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Rapid Determination of Ractopamine in Swine Urine Using Surface-Enhanced Raman Spectroscopy

Fuli Zhai, Yiqun Huang, Chunying Li, Xichang Wang, and Keqiang Lai*

College of Food Science and Technology, Shanghai Ocean University, No. 999 Hucheng Huan Road, LinGang New City, Shanghai, China 201306

ABSTRACT: Ractopamine is approved for use in swine to improve carcass leanness in the United States, but banned in the European Union and China because ractopamine residue may pose health risks. This study investigated the possibility of applying surface-enhanced Raman spectroscopy (SERS) for analysis of ractopamine in swine urine. Ractopamine (0.1−10 μg mL^{-1}) was added to urine samples collected from 20 swine to prepare a total of 240 samples. A simple centrifugation, a liquid−liquid extraction (LLE) method, and a more complicated method involving liquid−liquid extraction and solid-phase extraction (LLE-SPE) were used to extract ractopamine from urine samples. Principal component analysis (PCA) and partial least-squares (PLS) regression were used for spectral data analyses. Although no satisfactory result was obtained with the centrifugation method, ractopamine could be detected at levels of 0.8 and 0.4 μg mL^{-1} with the LLE and LLE-SPE extraction methods, respectively. The R^2 of the PLS model of actual ractopamine values versus predicted values was 0.74 for the LLE method and 0.73 for the LLE-SPE method. The SERS method with simple sample preparation has great potential for rapid analysis of ractopamine in swine urine.

KEYWORDS: ractopamine, surface-enhanced Raman spectroscopy, urine, gold substrate

INTRODUCTION

Ractopamine (Figure 1), a phenethanolamine β-adrenergic agonist, has demonstrated efficacy in increasing daily weight gain and protein synthesis rates while reducing lipid deposition in swine.[1] The residual ractopamine in pork products may pose health risks, particularly to people with asthma or cardiovascular disease.[2] Therefore, the use of ractopamine in swine is banned by the majority of countries in the world, such as the European Union, Japan, and China, although it has been approved for use in a few countries, such as the United States, Canada, Australia, Brazil, Mexico, and Thailand.[3] According to the U.S. Food and Drug Administration, ractopamine is approved for use in swine feed at a concentration level of 5−20 μg g^{-1}, and the maximum residue limit of ractopamine in swine liver is 0.15 μg g^{-1}.[4] As a controversial subject, ractopamine has been tied to various international trade barriers in the past decade.[5] In addition, due to its effects on promoting swine growth and increasing carcass leanness, it is not uncommon that ractopamine is intentionally added in swine feed in the countries that have banned the use of ractopamine in livestock.[6] In recent years, the detection of ractopamine in live swine or in pork products has been of great interest to government regulatory agencies and the food industry.

Conventional analytical methods of ractopamine in tissue and urine include ELISA,[7] HPLC,[8] LC-MS/MS,[9] and GC-MS.[10] A confirmatory method by regulatory agencies requires multiple extractions (liquid−liquid extraction (LLE) coupled with solid-phase extraction (SPE)) followed by HPLC with fluorescence detection.[11] Complicated and expensive sample preparation procedures with current official ractopamine analytical methods imply the urgent need for developing easier, faster, and highly sensitive methods to determine ractopamine residues in tissue or urine.[12,13]

Surface-enhanced Raman spectroscopy (SERS) is a novel analytical tool providing ultrasensitive detection and characterization of organic chemicals and microorganisms.[14] SERS technology overcomes the low sensitivity of traditional Raman spectroscopy through utilizing the benefits of tremendous enhancement of Raman scattering signals via adsorption of analytes onto roughened surfaces of nano metal substrates typically made from silver or gold.[15] With the aid of metallic nanostructures, Raman scattering signals of probed molecules within highly localized optical fields of metallic structures can be enhanced by >10^5 due to the effects of electromagnetic field and chemical enhancement.[16] Although there is still much to be learned about the basic mechanisms of SERS and applications of these nanosubstrates,[17] SERS technology has demonstrated great potential for trace analysis of prohibited or restricted chemicals (such as melamine, pesticides, and antibiotics),[18,19] microorganisms (such as anthrax and poxviridae virions),[20,21] and proteins.[22]

This study investigated the feasibility of using SERS technology coupled with a gold-coated nanosubstrate to detect and determine residual ractopamine in swine urine. Different sample preparation methods were used for analyses of ractopamine in urine with SERS technology in an attempt to simplify sample preparation. The methodology and study results could be extended to other similar studies and served as the basis for further study of applying the latest nanosubstrates in SERS analysis.

MATERIALS AND METHODS

Standard Solutions. A stock solution (100 μg mL^{-1}) was prepared by dissolving 2.5 mg of ractopamine (98.0%; Dr. Ehrenstorfer GmbH, Augsburg, Germany) in 25 mL of a 50% methanol (HPLC reagent; Sigma-Aldrich Chemicals, St. Louis, MO) aqueous solution. A

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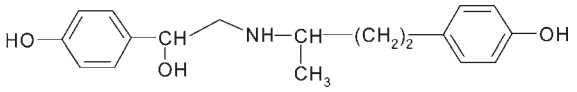

Figure 1. Molecular structure of ractopamine.

series of working standard solutions (0.1, 0.2, 0.4, 0.5, 0.8, 1, 2, 4, and 5 μg mL^{-1}) were then prepared by diluting the stock solution with 50% (v/v) methanol aqueous solution.

Urine Sample Preparation. Urine samples (n = 20) were collected from 20 pigs at three different time periods with 6−8 urine samples collected each time at a Shanghai local farm. These samples were kept frozen at −20 °C for no more than 1 week before being used for further analysis.

Each urine sample was centrifuged at 5000 rpm (TDL-5-A, Anting Scientific Instrument, Shanghai, China) for 5 min before being spiked with ractopamine to prepare a series of samples containing ractopamine ranging from 0.1 to 10 μg mL^{-1} (0.1, 0.2, 0.4, 0.5, 0.8, 1, 2, 4, 5, 8, and 10 μg mL^{-1}). Together with control samples (without spiked ractopamine), a total of 240 urine samples were prepared. For each urine sample, three different sample preparation methods were used before SERS analyses. Part of the samples were used immediately for SERS analysis without further extraction (treated only with simple centrifugation before the spike with ractopamine), and part of them treated with a LLE method or a more complicated method involving LLE and SPE (LLE-SPE).

LLE-SPE Method. A commonly applied LLE-SPE sample purification method for determination of ractopamine in urine with HPLC was used with some modifications.[8,23] Briefly, 5 mL of a urine sample was mixed well with 2 mL of sodium borate buffer (80 mmol L^{-1}, pH 10.3 ± 0.1, Sinopharm Chemical Reagent Co., Ltd., SCRC, Shanghai, China), extracted with 15 mL of ethyl acetate (analytical grade, SCRC) twice, and centrifuged at 5000 rpm for 10 min. The supernatant was evaporated to dryness with a rotary evaporator (R206B, Shanghai SENCO Technology Co., Ltd., Shanghai, China) at 42 °C and reconstituted in 4 mL of 1 mol L^{-1} acetic acid (analytical grade, SCRC).

The extracts were applied to cation exchange−solid phase extraction cartridges (PCX-SPE, 3 mL, 60 mg, Bonna-Agela Technologies, Tianjin, China) that were conditioned sequentially with 3 mL of methanol and 3 mL of deionized water. The columns were rinsed with 3 mL of 1 mol L^{-1} acetic acid and 6 mL of acetonitrile (HPLC reagent; Sigma-Aldrich Chemicals, St. Louis, MO). The analytes were eluted with 6 mL of 2% ammonia in ethyl acetate/methanol (30:70, v/v), evaporated at 42 °C with a rotary evaporator, and dissolved in 1 mL of a 50% (v/v) methanol aqueous solution.

LLE Method. The LLE method included only the LLE steps of LLE-SPE without further purification with PCX-SPE cartridges. The procedure of the LLE was the same as the relevant steps in the LLE-SPE method except that the reconstitution solution in the last step was 1 mL of a 50% (v/v) methanol aqueous solution instead of 4 mL of acetic acid. The LLE method was used to investigate the possibility of simplifying sample preparation to save time and cut costs.

Raman Instrumentation. Spontaneous Raman and SERS spectra were acquired using a Nicolet DXR microscopy Raman spectrometer (Thermo Fisher Scientific Inc., Waltham, MA) equipped with a 780 nm diode laser source. All spectra were recorded with 10× microscope objective (numerical aperture, 0.25) and 10 mW laser power from 600 to 1800 cm^{-1} with a spectral resolution of 5 cm^{-1}. Each spectrum was the average of two scans with exposure time of 20 s per scan. The instrument was calibrated using an internal silicon standard before measurements.

Gold-coated Klarite SERS-active substrates (Renishaw Diagnostics Ltd., Glasgow, U.K.) were used for SERS spectra collection. The active surface (4 mm × 4 mm) of Klarite has regular arrays of inverted square pyramid subunits, which are about 1 μm deep and 1.8 μm long, whereas

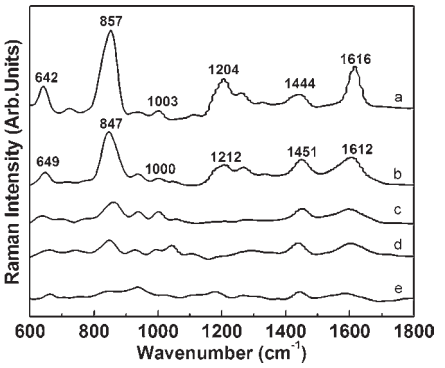

Figure 2. Raman spectra of solid ractopamine and representative SERS spectra of substrate and ractopamine standard solutions: a, solid; b, 5 μg mL^{-1}; c, 1 μg mL^{-1}; d, 0.1 μg mL^{-1}; e, substrate.

the distance between each subunit is about 0.4 μm.[18] To acquire SERS spectra, a standard solution or urine sample extraction (1.0 μL) was deposited directly onto the substrate. SERS spectra were recorded after the evaporation of the solvent at room temperature.

Data Analysis. Spectral data were preprocessed with binning, Gaussian smoothing, and second-derivative transformation (discrete point method) to remove background interference, separate the overlapped peaks, and increase the signal-to-noise ratio before being analyzed with partial least-squares (PLS) regression and principal component analysis (PCA) (DeLight 3.2; DSquared Development Inc., La Grande, OR).[24] For PLS model development, urine samples (spiked with different levels of ractopamine concentrations) from 20 pigs were divided into a calibration set and a prediction set as follows: urines samples from two pigs were randomly selected from each of the three batches of samples, and therefore urine samples from a total of six pigs were selected as prediction set, whereas the rest were used as the calibration set. Samples from the calibration set were used to build the PLS model (with leave-one-out cross-validation) between spectral data and ractopamine concentrations of urine extractions. Then the PLS model was used to predict the ractopamine concentrations for the samples in the prediction set on the basis of their spectral information. The predicted values were compared with the reference values. The higher the square of the correlation coefficient (R^2) and the ratio of performance to deviation (RPD) values, the better the predictability of the PLS model.[24] For the standard solutions, only leave-one-out cross-validation was used for PLS model development.

RESULTS AND DISCUSSION

Spectral Features of Ractopamine and Quantitative Analysis of Standard Solution. The Raman spectrum of ractopamine is shown in Figure 2. The three most prominent peaks at 1616, 857, and 642 cm^{-1} are assigned to C═C aromatic stretching vibration, C—H aromatic out-of-plane bending, and C—H wagging, respectively.[25,26] In addition, C—N stretching vibration, C—OH aromatic stretching vibration, and −CH_3/−CH_2/−CH bending are observed at 1204, 1003, and 1444 cm^{-1}, respectively.[26,27]

Figure 2 also shows some representative SERS spectra of ractopamine standard solutions. The SERS spectral features of ractopamine were basically consistent with those of their spontaneous Raman spectrum, although shifts of some characteristic bands and changes in peak intensity did occur in the SERS spectra of ractopamine compared to the Raman spectrum of ractopamine. The bonds of some functional groups of ractopamine molecules may be weakened or strengthened after adsorption of ractopamine molecules on the substrate surface, resulting in shifts of pertinent

characteristic bands and changes in peak intensity.[18] The characteristic peaks of ractopamine at around 847 and 1000 cm^{-1} were discernible at levels as low as 0.1 $\mu g\ mL^{-1}$. Some prominent bands of ractopamine, such as those around 649, 847, and 1612 cm^{-1}, exhibited an increase in the intensities of Raman scattering signals with an increase of ractopamine concentration, as clearly shown in the second-derivative transformation of SERS spectra of ractopamine standard solutions (Figure 3, top).

For ractoapmine standard solutions (0.1−5 $\mu g\ mL^{-1}$), a PLS model (n = 36) with five latent variables yielded the best result (R^2 = 0.94, RPD = 3.97) (Table 1). As shown in its PLS model (Figure 3, bottom), bands at around 845, 940, 1450, 1000, and 646 cm^{-1} accounted for the largest weights in the model, and these bands corresponded to the most prominent or strong absorption bands in the SERS spectra of ractopamine. This indicated a great potential of applying SERS with multivariate data analysis for quantitative analysis of ractopamine.

SERS Analysis of Urine Samples Prepared with LLE-SPE. Typical SERS spectra of ractopamine extractions from urine with LLE-SPE are shown in Figure 4. Compared to the SERS spectra of ractopamine standard solutions (Figure 2), the spectra of urine extractions had some prominent bands irrelevant to the ractopamine molecular vibrations. For example, the three most prominent bands at around 1348, 1026, and 770 cm^{-1} were mostly likely due to uric acid, one of the major components in urine.[28] Although LLE-SPE is a common sample preparation method for the determination of ractopamine in urine with the HPLC method, the extracted sample contained uric acid, which strongly interfered with the analysis of ractopamine using SERS methods. The other two major components of urine, urea and creatinine, did not seriously interfere with the SERS spectral features of ractopamine.

The prominent peaks at around 847 and 649 cm^{-1} in the SERS spectra of ractopamine standard solutions were still among the most prominent peaks in the SERS spectra of extraction of urine spiked with ractopamine, although these two bands shifted to 840 and 660 cm^{-1}, respectively, in the SERS spectra of ractopamine from urine. The other components in urine may interfere with the adsorption of ractopamine molecules on the Klarite substrate and cause the change in the interactions between ractopamine molecules and the adsorption sites, leading to the band shifts of the pertinent functional groups. The lowest level of ractopamine in urine that could be detected with SERS was about 0.4 $\mu g\ mL^{-1}$, which was higher than the 0.1 $\mu g\ mL^{-1}$ level for its standard solution counterpart. The loss of samples during ractopamine extraction led to an increase in the detection limit. Interference of the other components in urine extraction also adversely affected SERS analysis for ractopamine from urine.

The best PLS model (n = 112) developed through leave-one-out cross-validation for the determination of ractopamine in urine (spiked with ractopamine ranging from 0.4 to 8 $\mu g\ mL^{-1}$) extractions yielded an R^2 of 0.86 and an RPD of 2.63. When the PLS model was used to predict the samples (n = 48) in the prediction set, the resulting R^2 was 0.73 and the RPD was 1.93. This indicated the possibility of applying SERS with multivariate data analysis for the quantitative analysis of ractopamine, although the model predictability was far from satisfactory for practical uses.

SERS Analysis of Urine Samples Prepared with LLE. The SERS spectra of ractopamine LLE extractions showed consistency with those of ractopamine standard solutions, although inferences of other compounds in the urine extracts were quite obvious (Figure 5). A very strong band at 1008 cm^{-1} corresponds to the symmetrical C−N stretch of urea.[28] Due to the strong interference of urea, the intensity of characteristic bands of ractopamine was seriously weakened, but the band at about 846 cm^{-1} can still be discerned at the ractopamine level of 0.8 $\mu g\ mL^{-1}$ in urine.

The best PLS model (n = 98) for ractopamine extracted with LLE from urine samples (ractopamine concentration = 0.8−10

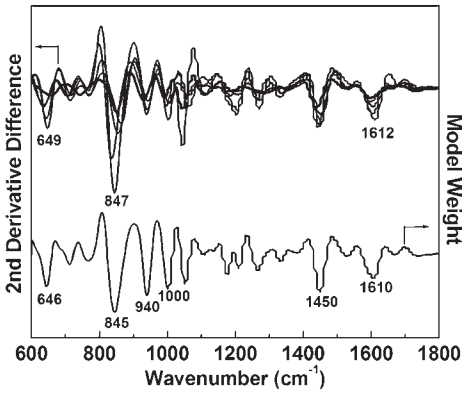

Figure 3. Second-derivative transformation of SERS spectra (top) and PLS model (bottom) for ractopamine standard solutions.

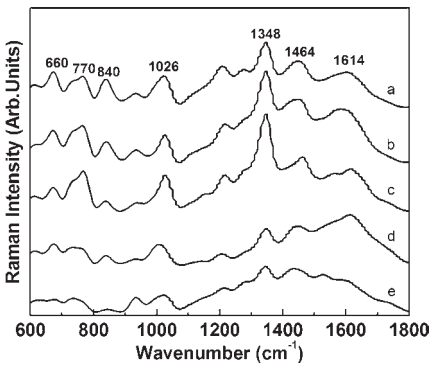

Figure 4. Representative SERS spectra of ractopamine extracted from urine with the LLE-SPE method: a, 8 $\mu g\ mL^{-1}$; b, 4 $\mu g\ mL^{-1}$; c, 1 $\mu g\ mL^{-1}$; d, 0.4 $\mu g\ mL^{-1}$; e, control.

Table 1. Results of PLS Models for Ractopamine Determination

		calibration				cross-validation		prediction		
	concn range ($\mu g\ mL^{-1}$)	LV[a]	n[b]	R^2	RPD[c]	R^2	RPD	n	R^2	RPD
standard solution	0.1−5	5	36	0.97	6.14	0.94	3.97			
LLE method	0.8−10	9	98	0.94	4.00	0.85	2.61	42	0.74	1.95
LLE-SPE method	0.4−8	8	112	0.93	3.68	0.86	2.63	48	0.73	1.93

[a] Number of latent variables used for PLS. [b] Number of samples used for PLS. [c] RPD, ratio of performance to deviation.

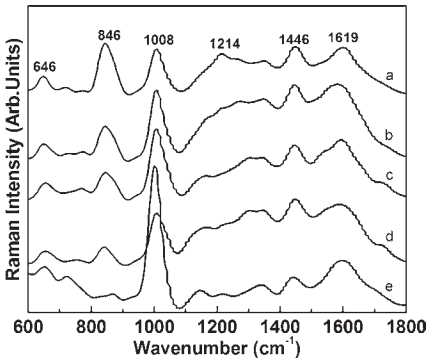

Figure 5. Representative SERS spectra of ractopamine extracted from urine with the LLE method: a, 10 μg mL^{-1}; b, 5 μg mL^{-1}; c, 2 μg mL^{-1}; d, 0.8 μg mL^{-1}; e, control.

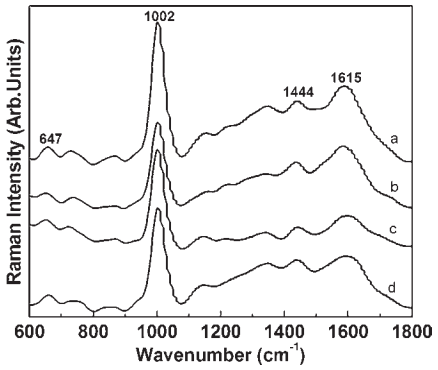

Figure 6. Representative SERS spectra of ractopamine in urine (after centrifugation): a, 10 μg mL^{-1}; b, 2 μg mL^{-1}; c, 0.8 μg mL^{-1}; d, control.

μg mL^{-1}) yielded an R^2 value of 0.85 and an RPD of 2.61 (Table 1). The PLS model predictability (n = 42, R^2 = 0.74, RPD = 1.95) for ractopamine in urine samples extracted with LLE was similar to that for urine samples exacted with LLE-SPE, although the presence of urea in the LLE extraction strongly interfered with the SERS spectral feature of urine extracted with LLE, making some characteristic bands of ractopamine weak and indiscernible.

SERS Analysis of Urine Prepared with Centrifugation. The SERS spectral features of the urine samples with only centrifugation were dominated by the molecular vibrations of urea, and the characteristic peaks of ractopamine could not be detected, even for urine samples spiked with ractopamine up to 10 μg mL^{-1} (Figure 6). In addition, the use of PCA was unable to differentiate between the control (n = 12) and the urine spiked with ractopamine (n = 48) (ractopamine concentration = 0.8−10 μg mL^{-1}). Again, the interferences of urea, uric acids, and other compounds in urine made it impossible to detect ractopamine without sample purification.

Effects of Sample Preparation Methods on the SERS Analysis. To determine ractopamine in urine with SERS, sample purification was required to alleviate the interferences of major components of urine, such as urea and uric acid. The LLE-SPE method is a commonly used sample purification approach for the determination of ractopamine in urine with chromatography-based analytical methods.[9,10] The SPE method is a powerful sample preparation technique for cleanup and enrichment of sample analytes in chromatographic analysis, but SPE is time-consuming and the disposable cartridges used are quite costly. Although SPE was an essential step for ractopamine analysis with the chromatographic method, this study demonstrated that the accuracy of the SERS method for quantitative analysis of ractopamine was not significantly affected by the elimination of the SPE step for urine purification. Compared to about 1 h for LLE-SPE method, the LLE method took <30 min. The use of the LLE method instead of the LLE-SPE method could simplify and speed the sample preparation for urine analysis besides cutting the cost of the PCX-SPE cartridge.

Challenges in the Application of SERS for Quantitative Analysis. The enhancement effect of surface-enhanced Raman scattering is affected by many factors, such as laser frequency, the surface structure of the substrate, and the molecular structures of the analytes.[16] It is very challenging to apply SERS for quantitative analysis because of the difficulty in obtaining reproducible SERS spectra. First, when SERS was applied to the analysis of ractopamine standard solutions, although the Klarite substrate had an orderly structure, the molecular orientations of ractopamine and their binding sites on the substrate varied, which caused inconsistency in SERS spectral features even for the same sample. Second, after deposition of a ractopamine standard solution onto a substrate, the ractopamine molecules would very likely unevenly spread across the rough substrate surface. This also contributed to the difficulty in acquiring reproducible SERS spectra. Third, other components in the tested biological sample (such as urine) or food components in pork would interfere with the analysis of the tested analyte. These components compete for the binding sites on the surface of substrate, making it physically difficult for the analyte to be effectively adsorbed onto the substrate. In addition, the SERS scattering signals of these components may cover or overlap with those of the analyte, making the feature peaks of the SERS spectrum of the analyte indiscernible.

Applications of SERS for the detection of residual drugs in biological or food samples are quite promising, although it is still very challenging to apply SERS for quantitative analysis.[29] However, the potential of applying SERS for quantitative analyses has been realized in more and more research fields.[30] With the aid of chemometric methods for spectral data analyses, as well as the rapid development of microscopy Raman spectrometer and nanosubstrates, SERS could serve as a rapid and accurate method for both qualitative and quantitative analyses of trace amounts of contaminants in complex biological samples and food matrices.

AUTHOR INFORMATION

Corresponding Author

*Phone: 86-21-6190-0363. Fax: 86-21-6190-0365. E-mail: kqlai@shou.edu.cn.

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