was cleaned using 10% EXTRAN® detergent, then the crystal was rinsed with distilled water and air-dried. Spectra were acquired using Spectrum® software (version 3.01.00; Perkin Elmer) (17).

Development of quantification models Partial least squares (PLS) and principal component regression (PCR) algorithms were used for quantitative analyses. Models were calibrated using Quant+ software (version 4.51; Perkin Elmer). Two versions (PLS1 and PLS2) of the partial least squares algorithm and PCR were used for correlation of spectral data with a set of honey samples containing different concentrations of oxytetracycline and sulfathiazole.

Before chemometric analysis, pre-treatments, and transformations using Quant+ software (version 4.51; Perkin Elmer) were performed on the spectral data. In addition to 5 points smoothing using the Savitzky-Golay method, (16) the first and second derivatives were applied to spectra preceded by normalization (standard normal SNV) (18) and autoscaling based on division of the absorbance of each spectrum by the standard deviation of all of the

spectra. Using SNV, autoscaling and derivation removed unwanted variations and baseline changes. The spectral region selected for building the calibration models spanned a range of 1,750 to 650 1/cm in which the highest correlation between spectral data and honey sample concentrations was located.

The best model was chosen based on the coefficient of determination (R^2) and standard error of calibration (SEC) values. The R^2 is expressed as the percent of variation in the values of the dependent variable (predicted antibiotic concentrations) that can be explained by variations in the value of the independent variable (real antibiotic concentrations). Thus, an R^2 of 1 indicates that 100% of the variation in dependent variable can be explained by the variation in x. The evaluation of the calibration is estimated by calculating the SEC obtained for each model. The equation for the SEC is:

$$SEC=\left(\left(\sum_{i=1}^{N}(C_i-C_i^*)^2\right)/(N-1-f)\right)^{1/2}$$

where Ci is the concentration real, Ci^* is the calculated value, N is the number of samples, and f is the number of factors in the calibration. The standard error of prediction (SEP) gives the estimation of the prediction performance during the step of validation of the calibration. The equation for the SEP is:

$$SEP=\left(\left(\sum_{i=1}^{M}(C_i-C_i^*)^2\right)/(M-1)\right)^{1/2}$$

where C_i is the concentration real, C_i^* is the calculated value, and M is the number of prediction samples (19). Higher R^2 values and smaller SEC values indicated better model calibration. The optimum number of calibration factors was selected based on the SEP, which should be minimized, and the R^2 value, which should be as close to a value of 1 as possible. In this study, an external validation method was used for assessment of the accuracy of calibration. This type of validation involves using a set of samples independent of the sample set used for calibration, and has been previously successfully applied (19,20). The calibration model was examined during a validation step using 6 external samples, obtained in the same way as described in the methodology for the set of calibration. The accuracy of the validation was assessed according to the SEP and R^2 values.

Development of qualitative models The SIMCA modeling technique was applied to spectra of the 3 honey sample class sets: 1) contaminated with oxytetracycline, 2) contaminated with sulfathiazole, and 3) uncontaminated samples. For development of classification models, AssureID software (version 4.1; Perkin Elmer) was used. Models showed adequate separation of classes, and proper recognition of validation honey samples was obtained over a spectral range of 1,450 to 600 1/cm, eliminating spectral information in the range of 4,000 to 1,450 1/cm. A filter to remove noise caused by CO_2 and humidity, 5-point smoothing, correction of the spectral baseline using a second order derivative, and use of a normalization MSC or multiplicative scatter correction were applied during collection of spectra.

ELISA testing A commercially available enzyme immunoassay (ELISA) kit (Ridascreen® Art. no. R3505, Art. no. R3004; R-Biopharm GmbH, Darmstadt, Germany) was applied for analysis of the OTC and SFZ levels in honey. The test procedure was carried out following instructions of the manufacturer. A calibration curve and results were obtained using Ridasoft.winNET® software (version 1.8; R-Biopharm GmbH).

Results and Discussion

Interpretation of MID-FTIR spectra Spectral of groups of honey samples uncontaminated and contaminated with sulfathiazole and oxytetracycline were used to determine regions of infrared energy absorption. HATR spectra of honey with OTC and corresponding bands representative of chemical groups from components present in honey samples are shown in Fig. 1. The 800 to 1,500 1/cm region corresponded to absorption zones for the fructose, glucose, and sucrose sugar constituents. The 900 to 750 1/cm region was anomeric and characteristic of saccharide configurations (21). Bands from 904 to 1,153 1/cm were assigned to the C-O and C-C stretching modes (22), and bands from approximately 1,474 to 1,199 1/cm were related to bending modes of O-C-H, C-C-H, and C-O-H angles. Bands observed at approximately 1,618 and 3,635 1/cm were attributed to water as O-H stretching vibrations occur at these wavelengths (23). An NH_3^+ stretching band from 3,000 to 2,700 1/cm was related to primary amino acids (24).

Spectra of honey samples exhibited absorption bands characteristic of different functional groups of the chemical composition of each honey sample (Fig. 1). These bands involved many of the general stretching, bending, and wagging motions of chemical bonds of functional groups. Therefore, a spectrum of a particular honey sample was considered as a fingerprint of the composition of the specific honey sample (14). Consequently, MID-FTIR (400-4,000 1/cm) spectroscopy coupled with multivariate analysis has been useful and successfully applied in different areas of the food industry including quality

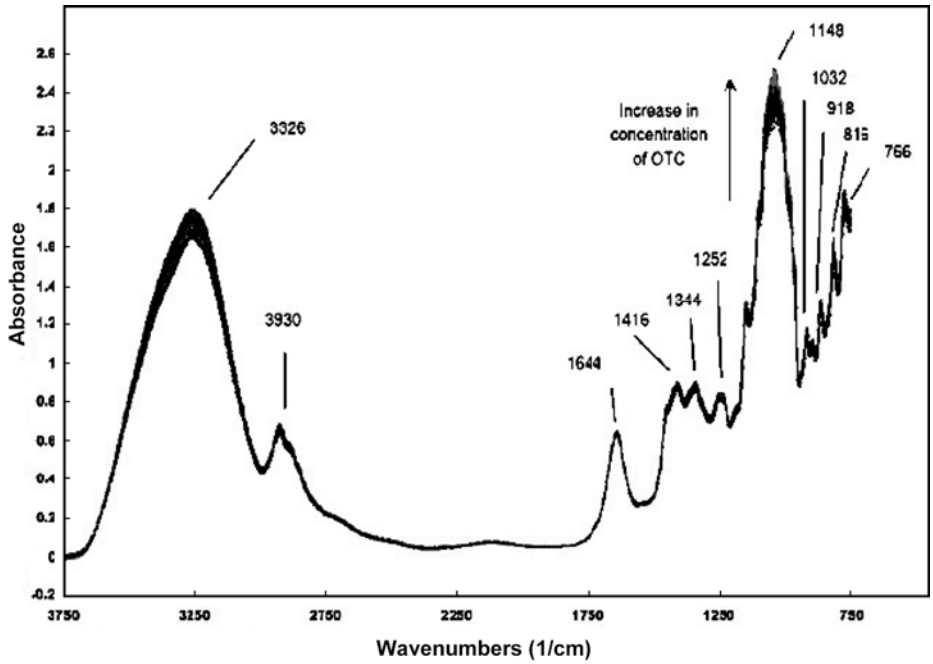

Fig. 1. Normalized spectra of honey samples fortified with oxitetracycline at concentrations of 2-1,000 μg/kg. Changes in the intensity of bands (absorbance change) with respect to an increase in OTC concentrations at different specific wave numbers (y axis).

control, adulterant detection, and origin denomination of different types of foods (25).

Spectra of samples with added antibiotics An example of MID-FTIR spectra of honey spiked with oxytetracycline antibiotic at different concentrations is shown in Fig. 1. The spectra revealed differences in absorbance values across the MID-FTIR (4,000 to 650 1/cm) region where these changes in absorbance emerged principally in the fingerprint region proportional to changes in concentrations of the antibiotic. Changes in the intensities of the bands corresponded to different vibrational modes of functional groups in the oxytetacycline added to honey samples. In order to mathematically correlate subtle differences of absorbance in the MID region with the concentration of the added antibiotic (14), multivariate techniques, such as PLS and PCR, were used. For construction of models, the band associated with water bonds was removed because regions in the spectrum with strong absorption values can exhibit nonlinearity with respect to Beer's law (26), and peaks with this feature should be excluded.

Development of calibration models An initial set of calibrations was performed for final models using the PCR, PLS1, and PLS2 algorithms to allow use of the Quant$^+$ software. The spectrum for each member of the calibration set containing honey contaminated with antibiotics and corresponding concentration data was used for construction of calibration models. Spectra were analyzed based on the lowest SEC and the highest R^2 values.

Calibration testing using PLS and PCR showed that PLS provided better performance in development of multivariate models that captured most of the variation across the selected spectral range. However, PCR did not consider reference values for construction of multivariate models while the PLS algorithm allowed construction of spectral components using reference value information (27). Thus, the PLS model performed better than the PCR model (14).

Graphs that demonstrated correlations between values present in honey samples and correlations predicted by models obtained using PLS1 for oxytetracycline and sulfathiazole are shown in Fig. 2. Points were close to the correlation line in these graphs, revealing the good predictive abilities of these models. The actual concentration refers to the concentration present in honey samples used to build the calibration models, and the predicted concentration refers to the concentration calculated using the model. Figure 2A shows the correlation for the set of calibration samples with added antibiotic OTC. Figure 2B shows the correlation obtained with chemometric model for the honey samples spiked with SFZ.

The optimal number of factors, R^2, and SEC values for calibration models used for both antibiotics with regard to contaminated honey samples are shown in Table 1. It has been suggested that the best parameters for evaluation of calibration and validation of multivariate models are SEC, SEP and R^2. Performance evaluation of calibration is estimated by computation of SEC values. Models with low SEC and high R^2 values indicate a degree of high correlation between spectral information and reference values (28,29).

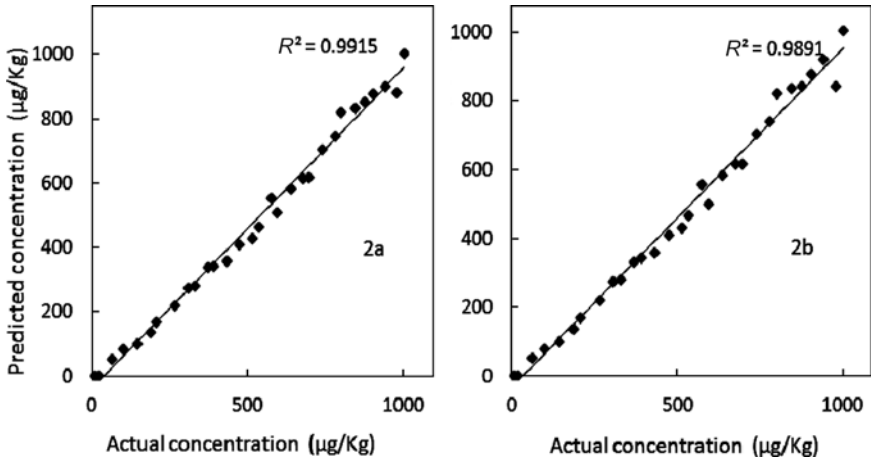

Fig. 2. Correlations obtained with concentrations (µg/kg) generated using chemometric model PLS1 (y axis, predicted concentration) and the concentrations present in honey samples (x axis, actual concentration). 2a corresponds to the calibration curve for the OTC value for antibiotics, indicating R^2 values. 2b corresponds to SFZ.

Table 1. Coefficient of determination and standard error of calibration values obtained for prediction models

Antibiotic	PLS1					PLS2				
	No. of factors[1)]	R^2 [2)]	R^2_v [3)]	SEC[4)]	SEP [5)]	No. of factors	R^2_v	R^2	SEC	SEP
Oxitetracycline	8	0.9915	0.9896	1.02	1.39	11	0.9828	0.970	1.75	2.62
Sulfathiazole	5	0.9891	0.9860	1.24	1.79	7	0.9804	0.9702	1.63	2.49

[1)]Optimal number of factors.
[2)]Coefficient of determination (R^2) should be as close to 1 as possible.
[3)]Coefficient of determination (R^2) for validation.
[4),5)]Standard error of the calibration (SEC), and standard error of prediction (SEP) values should be as low as possible.

Choosing the number of factors included in the calibration model is critical. A key objective of the factors is to transform data properties or random variables into a new and smaller data set. Transformed variables are called factors that contain the most relevant data for development of models from original information (30). R^2 values exceeded 0.98 for the calibration models developed using the PLS1 algorithm. However, models exhibited lower correlation values when the PCR algorithm was used for the 2 sets of samples contaminated with oxytetracycline and sulfathiazole. SEC values fluctuated between 1.02 and 1.75 for the PLS1 and PLS2 algorithms, respectively (Table 1).

External validation of the models was obtained independently with samples not used in the calibration stage, and the validation samples were prepared as described in the methods section. The results obtained in the validation stage indicated that chemometric methods could accurately predict the oxytetracycline and sulfathiazole content. SEP and R^2 values for predicted and actual values of external honey samples are shown in Table 1. These parameters were used for assessment of the analytical quality of models using the predicted and actual values. R^2 values between actual and predicted values of external honey samples exceeded 0.9860 for PLS1, while the lowest R^2 value was obtained using PCR. In addition, SEP values were 1.39 and 2.62 for PLS1 and PLS2, respectively. SEP indicated the ability of a model to predict unknown samples. According to Rohman and Che Man (31), high R^2 values and low SEP values indicate appropriate regression models.

Determination of the oxytetracycline and sulfathiazole contents in honey samples using ELISA ELISA was used for detection of oxytetracycline and sulfathiazole as a comparison with results obtained by the chemometric model developed using the PLS1 algorithm, which presented the smallest prediction errors. Comparison of the techniques was carried out over a concentration range of 7 to 18 µg/kg, which took into account the detection limit of the model (7 µg/kg) and the concentration range of the ELISA kit standards. The detection limit for chemometric models was established by successive calibrations considering the lowest concentration to which it was possible to validate the model. Five honey samples in duplicate contaminated with OTC and SFZ were used and, the results are shown in Table 2.

Commercial ELISA kits are routinely used in scanning techniques for detection of antibiotics. Results are considered presumptive because ELISA is a low specificity technique as groups of compounds are detected. Therefore, cross-reaction resulting in false positives can occur. Some false

Table 2. Calculation of sample concentrations using a chemometric model and the ELISA technique (Unit: ppb)

Sample concentration	Concentrations obtained with ELISA				Concentrations obtained with PLS1	
	OTC[1)]	CV[2)] (%)	SFZ[1)]	CV (%)	OTC	SFZ
9.24	6.22±0.17	2.67	8.31±0.11	1.29	7.51	11.60
11.09	8.14±0.60	7.43	14.11±0.46	3.24	8.90	13.27
13.51	9.23±0.32	3.50	12.76±0.14	1.12	15.77	11.63
15.22	10.03±0.06	0.60	18.7±0.55	2.95	18.03	16.09
17.06	>18	NA[3)]	19.2±0.12	0.64	15.40	15.56

[1)]Values are expressed for mean±SD for 2 samples.
[2)]Coefficient of variation between samples.
[3)]Data not available.

positives are acceptable using this technique, but the method should avoid or minimize the number of false negatives (7). Both techniques exhibited deviations from the actual concentrations in honey samples (Table 2). Comparison of R^2 values using both techniques showed that results using chemometrics were similar to results obtained using the reference ELISA screening technique (R^2=0.8577 for OTC and R^2=0.8216 for SFZ). On the other hand, advantages exist for using a chemometric method, such as this detection technique, instead of a biological method, such as ELISA. Chemometric methods can be used in higher concentration ranges. Moreover, because these methods do not involve biological materials such as enzymes, they do not depend on the activation temperature of enzymes. They are also less vulnerable to analytical error and do not depend on the small number of assays included in commercial kits.

In general, confirmatory analytical methodologies are costly in time, equipment, and chemicals, in addition to requiring long sample treatment procedures. Therefore, there is a need for screening methods that allow analysis of a large number of samples in a short period of time that are easy to use with low setup and running costs (32,33). These main requirements for a screening methodology were achieved by development of predictive multivariate models using MID-FTIR spectroscopy coupled with chemometrics, as was the case for MID-FTIR models developed in this study for detection and quantification of oxytetracycline and sulfathiazole amounts in honey samples.

Results for models using the pattern recognition technique (SIMCA) Models developed for each of the groups using SIMCA exhibited correct classification of honey samples and separation of models created for each class (Fig. 3).

Appropriate model use was reflected in results for interclass distances (ID) generated using AssureID software for obtained models. The ID value was defined as the geometric distance of the models (hiperboxes). An ID value >3.0 indicates that sample groups are suitably detached and, therefore, properly classified (34). The ID value for models generated using sets of honey samples spiked with antibiotics (SFZ and OTC) was 6.93. Distance values between models generated for all uncontaminated honey samples with respect to models for OTC and SFZ were 13.3 and 8.1, respectively.

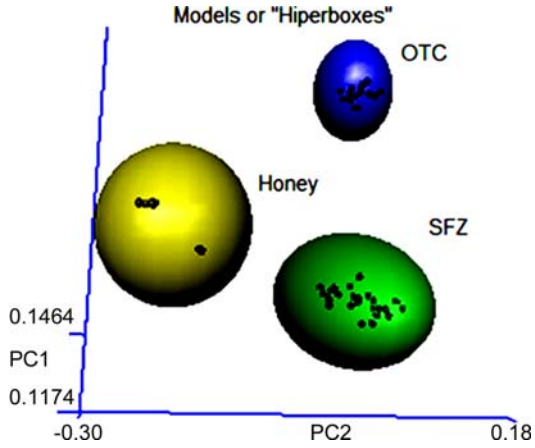

Fig. 3. Classification models obtained using SIMCA for classified groups of honey contaminated with OTC (top circle), SFZ (bottom right), and honey without antibiotics (bottom left). The number of principal components (PCs) developed a geometric structure.

For the SIMCA model, sensitivity was defined as the percentage of samples that were correctly recognized in their respective class (recognition rate). For models developed in this study, the recognition rate for SFZ was 100% (27/27) (samples used for construction model/ samples properly classified by the model). For OTC, the recognition rate was also 100% (27/27), and the recognition rate for honey without antibiotics was 100% (14/14).

The specificity for models using SIMCA was defined as the percentage of samples that belong to another class that are recognized as foreign (rejection rate). For models developed in this study using AssureID software, the rejection rate obtained for SFZ was 100% (41/41) (samples

Table 3. Total residual distance and honey samples used for SIMCA model validation

Material specified	Material identified	Total distance of the material specified[1)]	Residual distance[2)]
SFZ	SFZ	0.6867	0.9225
SFZ	SFZ	0.5725	0.7691
SFZ	SFZ	0.8238	1.2011
Honey	Honey	0.7958	1.1604
Honey	Honey	0.5616	0.8188
Honey	Honey	0.5825	0.8493
OTC	OTC	0.5566	0.8111
OTC	OTC	0.6129	0.9491
OTC	OTC	0.8863	1.354

[1)]Must be less than one.
[2)]Should be as low as possible.

belonging to other class/rejected samples). For OTC, the rejection rate was 100% (41/41), and the rejection rate for the group without antibiotics was also 100% (54/54). Results of the SIMCA model are shown in Table 3.

Model validation Each of 9 samples was classified correctly (Table 3) in testing the SIMCA model with the validation honey sample set. Model classification is properly validated when analyzed samples exhibit a total distance value less than 1, which indicates that an analyzed sample was classified correctly (16). Additionally, the residual distance should be as low as possible because a large residual distance indicates that an analyzed sample contained a source of variation that was not previously known. All 9 analytical samples used as the validation set exhibited total distance values <1 and low residual distances (Table 3).

In conclusion, the utility of an analytical method using infrared spectroscopy coupled with chemometric methods for detection and quantification of antibiotics in honey samples was shown. Honey samples contaminated with the oxy-tetracycline and sulfathiazole antibiotics that are commonly used for treatment of diseases in apiculture were analyzed. Use of a partial least square algorithm (PLS1) led to satisfactory detection of contamination levels as low as 7.51 μg/kg. Consequently, further work should investigate the detection limit of other contaminants that can be detected using these techniques. Chemometric models have already been used for detection of veterinary drug residues. Therefore, these techniques should be considered as an option for analysis of foods.

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Disclosure The authors declare no conflict of interest.

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