MALDI-TOF MS Quantification of Coccidiostats in Poultry Feeds

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a relatively new technique that is having a great impact on analyses. This study is the first to demonstrate the use of linear MALDI-TOF MS to identify and quantify coccidiostats in poultry feeds. 2,5-Dihydroxybenzoic acid (DHB) was found to be the best matrix. In MALDI-TOF MS, coccidiostats form predominantly $[M + Na]^+$ ions, with additional small amounts of $[M + K]^+$ and $[M - H + 2Na]^+$ ions, and no obvious fragment ions. Salinomycin and narasin were unstable in the concentrated DHB matrix solution but were stable when dried on the MALDI-TOF MS probe. A simple fast Sep-pak C18 cartridge purification procedure was developed for the MALDI-TOF MS quantification of coccidiostats in poultry feeds. The MALDI-TOF MS limit of detection for lasalocid, monensin, salinomycin, and narasin standards was 251, 22, 24, and 24 fmol, respectively. The method detection limit for salinomycin and narasin in poultry feeds was 2.4 μg/g.

Keywords: *Polyether ionophore; lasalocid; monensin; salinomycin; narasin; poultry feeds*

INTRODUCTION

Lasalocid, monensin, salinomycin, and narasin (Figure 1) are polyether ionophores and serve as the four major coccidiostats for prevention of coccidiosis in the poultry industry (Elliott et al., 1998). Their mode of action is attributed to their ionophoric properties (forming complexes with polar cations, i.e., K^+, Na^+, Ca^{2+}, and Mg^{2+}) and effects on cell membrane function (Pressman and Fahim, 1982; Braunius, 1985). Typical fortification levels of feeds range from 20 to 125 mg/kg, depending on the species treated (Braunius, 1985; Rodewald et al., 1994; Moran et al., 1994; Muldoon et al., 1995; Blanchflower and Kennedy, 1995; Elliot et al., 1998). Toxicity studies have shown that coccidiostats exert marked cardiovascular effects in experimental animals (Novilla, 1992; Novilla and Owen, 1994; Elliott et al., 1998) and might poison poultry or other animals (Rollinson et al., 1987; Salles et al., 1994; Andreasen and Schleifer, 1995). Although withdrawal periods from medicated feeds are required prior to slaughter, there is still concern over possible toxicological problems for humans consuming poultry meat. Therefore reliable analytical techniques are required for these compounds.

Several analytical techniques for the identity and quantification of coccidiostats were reviewed by Elliot et al. (1998). Of chemical methods for detection and quantification of coccidiostats, high-performance liquid chromatography (HPLC) with postcolumn derivatization (PCD) with vanillin (Rodewald et al., 1994; Moran et al., 1994, 1995) was the most common technique used. HPLC-PCD, with either a liquid−liquid or a silica gel solid-phase extraction, had a limit of detection of under 5 ng/g (Moran et al., 1994, 1995). HPLC electrospray mass spectrometry (MS) was another sensitive technique and offered the best possible confirmation of low levels of coccidiostats after a simple cleanup procedure (Blanchflower and Kennedy, 1995, 1996; Harris et al., 1998). The detection limit of this assay was 1 ng/g (Blanchflower and Kennedy, 1995, 1996).

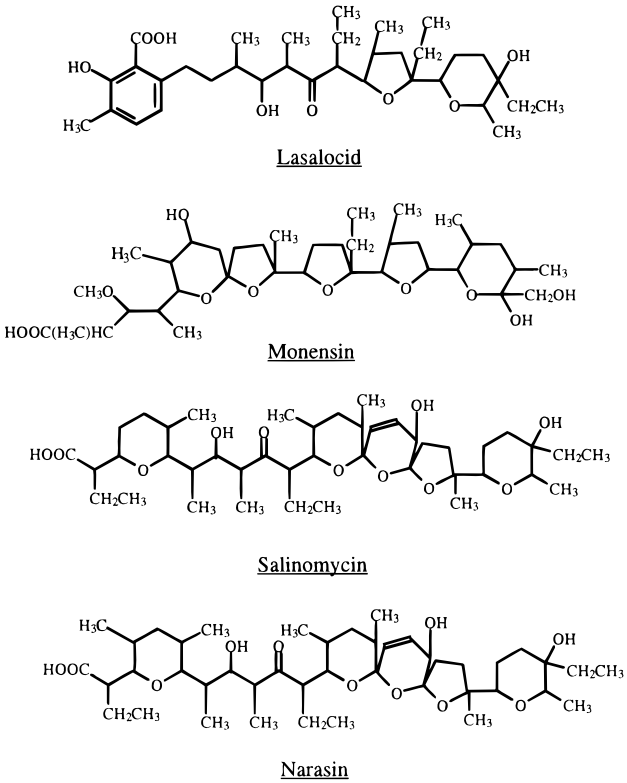

Figure 1. Structures of coccidiostats.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was first introduced in 1987 and originally developed for large biomolecules (Karas et al., 1987). The advantages MALDI-TOF MS has over other methodologies include speed of analysis, high sensitivity, wide applicability combined with a good tolerance toward contaminants,

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and the ability to analyze complex mixtures (Karas, 1996). The performance of MALDI-TOF MS allows for analysis of most molecules, except that simple linear instruments cannot tell the difference between isomers, which have identical mass. Presently, MALDI-TOF MS food-related applications are limited. The objective of this study was to explore and develop a simple fast protocol using MALDI-TOF MS to quantify the commonly used coccidiostats in poultry feeds.

MATERIALS AND METHODS

Materials and Reagents. Narasin (70 mg/kg) or salinomycin (60 mg/kg) medicated poultry feeds were obtained from Unifeed Grain Growers Ltd. (Okotoks, Alberta). Blank poultry feeds were from the Poultry Center, University of Alberta (Edmonton, Alberta). Lasalocid (sodium salt, 97%), monensin (sodium salt, 90−95%), salinomycin (96%), narasin (97%), maltotriose, and maltotetraose were purchased from Sigma Chemical Co. (St. Louis, MO). 2′,4′,6′-Trihydroxyacetophenone monohydrate (THAP), 3-aminoquinoline (3-AQ), and 2,5-dihydroxybenzoic acid (DHB) were obtained from Aldrich Chemical Co. (Milwaukee, WI). All water used was double-deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA). Individual stock solutions, lasalocid (1.34×10^{-4} M in methanol), monensin (1.16×10^{-4} M in acetonitrile), salinomycin (1.28×10^{-4} M in acetonitrile), and narasin (1.28×10^{-4} M in acetonitrile), were prepared and stored at 4 °C until used.

Extraction of Coccidiostats from Poultry Feeds. The feed samples were pulverized using a coffee grinder (Braun Inc., Woburn, MA) to obtain a homogeneous powder. Ground feed (1 g) was added to 10 mL of acetonitrile containing the internal standard monensin (1.45×10^{-5} M). The mixture was shaken with a horizontal shaker for 1 h and then filtered through Whatman no. 4 filter paper. Filtrate (2 mL) was loaded onto a Sep-pak C18 cartridge (Waters Corp., Milford, MA) at a flow rate of about 1 mL/min. Filtrate from the Sep-pak C18 cartridge was collected in a 10-mL volumetric flask. A further 4 mL of methanol/water (94:6) was used to elute the retained coccidiostats on the Sep-pak C18 cartridge at the same flow rate. This eluent was also collected in the 10 mL volumetric flask. The volume was then made up to 10 mL with methanol/water (94:6). This partially purified coccidiostat extract was kept at 4 °C for MALDI-TOF MS analysis.

MALDI-TOF MS. MALDI-TOF MS was performed using a Proflex III in linear positive mode (Bruker Analytical Systems Inc., Billerica, MA). Coccidiostats cocrystallized with matrixes on the probe were ionized by a nitrogen laser pulse (337 nm) and accelerated under 20 kV with time-delayed extraction before entering the time-of-flight mass spectrometer. When THAP and 3-AQ were used, the preparation of matrix and sample was the same as previously reported (Wang et al., 1999). When DHB was used, two types of matrix solutions were made. One contained 10−15 mg/mL DHB in 10% ethanol and the other 10−15 mg/mL DHB in 0.01 M NaCl in 10% ethanol. The ratio of matrix solution and sample was 1:1. For all matrixes either 1 μL of sample solution or 1.5 μL of a mixture of matrix and sample was applied to a probe. The MALDI-TOF MS sample was air-dried using a fan for 5 min. Laser strength was attenuated (the lower the attenuation, the higher the laser strength) to obtain the best signal-to-noise ratio and isotopic resolution. MALDI-TOF MS was calibrated with two-point external calibration using $[\text{maltotriose} + K]^+$ (exact isotopic mass = 543.13) and $[\text{maltotetraose} + K]^+$ (exact isotopic mass = 705.19) as calibrants, resulting in a mass accuracy ≤ 500 ppm. Any one spectrum or one MALDI-TOF MS run was acquired using 40 laser pulses at each of three randomly chosen spots per MALDI-TOF MS sample, for a total of 120 laser pulses, which took about 2 min. The major isotopic ^{12}C peak height or intensity was used for quantification. All statistics were carried out using Microsoft Excel 97.

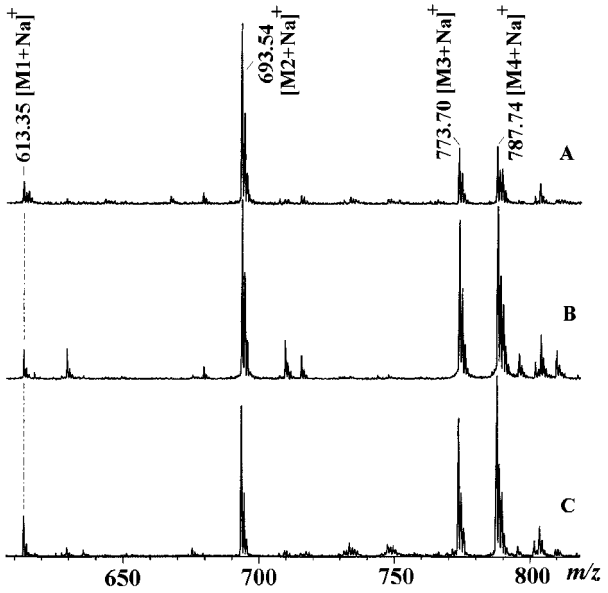

Figure 2. Performance of different matrixes for coccidiostat standards in MALDI-TOF MS: A, THAP; B, 3-AQ; C, DHB. A mixture of lasalocid (M1), monensin (M2), salinomycin (M3), and narasin (M4) was made by mixing equal amounts of stock solution (v/v). Note that only predominant sodium adduct peaks are labeled, while potassium adduct peaks (with 39 mass shift) and double sodium adduct peaks (loss of one proton, with 45 mass shift) are not labeled.

RESULTS AND DISCUSSION

It is possible to use MALDI-TOF MS to analyze any compound in a sample after the analytes of interest have been extracted from a sample matrix. Key concerns include the selection of matrixes, preparation of matrix and sample, signal-to-noise ratio, spot-to-spot repeatability, and response factors. Because of spot-to-spot variation, an internal standard is a requirement for quantification of analytes using MALDI-TOF MS.

Selection of Matrixes and Internal Standard. On the basis of our previous research, we chose three common matrixes, 2′,4′,6′-trihydroxyacetophenone monohydrate (THAP), 3-aminoquinoline (3-AQ), and 2,5-dihydroxybenzoic acid (DHB), to examine their applicability for desorption and ionization of coccidiostats using MALDI-TOF MS. All three matrixes produced good quality spectra for coccidiostats (Figure 2). Laser strength used for desorption and ionization of coccidiostats was matrix-related. 3-AQ required higher laser strength than THAP and DHB. Laser strength was selected based on good signal-to-noise ratio and the best resolution of analytes of interest (Wang et al., 1999). In all experiments, isotopic resolution was achieved for all matrixes. Compared to 3-AQ, THAP and DHB are good matrixes for coccidiostats since they produced good quality spectra with a relatively low laser strength. The response or peak intensity was another factor used to determine the best matrix. No matter which matrix was used, lasalocid exhibited only one-fifth or less the response of monensin under similar molar concentrations. Therefore, lasalocid was not the best internal standard to be selected for other coccidiostats in MALDI-TOF MS quantification. When THAP was used as matrix (Figure 2A), salinomycin and narasin showed one-third the response of monensin. In 3-AQ (Figure 2B) or DHB (Figure 2C), monensin, salinomycin, and narasin displayed similar responses in MALDI-TOF MS. Since DHB gave reasonable peak intensities for mon-

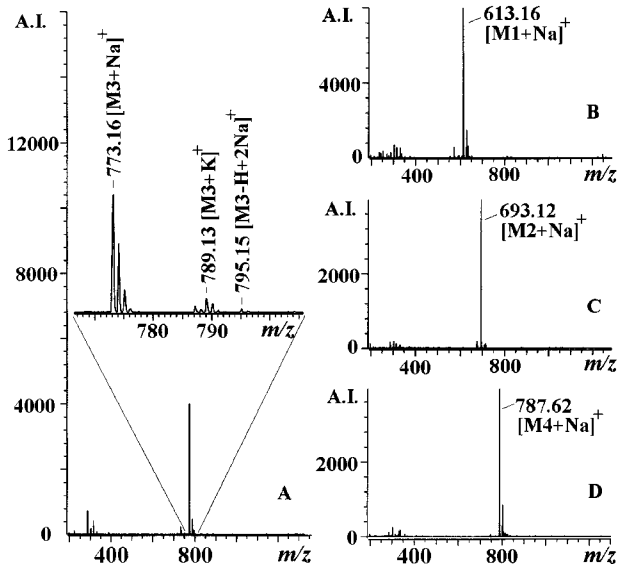

Figure 3. Ionization of coccidiostat standards in MALDI-TOF MS positive mode with DHB as matrix: A, salinomycin (M3) (3.20×10^{-5} M); B, lasalocid (M1) (1.34×10^{-4} M); C, monensin (M2) (2.87×10^{-5} M); D, narasin (M4) (3.20×10^{-5} M). Detailed ionization for salinomycin is labeled in spectrum A. For lasalocid, monensin, and narasin, only sodium adduct peaks are labeled.

ensin, salinomycin, and narasin under lower laser strength with good spot-to-spot repeatability and high sensitivity, it was selected as the best matrix for coccidiostats. All further research was carried out using DHB. Monensin, salinomycin, or narasin could serve as internal standards for each other using DHB.

Ionization and Linear Responses. In MALDI-TOF MS, salinomycin (Figure 3A), lasalocid (Figure 3B), monensin (Figure 3C), and narasin (Figure 3D) were predominately ionized as $[M + Na]^+$ ions, with additional small amounts of $[M + K]^+$ and $[M - H + 2Na]^+$ ions (Figure 3A). When an excess of sodium (0.01 M NaCl in DHB matrix solution) was applied to the preparation of MALDI-TOF MS samples, only sodium adduct ions, $[M + Na]^+$ and $[M - H + 2Na]^+$, were observed. For quantification, peak heights of $[M + Na]^+$ and $[M - H + 2Na]^+$ were summed if $[M - H + 2Na]^+$ was observed in a spectrum. There were no observed fragmentation or fragment ions for coccidiostats in MALDI-TOF MS. However, in HPLC electrospray MS, they do fragment; for example, salinomycin formed fragment ions at *m/z* 225 and 373 (Harris et al., 1998).

Coccidiostats, for example, monensin or narasin, contain molecular variations known as coccidiostat factors. Some minor factors are often with 14 mass lower or higher, occasionally 2 mass lower, than the major coccidiostats (Coleman et al., 1994; Rodewald et al., 1994). MALDI-TOF MS could easily detect all factors when the concentration of the total coccidiostats was high enough, for example, in the order of $\times 10^{-5}$ M. However, as expected, peak intensities from these minor factors were very low and 5% or in most cases much less than the peak height of the major coccidiostats. In this paper, MALDI-TOF MS quantification of coccidiostats focused on the major coccidiostats. For example, for narasin, only narasin factor A was determined, and for monensin, only monensin factor A was studied. These minor factors account for the slightly reduced purity of standards noted in the Materials and Methods section.

Since monensin, salinomycin, and narasin showed similar responses (Figure 2C), and the sample to be analyzed contained salinomycin or narasin, monensin was selected as an internal standard for quantification. Generally, peak intensity (total of all ions) or response of coccidiostats in MALDI-TOF MS was linearly correlated to their molar ratios in a MALDI-TOF MS sample. The linearity was separately determined for salinomycin (slope = 0.82, $R^2 = 0.98$) and narasin (slope = 1.15, $R^2 = 0.97$) in a range from 3.20×10^{-7} M (0.24 μg/mL) to 3.20×10^{-6} M (2.40 μg/mL) using monensin as an internal standard (2.87×10^{-6} M). The slopes were used as response factors to quantify salinomycin or narasin in poultry feeds and spiked samples.

Stability of Coccidiostats in MALDI-TOF MS Samples. Among the four coccidiostats, salinomycin and narasin tended to degrade in aqueous solutions. Davis et al. (1999) indicated that some coccidiostats, particularly salinomycin and narasin, were unstable in water/methanol (98:2) solution. However, no significant degradation was observed in pure acetonitrile or methanol for these compounds. When analyzed using MALDI-TOF MS, we found the preparation method of matrix and sample could result in the degradation of salinomycin and narasin. This might have occurred because the mixture of matrix (DHB) solution and coccidiostats was acidic, which could have caused salinomycin and narasin to dissociate. The stability of salinomycin and narasin was studied in two ways to determine a method to prepare stable MALDI-TOF MS coccidiostat samples for quantification. The first experiment was performed by applying the mixture to the probe at 0-, 30-, 60-, and 120-min intervals after coccidiostats had been mixed with matrix solution before acquiring MALDI-TOF MS data. The second experiment involved applying the mixture to the probe immediately after the coccidiostats had been mixed with matrix solution, followed by acquiring MALDI-TOF MS data at 0-, 30-, 60-, and 120-min intervals. (Note that it took 5 min for the mixture to dry on the probe.) Figure 4 shows the spectra of the four coccidiostats under these conditions. Peak intensities of lasalocid and monensin (Figure 4) remained constant, which indicated they were stable under either condition. However, peak intensities of salinomycin and narasin (Figure 4A) decreased significantly at each interval under the condition of the first experiment. An attempt was made to study details of the degradation since several peaks at masses between 490 and 590 were generated as the peak intensities of salinomycin and narasin decreased (spectra not shown). However, the degradation of salinomycin and narasin was complex and peaks between *m/z* 490 and 590 tended to degrade further with increased time. Nevertheless, if salinomycin and narasin were dried on the probe immediately after mixing with matrix, the peak intensities (Figure 4B) stayed constant. All the linear responses and quantifications of coccidiostats in this study were carried out in this manner with 0.01 M NaCl in the matrix solution. It also seemed that salinomycin and narasin were slightly more stable with addition of the sodium salt (data not shown).

MALDI-TOF MS Quantification of Coccidiostats. One of the advantages of MALDI-TOF MS is its tolerance of contaminants. Crude sample extracts can be often applied to MALDI-TOF MS directly without purification, and the analytes of interest can still be

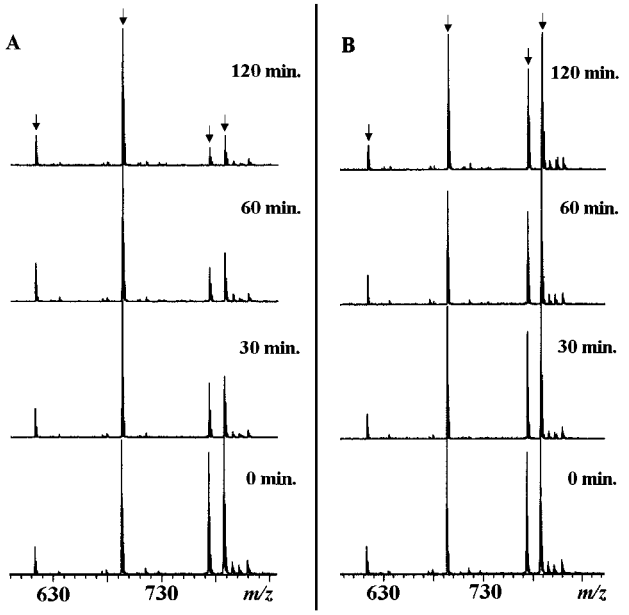

Figure 4. Stability of coccidiostats in MALDI-TOF MS samples: A, spectra of coccidiostats by applying the mixture to the probe at different intervals (0, 30, 60, 120 min) after coccidiostats had been mixed with matrix solution and followed directly by acquisition of MALDI-TOF data; B, spectra of coccidiostats applied to the probe and dried immediately after coccidiostats had been mixed with matrix solution, followed by acquisition of MALDI-TOF data at different intervals (0, 30, 60, 120 min). A mixture of lasalocid, monensin, salinomycin, and narasin was made by mixing equal amounts of stock solutions (v/v). Arrows, from left to right, indicate lasalocid, monensin, salinomycin, and narasin sodium adduct peaks.

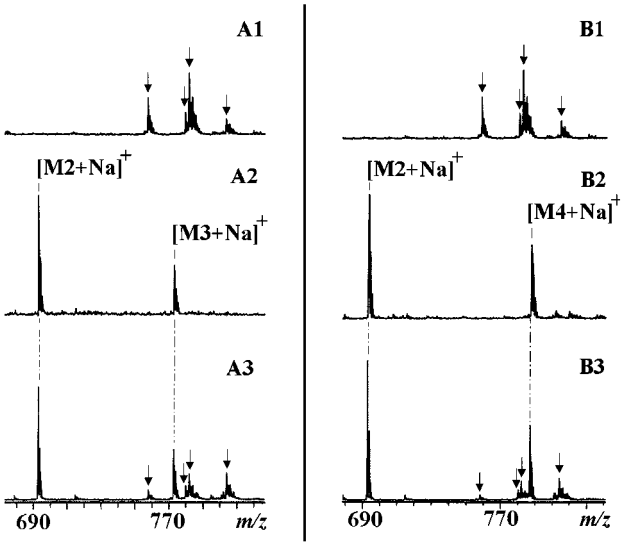

Figure 5. MALDI-TOF MS spectra of coccidiostats from poultry feeds: A1 and B1, interfering compounds eluted with chloroform/methanol (95:5); arrows, from left to right, indicate interfering compounds at *m/z* 758, 780, 782, and 804, respectively; A2 (salinomycin, M3) and B2 (narasin, M4), purified coccidiostats from poultry feed samples (using Sep-pak C18 cartridge purification procedure); M2, internal standard monensin; A3, salinomycin medicated poultry feed crude extracts; B3, narasin medicated poultry feed crude extracts.

determined (Abell and Sporns, 1996; Wang et al., 1999; Driedger and Sporns, 1999). Coccidiostats (Figure 5A3,B3) in crude extracts or acetonitrile extracts were identified. The extracts also contained a group of unidentified compounds (at *m/z* 758, 780, 782, and 804; Figure 5A1,A3, B1,B3). These compounds were close enough in mass to interfere with the detection of salinomycin (Figure 5A3) or narasin (Figure 5B3) or possibly suppress the coccidiostat molecular ions. Therefore, a simple fast protocol was developed to remove interfering compounds using a Sep-pak C18 cartridge. After crude extracts were loaded on the cartridge, interfering compounds were retained on the cartridge. Some coccidiostats passed through the cartridge and some were also retained. With elution of an additional 4 mL of methanol/water (94:6), all coccidiostats were eluted from the cartridge. Because of this selective elution, the use of other C18 cartridges could required a modified elution protocol. After the cartridge purification procedure, coccidiostats and interfering compounds were separated, and MALDI-TOF MS could generate clean spectra for coccidiostats of poultry feed extracts (Figure 5A2,B2). The interfering compounds, retained on the cartridge, were eluted with chloroform/methanol (95:5), and the spectra are shown in Figure 5A1,B1. The recovery of the purification procedure for coccidiostats was studied with standards. A 2-mL mixture of coccidiostats (monensin $= 2.1 \times 10^{-5}$ M, salinomycin $= 2.1 \times 10^{-5}$ M, and narasin $= 2.1 \times 10^{-5}$ M) was loaded on the cartridge and carried through the entire procedure. After 3 mL of methanol/water (94:6) washing, no coccidiostat standards could be detected in the eluent by MALDI-TOF MS. Because the concentration of coccidiostats in sample extracts was lower than that of the above standard mixture, it was assumed that all sample coccidiostats were recovered since the cartridge could not be overloaded.

All poultry feed samples and spiked samples were analyzed using the developed method, and the results are presented in Table 1. Monensin (1.45×10^{-5} M) was used as an internal standard and added to the extraction solvent (acetonitrile). Medicated poultry feed contained 60 μg/g salinomycin or 70 μg/g narasin, and the MALDI-TOF MS assay for salinomycin or narasin in medicated samples found 60.3 and 66.0 μg/g, respectively (Table 1). The feasibility of the method was further confirmed by spiking experiments. Blank poultry feed was spiked at two concentrations and medicated samples were spiked at one concentration of salinomycin or narasin (Table 1). Recovery for salinomycin ranged from 99% to 102%, and recovery for narasin was from 92% to 96%. All these data indicate that MALDI-TOF MS coupled with the Sep-pak C18 cartridge purification procedure can be used to accurately determine coccidiostat levels in poultry feeds.

Table 1. MALDI-TOF MS Quantification of Coccidiostats in Poultry Feeds

sample	spike level (μg/g)	assay[a] (mg/g)	RSD[b] (%)	recovery (%)
	Salinomycin Assay			
blank sample	58.7	59.1	4.1	101
	39.2	40.0	5.0	102
medicated sample[c]	0.0	60.3	3.9	101
	23.7	83.4	7.0	99
	Narasin Assay			
blank sample	56.8	52.3	2.6	92
	37.8	36.4	2.6	96
medicated sample[d]	0.0	66.0	4.3	94
	24.5	88.4	4.8	93

[a] Mean of three extracts ($n = 3$); each extract value was determined using 3 MALDI-TOF MS runs, or a total of 3×3 or 9 total MALDI-TOF MS runs. [b] Relative standard deviation of three extract values. [c] Contained salinomycin (60 μg/g). [d] Contained narasin (70 μg/g).

The MALDI-TOF MS limit of detection (S/N ≥ 3) for standard lasalocid, monensin, salinomycin, or narasin was 251 fmol (0.205 μg/mL), 22 fmol (0.020 μg/mL), 24 fmol (0.024 μg/mL), and 24 fmol (0.025 μg/mL), respectively. The low limit of detection indicates that MALDI-TOF MS is a very sensitive technique for identification of coccidiostats. The method detection limit (S/N ≥ 3) in this study for salinomycin and narasin was 2.4 or 2.4 μg/10 mL in the extraction solvent. This limit of detection was about the same level (2.5 μg/g) as that from HPLC-PCD with vanillin for poultry feeds (Rodewald et al., 1994). However, as stated earlier, the method detection limit could be 5 or 1 ng/g if coccidiostat extracts were purified and concentrated before HPLC-PCD (Moran et al., 1994, 1995) or HPLC electrospray MS analyses (Blanchflower and Kennedy, 1995, 1996). Of note, for both HPLC-PCD and HPLC electrospray MS, the concentration of working standards was in a range of about 0.10−1.0 mg/mL, which is about the same level as used in our study. Theoretically, the detection limit reported in this study could also be improved by concentrating sample extracts and/or increasing sample size in the extraction procedure.

In conclusion, MALDI-TOF MS is a simple technique to identify and quantify coccidiostats without derivatization or without HPLC separation before use of a mass spectrometer. MALDI-TOF MS can serve as a valuable rapid and sensitive technique for quantification of coccidiostats in poultry feeds with minimum sample purification. It is also likely that MALDI-TOF MS has the potential to analyze coccidiostats in other complex sample matrixes and to be used as an alternative to HPLC-PCD and HPLC electrospray mass spectrometry.

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