Stable Isotope Labeling by Carbon-13 in Bacteria Culture for the Analysis of Residual Avermectin Using Stable Isotope Dilution Liquid Chromatography Tandem Mass Spectrometry

Kodai BEPPU*, Daichi SAITO*, Yoshio MUGURUMA*, Miki TAKAHASHI*,
Shuichi HARADA**, Koichi INOUE**+

*College of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1
Nojihigashi, Kusatsu, Shiga 525-8577, Japan

** Hayashi Pure Chemical Ind., Ltd, 3-2-12, Uchihiranomachi Chuo-ku, Osaka,
540-0037, Japan

Author for correspondence: Koichi Inoue, Ph.D.
College of Pharmaceutical Sciences, Ritsumeikan University, 1-1-1 Nojihigashi, Kusatsu,
Shiga 525-8577, Japan

TEL: +81-77-561-5838 (FAX: 561-2564) E-mail address: kinoue@fc.ritsumei.ac.jp
Abstract

This study describes the development of a new stable isotope labeling by carbon-13 in bacteria culture (SILCB) for the analysis of residual antibiotics via liquid chromatography with tandem mass spectrometry (LC-MS/MS). Stable isotope dilution (SID) LC-MS/MS with QuEChERS was employed to determine avermectin, particularly avermectin B1a (AV-a) and B1b (AV-b), based on completely $^{13}$C-labeled internal standards ($^{13}$C-ISs) obtained from the SILCB. Our SILCB was developed from an optimal inorganic medium using $^{13}$C$_6$-glucose for Streptomyces avermitilis (14893 strain). A rough extract containing $^{13}$C-ISs was purified via high-speed countercurrent chromatography with a volatile two-phase solvent system composed of $n$-hexane/ethyl acetate/methanol/0.5% formic acid in water (7/3/5/5, V/V). The purified $^{13}$C-ISs were evaluated to confirm the presence of completely $^{13}$C-labeled ions with $m/z$ 938 > 326 and $m/z$ 923 > 309 for AV-a and AV-b, respectively. The QuEChERS approach with the $^{13}$C-ISs procedure achieved acceptable recovery rates in beef meat samples of 99.5% - 100.0% (RSD<2.0%, n=6). For the analysis of residual antibiotics in foodstuffs by SID-LC-MS/MS and QuEChERS, the SILCB represents a significant improvement over previous methods suffering from cumbersome sample preparation and matrix effects.

KEY WORDS: stable isotope labeling-culture, avermectin, residue assay, LC-MS/MS
Introduction

A regulation that establishes the maximum permitted content of residual antibiotics in foodstuffs is required to prevent the emergence of drug resistance and food contamination.\textsuperscript{1, 2} Currently, there is open debate in the Ministry of Health, Labour and Welfare in Japan, as well as internationally, on the maximum residue limits (MRLs) for antibiotics, which would be stipulated in a positive list system for various veterinary foods.\textsuperscript{3, 4} The continuous implementation of regulatory systems, such as MRLs, must be accompanied by the development of analytical methods for the precise evaluation of various antibiotics in foodstuffs. Moreover, due to globalization, the demand for a simple, efficient, and reliable assay of residual antibiotics is expected to increase to ensure sustainability and equitable food safety.\textsuperscript{5, 6}

The determination of antibiotics in various foodstuffs based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been previously described.\textsuperscript{2} It is worth mentioning that 93.0% of the reports on LC-MS/MS techniques for foodstuff analysis after extraction and/or purification used an electrospray ionization (ESI) source in the positive ion mode and/or negative ion mode.\textsuperscript{2} Thus, LC-MS/MS has already become a powerful tool for the qualitative and quantitative detection of various chemical residues in high-matrix food samples.\textsuperscript{7-9} However, it should be noted that the analysis of very trace amounts of antibiotics in matrix samples, such as meat, egg, honey, and milk, exhibits generally lower precision and unstable repeatability compared with the idealized values of recovery test.\textsuperscript{10} LC-MS/MS coupled with ESI will become standard analytical techniques for the detection of numerous residual chemicals in foodstuffs with high sensitivity and selectivity. However, some problems
still exist, particularly with regard to the matrix effect (ME), which can cause ionization suppression and/or enhancement.11, 12 To circumvent the ME problem, the use of optimal internal standards (ISs, usually stable-isotope labeling) is important. However, its application is sometimes limited by its cost or availability.13, 14 Thus, in this study, a method for the stable isotope dilution (SID) LC-MS/M analysis of residual antibiotics is developed. In the new method, which is referred to as stable isotope labeling by carbon-13 in bacteria culture (SILCB), complete 13C-labeling (stable-isotope labeling) of internal standards (13C-ISs) is performed as a platform for the analysis of residual antibiotics. The use of 13C-ISs by coelution in the SID-LC-MS/MS system allows a quantitative determination of antibiotics while solving the ME problem.

To use the SILCB, the present study investigated the quantitation of avermectin B1a (AV-a) and B1b (AV-b) in beef meat samples via SID-LC-MS/MS. Avermectin (AVM), which are macrocyclic lactones used as veterinary drugs (antibiotics), are derived from the soil bacterium Streptomyces avermitilis (Fig. 1). AVM consists of a mixture of AV-a (at least 80%) and AV-b (not more than 20%). The MRLs of AVM in beef meat have been established at 0.01-0.5 μg/g by the Commission Regulations in the European Union.15 In Japan, the MRLs of AVM are established for the sum of AV-a, AV-b, and a trace metabolite, 8,9-Z-avermectin B1a, and range from 0.005 to 0.5 μg/g (0.02 μg/g in beef meat).16 For the LC-MS/MS assay of very trace amount of AVM in foodstuffs, [M+NH₄]⁺ is selected as the monitoring ion in ESI-positive mode because of the unstable and low response of the [M+H]⁺ and [M+Na]⁺ adduct ions of AVM due to ionization suppression or enhancement.17 In addition, for the sample preparation of AVM from beef meat, cumbersome and time-consuming procedures, such as liquid-liquid
extraction (LLE) and solid-phase extraction (SPE), are often required. To circumvent these issues, we adopted this new SILCB using QuEChERS-based SID-LC-MS/MS for the analysis of residual AVM in beef meat samples.

**Experimental**

**Reagents and chemicals**

AVM standard was obtained from Hayashi Chemical Co., Inc (Osaka, Japan). Glucose for stable isotope labeling (U-\(^{13}\)C\(_6\), 99%) was obtained from Cambridge Isotope Laboratories, Inc. (MA, USA). Acetonitrile, methanol, \(n\)-hexane, ethyl acetate, toluene, formic acid (FA, for HPLC 99%), ammonium formate, soluble starch, sodium chloride (NaCl), ammonium sulfate ((NH\(_4\))\(_2\)SO\(_4\)), iron (II) sulfate heptahydrate (FeSO\(_4\)·7H\(_2\)O), zinc sulfate (ZnSO\(_4\)), and manganese (II) chloride tetrahydrate (MnCl\(_2\)·4H\(_2\)O) were obtained from Fujifilm Wako Chemical Co., Inc. (Osaka, Japan). Glucose, agar powder, dried yeast extract, casein peptone, dipotassium hydrogen phosphate (K\(_2\)HPO\(_4\)), calcium carbonate (CaCO\(_3\)), and magnesium sulfate heptahydrate (FeSO\(_4\)·7H\(_2\)O) were obtained from Nacalai Tesque (Osaka, Japan). The purified water used for the mobile phase and sample preparation was obtained from a PURELAB Flex 5 system (ELGA, London, UK). Beef meat samples were commercially purchased from local Japanese grocery stores in 2020–2021. A stock solution of AVM in acetonitrile was prepared and stored at \(-20^\circ\)C. It was diluted as required using distilled water/acetonitrile (12/88, \(V/V\)).

**LC-MS/MS condition and equipment for the analysis of AVM**
The UHPLC system was comprised of an ACQUITY ultra-performance liquid chromatography system (H-class; Waters, MA, USA). Reversed-phase (RP) analysis was conducted using a TSK-GEL ODS-100Z column (2.0 × 150 mm, 3.0 μm, Tosoh Co. Inc., Tokyo, Japan) at 40°C. An injection volume of 10 μL was used, and the total runtime was 10 min. The mobile phase comprising solvent A (0.5 mM ammonium formate in water) and solvent B (0.5% FA in acetonitrile) was delivered at a flow rate of 0.2 mL/min. The gradient elution settings were as follows: solvent A/B = 12/88 (0 min), 8/92 (6 min), 2/98 (6.01-7 min), and 12/88 (7.01-10 min). A Waters Xevo TQD triple quadrupole mass spectrometer was operated with an ESI source in the positive mode. The cone voltage and collision energy were set to 20 V and 25 or 30 eV, respectively. The ionization source conditions were as follows: capillary voltage 2.0 kV; extractor voltage 3 V; RF lens voltage 2.5 V; source temperature 150°C; and desolvation temperature 400°C. The cone and desolvation gas flows were 50 and 800 L/h, respectively, and were obtained using a nitrogen source (N² Supplier Model 24S, Anest Iwata Co., Yokohama, Japan).

*SILCB for the preparation of the \textsuperscript{13}C-ISs of AV-a and AV-b*

* *avermitilis* (14893 strain) was obtained from Nite Biological Resource Center (Chiba, Japan) and cultivated in an ager growth medium consisting of 4 g/L soluble starch, 20 g/L agar powder, 1 g/L K₂HPO₄, 1 g/L MgSO₄·7H₂O, 1 g/L NaCl, 2 g/L (NH₄)₂SO₄, 2 g/L CaCO₃, 0.001 g/L FeSO₄·7H₂O, 0.001 g/L MnCl₂·4H₂O, and 0.001 g/L ZnSO₄·7H₂O at room temperature. After 7 days, *avermitilis* in the medium was scraped off with a platinum ear in aseptic condition and suspended in 6 mL of liquid biosynthetic medium consisting of 3.3 g/L
U$_{\text{13}C}^\text{6}$, 1 g/L K$_2$HPO$_4$, 1 g/L MgSO$_4$·7H$_2$O, 2 g/L (NH$_4$)$_2$SO$_4$, 2 g/L CaCO$_3$, 0.001 g/L FeSO$_4$·7H$_2$O, 0.001 g/L MnCl$_2$·4H$_2$O, and 0.001 g/L ZnSO$_4$·7H$_2$O. These separated cultures were incubated in a shaking incubator in the dark at 200 rpm and 30°C for 6 days.

After incubation of the culture tube, the cultivation fluid was added to a centrifuge tube containing 6 mL of ethyl acetate/toluene and zirconia balls. The mixture was shaken using a Geno/Grinder (SPEX Co., Inc., Kobe, Japan) for 10 min and then centrifuged at 3000 rpm for 10 min using a Hitachi Himac CF15RN (Tokyo, Japan) centrifuge. The organic layer was separated and transferred to a flask. This procedure was performed three times. The extraction solution was evaporated to dryness at 45°C and redissolved in 1 mL of water/acetonitrile (12/88, V/V).

Purification of the $^{13}$C-ISs of AV-a and AV-b

The $^{13}$C-ISs of AV-a and AV-b were purified via high-speed countercurrent chromatography (HSCCC) with a volatile two-phase solvent system composed of $n$-hexane/ethyl acetate/methanol/0.5% FA in water (7/3/5/5, V/V).$^{19}$ HSCCC was performed using an Easy-Prep CCC (multilayer coil planet centrifuge, Kutsuwa Co., Ltd., Hiroshima, Japan) with an orbital radius of 7.6 cm that produces a synchronous type-J planetary motion with a maximum speed of 1500 rpm. This centrifuge was equipped with three column holders and three multilayer coiled columns. Each multilayer coiled column on the holder consisted of nine coiled layers of 1.6 mm i.d. polytetrafluoroethylene tubing with a capacity of approximately 115 mL. All three columns and other tubes were connected in series to provide a total capacity of 75 mL. The fraction systems (PU 714M LC pump, UV702 detector, SC 762 system
controller and PLC 761 fraction collector) were obtained from GL Science Co. (Tokyo, Japan). Separation was performed in the RP (head-to-tail mode), with the column initially filled with the less-polar upper stationary phase. Then, the $^{13}$C-ISs were dissolved in 2.5 mL of each phase. Finally, the supernatants were loaded onto the column. The column was rotated at 1000 rpm while the mobile phase was pumped into the head of the column at a flow rate of 0.5 mL/min. The retention of the stationary phase was determined to be 84%.

**QuEChERS for the sample preparation of AV-a and AV-b from beef meat**

Beef meat sample (2.0 g) was added to acetonitrile (4 mL) containing 100 μL of $^{13}$C-ISs (mixed $^{13}$C$_{48}$-AV-a and $^{13}$C$_{47}$-AV-b), and the mixture was homogenized for 1–2 min in a centrifuge tube, followed by centrifugation at 10000 rpm for 15 min. The supernatant was transferred to another plastic tube. This procedure was performed twice. Then, 1095 mg of a sorbent for dispersive SPE (Supel™ QuE PSA/ENVI-Carb Sigma-Aldrich, Merck Co., Tokyo, Japan) was added, and this sample was shaken using a Geno/Grinder for 5 min. Then, it was centrifuged at 10000 rpm for 10 min and transferred to a flask. This extraction solution was evaporated to dryness at 45°C and dissolved in 1 mL of water/acetonitrile (12/88, V/V).

**Results and Discussion**

*LC-MS/MS analysis of AVM*

The ionization and chromatographic conditions for the LC-MS/MS analysis of AVM were optimized according to a previous report. To analyze AVM using the multiple reaction monitoring (MRM) mode, the full scan and product ion spectra of AV-a and AV-b were
investigated under the LC conditions, which are described in the experimental section. These analytes could be detected under ESI conditions using 0.1% FA in a mobile phase of water/acetonitrile. On the basis of previously reported results, the [M+Na]+ adduct ions of AV-a and AV-b were most likely unstable and yielded low responses. Previous reports suggested that [M+NH₄]+ adduct ions were useful precursor ions to detect AV-a and AV-b in an ESI-positive mode in a mobile phase consisting of solvent A (0.5 mM ammonium formate in water) and solvent B (0.5% FA in acetonitrile). Therefore, we decided to monitor AV-a and AV-b by RP mode using TSK-GEL 100Z in the gradient mode and to determine the limit of detection (S/N=3, LOD = 0.15 and 1.2 ng/mL based on the AVM standard solution) and limit of quantification (S/N=10, LOQ = 0.3 and 2.4 ng/mL based on the AVM standard solution) values and calibration curves (in a range of the LOQ value to 1.5 μg/mL, r² > 0.999) for the developed LC-MS/MS assay. The obtained optimal conditions for LC-MS/MS are presented in Table 1. In addition, detailed results, as well as the MS/MS spectra, MRM chromatograms, and calibration curves, are presented in Fig. S1. The optimal LC-MS/MS condition and validation were applied to the analysis of AVM.

**Optimization of the SILCB for obtaining 
^{13}C-ISs of AV-a and AV-b**

In general, AVM can be obtained from cultured microorganisms, such as *S. avermitilis*. Thus, a high-yielding mutant of AVM has been extensively studied under various conditions. In this study, we investigated the use of 
^{13}C-ISs, which was obtained from stable isotope labeled *S. avermitilis*, in the SID-LC-MS/MS assay. Basu et al. used a stable isotope labeling by amino acids in cell culture (SILAC) for SID-LC-MS/MS assay. This stable isotope
labeling method with amino acids using deuterium (²H) and/or nitrogen-14 (¹⁴N) have been employed for MS techniques, such as proteomics and metabolomics.²⁷,²⁸ Moreover, the SILAC method, generally using ²H, circumvents the ME problem. In efficient LC separations, ²H-ISs and their analytes are have different retention times. For instance, Berg and Strand studied the use of ¹³C- and ²H-ISs for the LC-MS/MS analysis of amphetamine and methamphetamine as drugs.²⁹ The results revealed that ¹³C-ISs were coeluted with their analytes under different chromatographic conditions, whereas ²H-ISs were slightly separated from their analytes.²⁹ As discussed by these authors, unfortunately, there are few commercially available ¹³C-ISs. Therefore, the development of a synthetic method to increase the supply of ¹³C-ISs, particularly for antibiotics, could be the solution to the ME problem in the LC-MS/MS analysis of drugs in biological samples. In this context, a completely ¹³C-labeled glucose, namely U-¹³C₆, is already well established and applied.³⁰,³¹ In this study, we investigated the optimal minimum media using glucose for S. avermitilis (Fig. 2). The result revealed that the response of extracted AVM in LC-MS/MS using condition A was higher than that of various inorganic matters. In condition A, the proliferation of S. avermitilis was clearly observed (Fig. S2). Thus, we then exchanged glucose with U-¹³C₆ in condition A and analyzed the extracted AVM via LC-MS/MS. The MS spectra of completely ¹³C-labeled AV-a (¹³C₄₈; m/z 938) and AV-b (¹³C₄₇; m/z 923) were recorded, and the precursor ions of non-labeled AV-a (m/z 890) and AV-b (m/z 876) were not observed (Fig. 3). Meanwhile, the production of completely ¹³C-labeled AV-a and AV-b from S. avermitilis yielded heterogeneous mixtures in a rough-extract, and the ¹³C-analogs required purification to be used for the SID-LC-MS/MS assay. Thus, HSCCC was employed to completely purify ¹³C-labeled ¹³C₄₈-AV-a and ¹³C₄₇-AV-b from the rough extract. On the basis
of previous reports, the optimal K value of non-labeled AV-a and AV-b was evaluated using a two-phase solvent system, which should satisfy the following requirements: (1) the settling time of the two-phase solvent system with samples should be less than 30 s, (2) the K value of the targeted compound should be close to 1.0, (3) the separation factor should be greater than 1.5, (4) the volumes for each phase in the two-phase solvent system should be similar, and (5) the two-phase solvent system should be volatile. We utilized n-hexane/ethyl acetate/methanol/0.5% FA in water (7/3/5/5, V/V) to satisfy conditions of (1), (4), and (5). Using this two-phase solvent system, the retention of the stationary phase in the HSCCC column was about 84%. The total separation time was about 6.6 h, and the total elution volume was 600 mL for the HSCCC system for the purification of completely $^{13}$C-labeled $^{13}$C$_{48}$-AV-a and $^{13}$C$_{47}$-AV-b (Fig. S3). The MRM chromatograms of completely $^{13}$C-labeled $^{13}$C$_{48}$-AV-a and $^{13}$C$_{47}$-AV-b were presented in Fig. 4. These chemicals (about 3.8 μg) were obtained from 540 mL (6 mL × 90 tubes).

Analysis of residual AVM in beef meat samples via QuEChERS-based SID-LC-MS/MS

For the analysis of residual AVM from animal foodstuffs via LC-MS/MS, various step are performed prior to quantification to minimize the ME and increase selectivity, including LLE and/or SPE, to clean up the extracts and preconcentrate the AVM. Our previous protocol for the analysis of AVM in beef meat samples is presented in Fig. 5-(a). Recovery rates ranging from 70.8% to 117.1% were obtained (RSD < 15%, n=10). In addition, the sample preparation took about 6 h. Therefore, we were interested in optimizing the sample preparation protocol for the analysis of AVM in matrix samples via LC-MS/MS. Recently, the QuEChERS approach
was reported for the sample preparation for the analysis of AVM in foodstuffs via LC-MS/MS which achieved a recovery rate of 93.4–111.8% (RSD < 15%, n=18). For the QuEChERS-based LC-MS/MS assay, the ionization and sample preparation protocol should be validated to achieve maximum sensitivity and selectivity of analytes for a satisfactory performance (recovery test) without ME. This is particularly important for the analysis of very trace amounts of residual antibiotics from high-matrix animal foodstuffs via LC-MS/MS, for which rough QuEChERS and absolute calibration are insufficient. Indeed, the the QuEChERS protocols were applied for the analysis of phthalate esters in capsanthin and alkaloids in tobacco samples. Thus, in this study, a QuEChERS protocol (Fig. 5-(b)) for sample preparation was investigated for an advanced validation of the method and operation time reduction. The use of the QuEChERS approach and the IS method with completely $^{13}$C-labeled AVM enabled the analysis of AVM via SID-LC-MS/MS in beef meat samples as high-matrix foodstuff. This protocol was presented in Fig. 5-(b), which demonstrates that operation time was successfully reduced to 1 h. The results of the validation are presented in Table 2. Typical MRM chromatograms of AVM in beef meat samples are illustrated in Fig. 6. The proposed method quantifies the presence of AV-a and AV-b at two concentration levels (MRL and 10 × MRL, n=6) of AVM (Table 2), indicating that it can be applied in routine residue assay (99.5-100.0%, RSD<2.0%) to confirm the presence of AVM in beef meat samples.

**Conclusions**

In this study, we described the development of a very simple, useful, and reliable assay of AVM in beef meat samples via QuEChERS-based SID-LC-MS/MS. We evaluated the
suitability of complete $^{13}$C-labeling IS calibration for a precise, quantitative, and repeatable performance compared with absolute calibration. The present SILCB method prevents cumbersome sample preparation and ME in the LC-MS/MS assay of antibiotics. The applicability of the upgrade large-scale and efficient SILCB method for the residue assay of various antibiotics in foodstuffs via QuEChERS-based SID-LC-MS/MS will be the subject of future work.

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**Figure legends**

Fig. 1. Chemical structures of avermectin (AVM)

CAS: 56195-55-3/65195-56-4, Molecular Formula: C_{48}H_{72}O_{14}/C_{47}H_{70}O_{14}

Fig. 2. Investigation of condition for producing AVM by LC-MS/MS responses.

*S. avermitilis* in the medium was scraped off with a platinum ear in aseptic condition, and suspended in 6 mL of the liquid biosynthetic medium consisted of 3.3 g/L glucose, 1 g/L K_2HPO_4, 1 g/L MgSO_4·7H_2O, 1 g/L NaCl, 2 g/L (NH_4)_2SO_4, 2 g/L CaCO_3, 0.001 g/L FeSO_4·7H_2O, 0.001 g/L MnCl_2·4H_2O, and 0.001 g/L ZnSO_4·7H_2O.

Fig. 3. LC-MS/MS spectra of complete ^13^C-labeling ^13^C_{48}-AV-a and ^13^C_{47}-AV-b from *S. avermitilis*.

ESI-positive mode and full scan ranged from *m/z* 800 to 1000.

(a) Retention time for AV-a is ranged from 5.2 to 6.0 min.

(b) Retention time for AV-b is ranged from 4.2 to 5.0 min.

Fig. 4. LC-MS/MS MRM chromatograms of purified complete ^13^C-labeling AVM based on HSCCC.

(a) Purified fraction of ^13^C_{48}-AV-a (*m/z* 938>305, retention time 5.6 min) by HSCCC.

(b) Purified fraction of ^13^C_{47}-AV-b (*m/z* 923>309, retention time 4.6 min) by HSCCC.

Fig. 5. Protocols for sample preparation of AVM in beef meat samples based on LC-MS/MS
assay.

(a) Previous protocol based on LLE is expressed for LC-MS/MS.\(^{18}\)

(b) Developed protocol based on QuEChERS is expressed for SID-LC-MS/MS.

Fig. 6. Typical MRM chromatograms of AVM (MRL) in beef meat samples for recovery test.

(a) Recovery test of AVM for MRL (0.02 μg/g).

(b) Recovery test of AVM for 10×MRL (0.2 μg/g).

Monitoring ions: \(m/z\) 890>305 for AV-a, \(m/z\) 938>326 for \(^{13}\text{C}_{48}\text{-AV-a}\), \(m/z\) 876>305 for AV-b, \(m/z\) 923>309 for \(^{13}\text{C}_{47}\text{-AV-b}\).
Table 1 LC-MS/MS condition, LOD and LOQ for analysis of AVM such as AV-a and AV-b

| Analytes                  | Abbreviated name | Molecular mass (Da) | Precursor ion (m/z) | Cone voltage (V) | Product ion (m/z) | Collision energy (eV) | LOD (ng/mL) | LOQ (ng/mL) |
|---------------------------|------------------|---------------------|---------------------|------------------|-------------------|-----------------------|--------------|--------------|
| Avermectin B1a            | AV-a             | 873.07              | 890                 | 20               | 305               | 30                    | 0.15         | 0.3          |
| Completely 13C-labeled avermectin B1a | 13C$_{13}$-AV-a | 921.07              | 938                 | 20               | 326               | 25                    |              |              |
| Avermectin B1b            | AV-b             | 859.05              | 876                 | 20               | 291               | 25                    | 1.2          | 2.4          |
| Completely 13C-labeled avermectin B1b | 13C$_{13}$-AV-b | 906.05              | 923                 | 20               | 309               | 25                    |              |              |
Table 2 Recovery test for MRL levels of AVM in beef meat samples using IS, and absolute calibrations.

| Analytes | Concentration levels (μg/g) based on AVM standard | Recovery values (%) based on fully 13C-labeling IS calibration | RSD (%), n=6 | Recovery values (%) based on absolute calibration | RSD (%), n=6 |
|----------|-------------------------------------------------|-------------------------------------------------------------|------------|-------------------------------------------------|------------|
| AV-a     | 0.02 (for MRL)                                  | 99.5                                                        | 2.0        | 50.3                                            | 11.0       |
| AV-b     | 99.9                                            | 1.1                                                         | 59.7       | 7.0                                             |
| AV-a     | 99.9                                            | 0.1                                                         | 39.2       | 1.6                                             |
| AV-b     | 100.0                                           | 0.1                                                         | 21.2       | 3.5                                             |
R=C₂H₅: avermectin B1a (AV-a), C₄₈H₇₂O₁₄
R=CH₃: avermectin B1b (AV-b), C₄₇H₇₀O₁₄
Content ratio of AV-a $\geq 80\%$, AV-b $\leq 20\%$
### Minimum media for *Streptomyces avermitilis*

| Inorganic matter | Full added | A | B | C | D | E | F | G | H |
|------------------|------------|---|---|---|---|---|---|---|---|
| NaCl             | O          | - | O | O | O | O | - | - | O |
| MgSO₄·7H₂O       | O          | O | - | O | O | - | O | - | O |
| FeSO₄·7H₂O       | O          | O | O | O | - | O | O | O | - |
| MnCl₂·4H₂O       | O          | O | O | O | O | - | O | - | - |

**Fig. 2**

The figure shows the ratio of AV-a/AV-b peak responses for full added media. The graph indicates the minimum media requirements for *Streptomyces avermitilis* with different inorganic matter additions.
Fig. 3
Fig. 4

(a)  
- $m/z$ 890 > 305, $2.2 \times 10^5$
- $m/z$ 938 > 326, $2.2 \times 10^5$
- $m/z$ 876 > 305, $2.2 \times 10^5$
- $m/z$ 923 > 309, $2.2 \times 10^5$

(b)  
- $m/z$ 890 > 305, $1.1 \times 10^5$
- $m/z$ 938 > 326, $1.1 \times 10^5$
- $m/z$ 876 > 305, $1.1 \times 10^5$
- $m/z$ 923 > 309, $1.1 \times 10^5$
Fig. 5
Fig. 6

(a) 

- $m/z 890 > 305 \quad 8.7 \times 10^4$
- $m/z 938 > 326 \quad 1.8 \times 10^4$
- $m/z 876 > 305 \quad 7.6 \times 10^3$
- $m/z 923 > 309 \quad 1.5 \times 10^4$

(b) 

- $m/z 890 > 305 \quad 6.4 \times 10^5$
- $m/z 938 > 326 \quad 1.2 \times 10^4$
- $m/z 876 > 305 \quad 5.1 \times 10^4$
- $m/z 923 > 309 \quad 1.3 \times 10^4$
Bacteria Culture

SID-LC-MS/MS

QuEChERS

13C-labeled antibiotics

Detection intensity

AV-a

13C_{48}-AV-a

13C_{47}-AV-b

AV-b

Δ48

Δ47

m/z

t_R