LC-MS/MSによる鶏組織およびその加工品中の7種の抗ウイルス剤一斉分析法

誌名	食品衛生学雑誌
ISSN	00156426
著者名	朝倉,敬行 北村,真理子 安本,三穂 竹内,理貴 中里,光男 安田,和男
発行元	日本食品衛生学会
巻/号	63巻1号
巻号補足	
掲載ページ	p. 1-11
発行年月	2022年2月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

Original Paper

Simultaneous Analysis of 7 Antiviral Agents in Chicken Tissues and Processed Products by LC-MS/MS

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Since amantadine, rimantadine, arbidol, laninamvir, oseltamivir, peramivir, and zanamivir may be used as antiviral agents to treat avian influenza, we herein developed a simultaneous assay using LC-MS/MS. This method was applied to chicken products (including yakitori (grilled chicken), fried chicken, chicken steak, and boiled eggs) as well as chicken tissues (muscle, fat, the liver, gizzards, and heart) and eggs.

Samples were extracted with methanol–water (9 : 1), purified by a tandem column with an InertSep® MAX cartridge (upper part) and InertSep® MCX cartridge (lower part), and then measured by LC-MS/MS. The sample matrix had no effect on the identification of compounds. Chromatographic separation was performed on a ZIC-HILIC column using a mobile phase of 1% acetic acid solution and 1% acetic acid solution in acetonitrile, resulting in complete separation and other obstructive peaks from the sample matrices. An external solvent calibration curve was used for quantification.

The application of the method to 6 samples of chicken tissues and eggs achieved good results of between 77.9 and 97.5% for trueness and between 1.7 and 9.2% for concurrent accuracy. The method was also applied to 9 samples of processed products, including grilled chicken and fried chicken, and achieved good results with true percentages ranging between 72.6 and 99.2% and concurrent accuracies between 3.0 and 11.2%. Therefore, the developed method may also be applied to processed products.

The limit of quantification (LOQ) of the developed method was 0.01 mg/kg.

The method was then applied to 42 types of commercial processed products, including yakitori, fried chicken, steamed chicken, chicken steak, and boiled eggs, and no antiviral agents were detected.

Collectively, the present results confirmed that the method developed herein is applicable to not only chicken tissues, but also their processed products.

(Received June 6, 2021 Accepted November 18, 2021)

Key words: antiviral agent; chicken tissue; chicken product; LC-MS/MS; Highly Pathogenic Avian Influenza

Introduction

The "Act on Domestic Animal Infectious Diseases Control" designated 28 diseases, such as foot-and-mouth disease, classical swine fever, and highly pathogenic avian influenza, in Japan. Among these diseases, highly pathogenic avian influenza (hereinafter referred to as "avian influenza") has become a major threat to human health both socially and in terms of food hygiene. In 1997, an outbreak of avian influenza occurred in Hong Kong, during which human infection was confirmed for the first time, and the disease was recognized as one of the zoonoses. Avian influenza then spread primarily in Asia and became a major social issue as a new type of influenza. In 2004, avian influenza was also detected in Japan.

Avian influenza is dealt with in Japan in accordance with the "Specific Livestock Infectious Disease Control Guidelines for Highly Pathogenic Avian Influenza and Low Pathogenicity Avian Influenza"[*1]. These guidelines state that slaughtering and incineration are to be performed along with shipping restrictions on poultry from source farms. Vaccines and various antiviral agents may be used overseas to prevent and treat the disease; however, they are prohibited in Japan because of concerns

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[*1] Announcement by the Minister of Agriculture, Forestry and Fisheries "Specific Domestic Animal Infectious Disease Quarantine Guidelines for Highly Pathogenic Avian Influenza and Low Pathogenic Avian Influenza" (July 1st, 2020) https://www.maff.go.jp/j/syouan/douei/katiku_yobo/k_bousi/attach/pdf/index-30.pdf

regarding the emergence of drug-resistant viruses due to the use of anti-influenza virus agents for humans in animals (livestock). Some viruses have already acquired resistance to amantadine[1].

Although vaccines have been used to treat avian influenza in China[2,3], infection by the virus was not completely avoided, only its onset was suppressed[4]. Anti-influenza virus agents may also be used as preventive agents even before the onset of symptoms, and there are cases in which antiviral agents, which are considered to have been actually used as preventive agents in Chinese poultry farms, were detected in chicken meat[5,6]. In Japan, an anti-influenza virus agent for humans was administered to chickens and therapeutic effects were observed[7].

Analyses of antiviral agents have so far focused on the *in vivo* behaviors of drugs, mostly using human and animal sera[8,9], plasma[10)–14], urine[10,12,13,15], saliva[13], and the liver[16] as samples. In the case of analyses of foods, an analytical method using LC-MS/MS was reported by Chan et al.[17] and Tsuruoka et al.[18]; however, the number of antiviral agents selected as analytes was very small, namely, only one or a few items. Berendsen et al.[19] developed an analytical method for multiple antiviral agents using LC-MS/MS, but the method also included antiviral agents other than anti-influenza agents. Furthermore, the study subject was chicken muscle only and a few of the agents tested targeted internal organs or eggs used for fried chicken or yakitori (grilled chicken), which are chicken products.

Therefore, we herein developed an LC-MS/MS method for the analysis of seven antiviral agents: amantadine which has been detected in chicken muscle[5], oseltamivir and zanamivir, which are widely used to treat human influenza, recently developed peramivir and laninamivir, and rimantadine and arbidol, which are used in other countries. We also applied this method to processed chicken products, which are consumed in large quantities in Japan, including fried chicken, boiled eggs, and yakitori containing chicken organs.

Materials and Methods

1. Samples

Chicken tissues (muscle, fat, the liver, gizzards, heart, and eggs) and chicken products (including yakitori, steamed chicken, fried chicken, and chicken soboro (ground chicken)) were purchased from a market in Tokyo, Japan. Chicken tissues and eggs were minced in a food processor. Processed products were also minced with a food processor after the removal of batter and sauces. These samples were stored at −20℃ until analyzed.

2. Regents

Standard solutions: amantadine hydrochloride (purity: 82.5%) was purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan), oseltamivir (<98.0%), arbidol (<98.0%), zanamivir (<98.0%), 1-peramivir (<98.0%), and laninamivir (<98.0%) from Toronto Research Chemicals, Inc. (Toronto, Canada), and 1-(1-adamantyl) ethylamine hydrochloride (rimantadine hydrochloride) (<98.0%) from Sigma-Aldrich (St. Louis, MO, USA).

The structural formula of each of the antiviral agents tested is shown in Fig. 1.

Stock standard solutions (100 mg/L) of amantadine, rimantadine, arbidol, oseltamivir, and peramivir were prepared by dissolving 10 mg of each powder in 100 mL of methanol. A stock standard solution (100 mg/L) of zanamivir was prepared by dissolving 10 mg of powder in 100 mL of water. A stock standard solution (100 mg/L) of laninamivir was prepared by dissolving 10 mg of powder in 100 mL of methanol–water (1 : 1).
Stock solutions were stored in the dark at 4℃.

Mixed standard solution: a mixed standard solution (10 mg/L) was prepared by transferring 10 mL each of the stock standard solutions into a 100-mL volumetric flask and diluting with methanol.

Solid-phase extraction columns: InertSep® MCX (500 mg/6 mL) and InertSep® MAX (500 mg/6 mL) were purchased from GL Sciences Inc. (Tokyo, Japan).

Other reagents:
Methanol and acetonitrile of LC/MS grade were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), Methanol of HPLC grade, acetic acid of HPLC grade, and ammonia solution (25%) were purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Reagents for sample pretreatment and mobile phase ultrapure water were obtained using Milli-Q Integral (Merck KGaA, DA, Germany).

3. Apparatus

The polytron homogenizer model Multi Disperser PB-95 by SMT Co., Ltd. (Osaka, Japan), the refrigerated centrifuge model 5930 by Kubota Corporation (Tokyo, Japan), the rotary evaporator model N-1110 by Tokyo Rikakikai Co., Ltd. (Tokyo, Japan), the high-performance liquid chromatograph model LC-20 by Shimadzu Corporation, and the mass spectrometer model API-5500QTRAP by Sciex Pte. Ltd. were used.

4. Measurement conditions

Analyses were performed using LC-MS/MS instrumentation consisting of a QTRAP 5500 system (Sciex, MA, USA) equipped with a Prominence 20A series LC system (Shimadzu Corp.). The analytical column selected was ZIC-HILIC (2.1 mm i.d. ×150 mm, 3.5 m) from Phenomenex Co., Inc. (CA, USA). The mobile phase flow rate was set to 0.2 mL/min. Mobile phases A and B were 1% acetic acid solution and 1% acetic acid solution in acetonitrile, respectively.

A mixture of mobile phase solutions A and B at a ratio of 80 : 20 was initially flowed for 5 min, and the concentration of acetonitrile was then increased to 50% over 10 min and maintained at this level for 10 min. The mobile phase was immediately changed back to the initial conditions and then re-equilibrated for 5 min. The total run time for the analysis was 30 min. The injection vol-

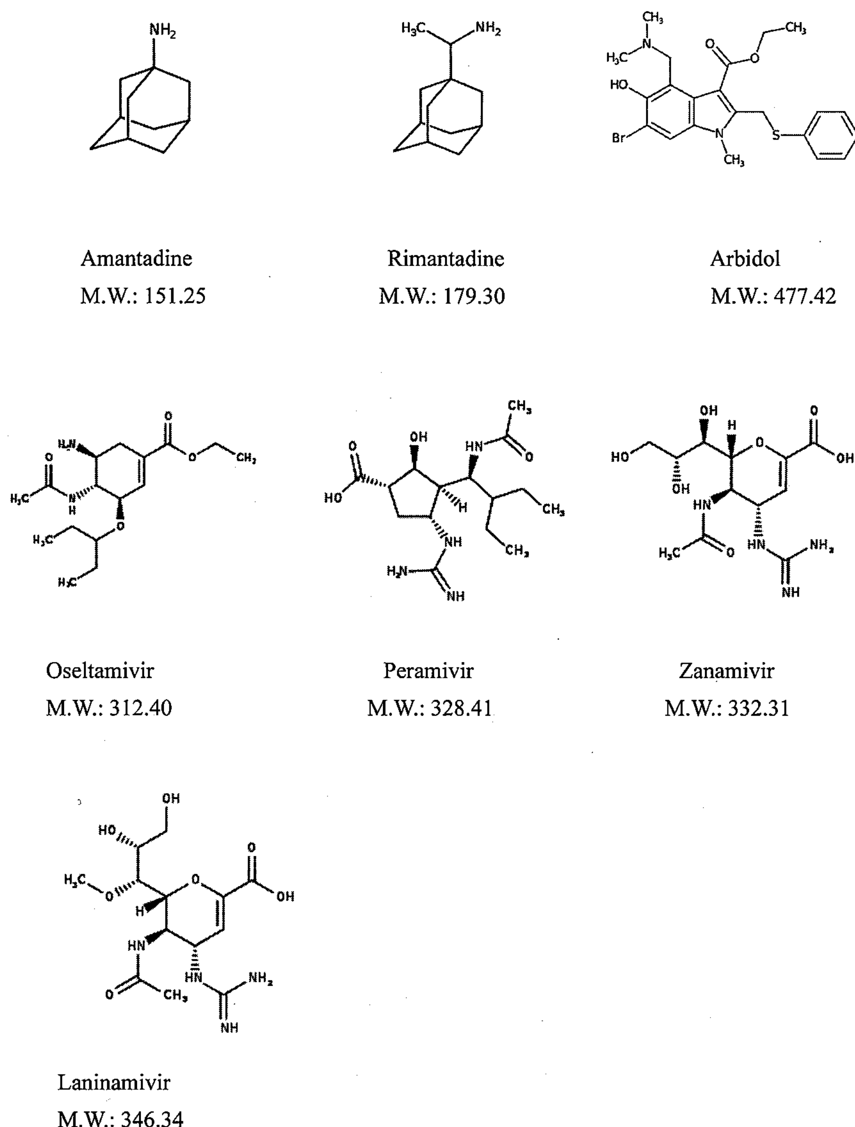


Fig. 1. Structures of analytes

ume was 10 L. The mass spectrometer was operated in the positive polarity selected reaction monitoring (SRM) mode with an electrospray ionization (ESI) interface. The operational conditions of LC-MS/MS were as follows: ion spray voltage: 5,500 V; curtain gas (N_2) pressure: 50 psi; collision gas pressure: 8 psi; temperature: 400℃; ion source gas 1 pressure: 50 psi; ion source gas 2 pressure: 50 psi.

5. Calibration standards

Standard solutions for calibration curves (0.1, 0.5, 1, 5, and 10 ng/mL) were prepared by diluting working standard solutions with acetonitrile–methanol–1% acetic acid solution (8 : 1 : 1).

6. Preparation of Test Solutions

6.1 Extraction

Ten grams of a sample was weighed in a 100-mL polypropylene centrifuge tube. After the addition of 50 mL methanol–water (9 : 1), the sample was homogenized for 1 min, centrifuged at 1,880×g at 4℃ for 10 min, and the resulting supernatant was transferred to a 200-mL volumetric flask.

The residue was homogenized with 50 mL of methanol–water (9 : 1) and centrifuged at 1,880×g for 10 min. The collected supernatant was combined with the previous supernatant, and methanol–water (9 : 1) was then added to a total volume of exactly 200 mL and used as the extraction solution.

6.2 Purification

InertSep® MAX (500 mg/6 mL) and InertSep® MCX (500 mg/6 mL) cartridges were each preconditioned with 10 mL of methanol. Two cartridge columns were connected with the InertSep® MAX cartridge on top (tandem column).

Ten milliliters of extraction solution obtained in 6.1 was loaded on the tandem column. Twenty milliliters of methanol was flowed through. The target compounds were passed through the Inertsep® MAX cartridge and retained on the Inertsep® MCX cartridge. The Inertsep® MAX cartridge was removed. The remaining InertSep® MCX cartridge was washed with 10 mL each of metha-

nol containing 1% formic acid, water, and methanol. Antiviral agents were then eluted with 10 mL each of 25% ammonia solution–methanol (1 : 19) and 25% ammonia solution–methanol (1 : 9). These eluates were collected into a flask. The eluate was evaporated. The residue was redissolved in 5.0 mL of acetonitrile–methanol–1% acetic acid solution (8 : 1 : 1) and used as the test solution.

Results and Discussion

1. Analysis Conditions

1.1 MS Conditions

MS conditions for analyzing each antiviral agent by LC-MS/MS were considered with reference to previous studies[20)–22)]. MS was performed in the positive ionization mode using ESI. Infusion measurements were performed in order to select the measurement mode, and proton adduct molecules were then strongly detected in all antiviral agents. Analysis conditions in the SRM mode were investigated using each proton adduct molecule as a precursor ion. Product ions were detected for each of the precursor ions by collision-induced dissociation. Table 1 shows the optimized conditions for each monitoring ion.

Table 1. SRM parameters of analytes

	precursor *m/z*	product *m/z*	DP※1 (V)	CE※2 (eV)	CXP※3 (V)
Amantadine	152.5	134.9[a)]	96	18	6
	152.5	79.0[b)]	96	39	12
Rimantadine	180.1	163.1[a)]	96	23	14
	180.1	120.1[b)]	96	37	14
Arbidol	479.0	434.0[a)]	66	23	20
	479.0	280.9[b)]	66	45	14
Laninamivir	347.1	60.1[a)]	136	21	8
	347.1	121.0[b)]	136	35	28
Oseltamivir	313.1	166.1[a)]	96	23	14
	313.1	120.1[b)]	96	37	12
Peramivir	329.2	270.1[a)]	81	25	14
	329.2	59.1[b)]	81	40	14
Zanamivir	333.1	60.0[a)]	126	21	8
	333.1	121.0[b)]	126	37	16

※1 Declustering potential ※2 Collision energy ※3 Collision cell exit potential

a) Used for a quantitative analysis b) Used for a qualitative analysis

1.2 LC Conditions

Reverse-phase columns (ODS) have frequently been used as LC columns in analyses of antiviral agents[20)–23)]. Therefore, we examined several common ODS columns. However, their retention times varied from quick to slow, and the peak shapes of some compounds were not ideal due to peak tailing. Therefore, difficulties were associated with simultaneously analyzing the seven different antiviral drugs using ODS-based columns. Since the target antiviral agents were basic or amphoteric substances, the use of sulfone-modified cation exchange columns was considered for the analysis. The range of retention times for cation exchange columns is generally narrower than that of ODS columns; however, the peak shapes of laninamivir and zanamivir were poor.

Therefore, we selected Hydrophilic Interaction Chromatography (HILIC) columns with hydrophilic interactions, which have come into use recently for the analysis of polar substances. Acetonitrile–water, which is widely used in HILIC analyses, was used in the mobile phase. Since the elution behavior of HILIC columns differs depending on the type of stationary phase, we examined the detection results of each antiviral agent in accordance with the operational conditions shown above us-

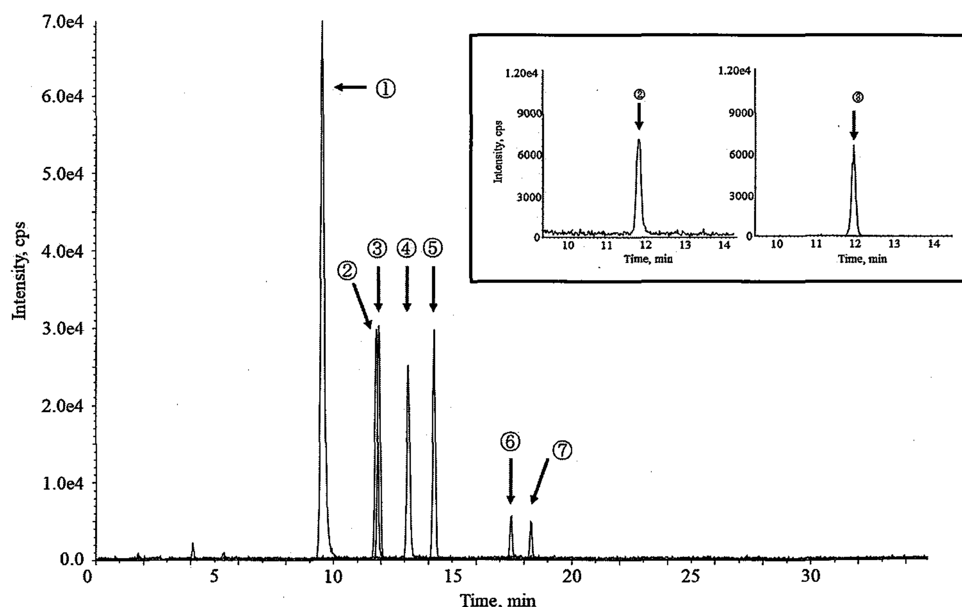


Fig. 2. TIC chromatogram of antiviral agent standard solutions (concentration: 0.005 mg/L)

① Arbidol, ② Rimantadine, ③ Oseltamivir, ④ Amantadine, ⑤ Peramivir, ⑥ Laninamivir, ⑦ Zanamivir. Insets show expanded views of the rimantadine (②) and oseltamivir (③) peaks (concentration: 0.001 mg/L).

ing HILIC columns, such as Inertsil Amide, Inertsil HILIC (2.1 mm i.d. ×150 mm, 3 m, GL Science Inc., Tokyo, Japan), Triart Diol-HILIC (2.1 mm i.d. ×150 mm, 3 m, YMC Co., Ltd., Kyoto, Japan), PC HILIC (2.0 mm i.d. ×150 mm, 3 m, Osaka Soda, Osaka, Japan), and ZIC-HILIC (2.1 mm i.d. ×150 mm, 3 m, Merck KGaA, DA, Germany). The results obtained showed optimal separation, peak shapes, and MS sensitivity with ZIC-HILIC.

Regarding the solvent system for the mobile phase, acetonitrile and methanol were tested. Various tests were conducted using formic acid, acetic acid, and formic acid ammonium, which are considered to be optimal for promoting the ionization of seven types of antiviral agents, and are widely used as mobile phase additives in LC-MS/MS analyses. Acetic acid was selected as the additive because it showed the best elution characteristics. We then examined its concentration based on comparisons of combinations of acetic acid at three different concentrations (0.5, 1, and 2%) and organic solvents i.e., acetic acid-containing acetonitrile and methanol at the same concentration. The results obtained revealed the best peak shape and sensitivity when gradient elution was performed using 1% acetic acid solution containing acetonitrile–1% acetic acid solution as the mobile phase. Based on these results, we selected ZIC-HILIC as the analytical column and employed the conditions of this gradient analysis of 1% acetic acid solution containing acetonitrile–1% acetic acid solution for the mobile phase. Figure 2 shows the total ion chromatograms of the mixed standard solutions of the seven antiviral agents. The retention times of rimantadine and oseltamivir were approximate, and good separation in SRM was also observed. Moreover, the coefficient of determination (R^2) was better than 0.999 for all antiviral agents when the calibration curve was generated under the prepared conditions.

2. Preparation of the Test Solution

2.1 Extraction Method

In addition to acetonitrile, methanol, and their polar solvents prepared by addition of water or acid[23)–25)], McIlvaine buffer[18)] has been used to extract basic antiviral agents from foods. Although most of the antiviral agents analyzed in the present study were basic substances, peramivir, zanamivir, and laninamivir are amphoteric substances that have carboxyl groups in their molecular structures. Therefore, we investigated a solvent system that efficiently extracts antiviral agents with markedly different chemical properties. We spiked 0.1 g each of the antiviral agents into 10 g of a paste-like chicken muscle sample using methanol, acetonitrile, ethyl acetate, acetone, acetonitrile-hexane mixed solution, and methanol–acetonitrile mixed solution as extraction solvents, and the extraction rate of each sample was examined and compared.

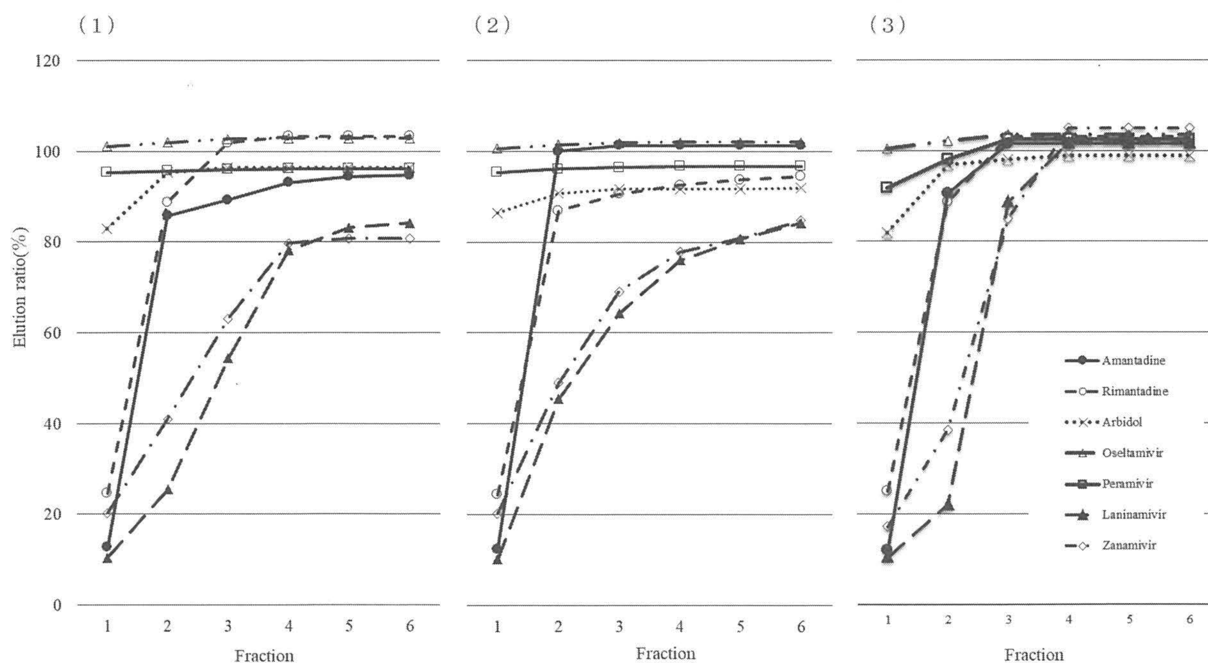


Fig. 3. Recovery of antiviral agents from each fraction of different solvents used for elution from the InertSep® MCX cartridge

Five milliliters of standard solution (0.1 mg/L in methanol) was loaded onto the InertSep® MCX cartridge and washed with 1% HCOOH–methanol, water, and methanol. It was then eluted with solvents at various ratios of 25% ammonia–methanol. Five milliliters of each fraction was collected and the recovery of each antiviral agent was assessed. The ratios of 25% ammonia–methanol used for elution are as shown in (1) to (3) below. n=3

(1) Collected six fractions of 5 mL each of 25% ammonia–methanol (1 : 19).

(2) Collected six fractions of 5 mL each of 25% ammonia–methanol (1 : 9).

(3) Collected two fractions of 5 mL each of 25% ammonia–methanol (1 : 19), and then four fractions of 5 mL each of 25% ammonia–methanol (1 : 9).

The results obtained showed a relatively good recovery rate of 80% or higher with methanol and acetone. Therefore, we investigated the extraction ratio for methanol, acetone, and mixed solutions of methanol and acetone with water, i.e., methanol–water (95 : 5), methanol–water (9 : 1), methanol–water (8 : 2), acetone–water (95 : 5), acetone–water (9 : 1), and acetone–water (8 : 2), and a good recovery rate of 85% or higher was achieved for all antiviral agents with methanol–water (9 : 1). However, since each antiviral agent has a different substituent, the effects of pH on the extraction solvent were investigated.

No significant differences were observed in recovery rates due to the effects of pH; therefore, we selected methanol–water (9 : 1) as the extraction solvent.

2.2 Purification by the Solid-phase Extraction Column

This analysis targeted not only chicken tissues, but also chicken products and eggs. However, since there was no defatting process in the extraction procedure, food-derived low polarity components, such as fat, were expected to affect the results obtained.

The purification of antiviral agents is generally performed using the reverse-phase solid-phase extraction column C18[23] or cation exchange cartridge columns in many cases in previous studies that targeted basic compounds, such as amantadine or rimantadine, as analytes[20), 25)].

We also considered the use of InertSep® C18 (500 mg/6 mL), a reverse-phase type cartridge, and OASIS® HLB (500 mg/6 mL), a polymer type cartridge, which are commonly used solid-phase extraction columns. One milliliter each of the 0.1 mg/L mixed standard solution of antiviral agents prepared with acetonitrile and methanol was loaded onto each of the columns. Antiviral agents were barely retained in any of the columns.

Since most of the antiviral agents analyzed in the present study were basic substances or amphoteric substances, the purification effect was considered to be greater when ion exchange columns were used. Therefore, we investigated the behavior of each antiviral agent using the InertSep® MCX cartridge, a strong cation-exchange SPE cartridge. Five milliliters of the 0.1 mg/L mixed standard solutions of antiviral agents prepared with methanol was loaded onto the InertSep® MCX cartridge, and all antiviral agents were retained well. To remove ionic impurities, the InertSep® MCX cartridge was washed with 10 mL each of 1% formic acid-methanol, water, and methanol, and none of the antiviral agents were detected in washing fluids; therefore, they were sufficiently retained.

We investigated elution from the InertSep® MCX cartridge. Using ammonia solution as the elution solvent, we examined the elution behavior of 5 mL each of 25%

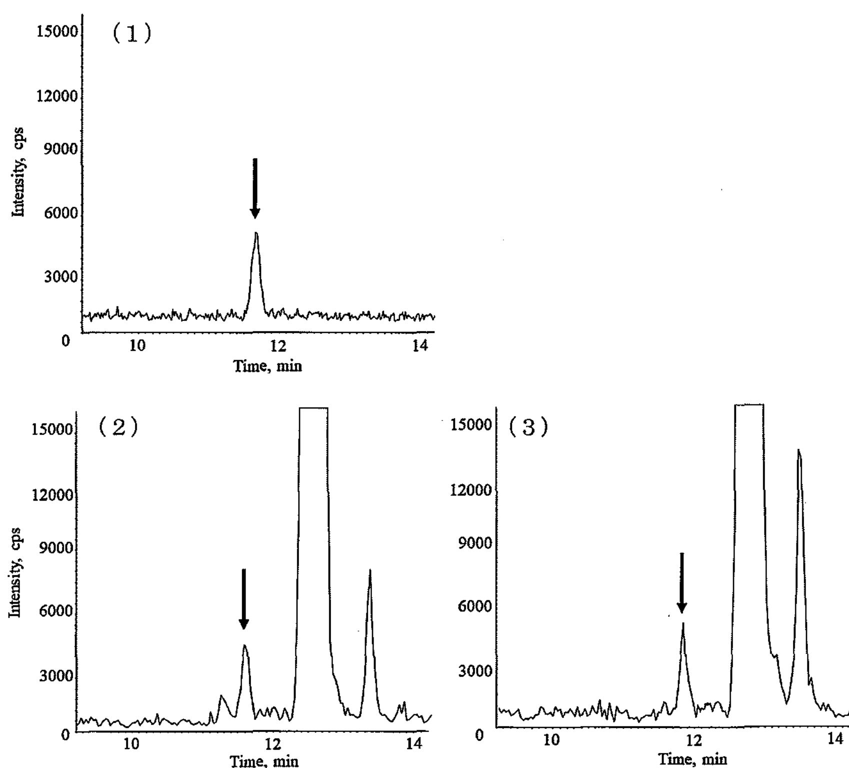


Fig. 4. Effects of purification using the tandem column on SRM chromatograms of rimantadine in the sample solution extracted from chicken fat

(1) Rimantadine standard solution (0.001 mg/L).
(2) Purification with the InertSep MCX® Cartridge.
(3) Purification using the tandem column of the InertSep MCX® cartridge and InertSep MAX® cartridge.

ammonia solution-methanol mixed solutions with various mixing ratios. As shown in Fig. 3, 25% ammonia solution–methanol (1 : 19) (Fig. 3(1)) and 25% ammonia solution–methanol (1 : 9) (Fig. 3(2)) failed to elute satisfactory amounts of laninamivir and zanamivir. Therefore, the elution behaviors of all antiviral agents were investigated with various concentrations of the 25% ammonia solution used in 25% ammonia solution-methanol. The results obtained showed that the best recovery rates of antiviral agents from the InertSep® MCX cartridge were obtained by initially eluting with 10 mL of 25% ammonia solution-methanol (1 : 19), and then with 10 mL of 25% ammonia solution–methanol (1 : 9) (Fig. 3(3)). The change in the polarity of the solvent in the column was considered to be caused by the change in elution behavior because laninamivir and zanamivir are amphoteric substances.

We attempted to apply the method to an actual sample of chicken fat. However, the matrix effect was observed for rimantadine, oseltamivir, and zanamivir because purification using the InertSep® MCX cartridge alone was found to be insufficient. To enhance the purification effect, additional measures were considered. Since analytes in the present study were basic and amphoteric substances, ionic substances may interfere with the analysis. Therefore, the InertSep® MAX cartridge, a strong ion exchange system, was selected as an additional column.

One milliliter of the 0.1 mg/L mixed standard solution prepared with methanol–water (9 : 1) was added to 10 mL of methanol–water (9 : 1). This solution was loaded onto the InertSep® MAX cartridge. Some antiviral agents were retained. A further 20 mL of methanol was flowed through and all of the antiviral agents were eluted. When the eluate from the InertSep® MAX cartridge was loaded onto the InertSep® MCX cartridge, all of the antiviral agents were retained. The same method was applied to the extraction solution from chicken fat spiked with antiviral agents at 0.1 mg/L.

The recovery rates of antiviral agents were sufficient. A yellow band remained on the InertSep® MAX cartridge.

Purification using this tandem column resulted in smaller peaks of impurities that may interfere with analyses in the chromatogram of SRM, as shown in Fig. 4. The results revealed that purification may be performed using the tandem column consisting of an InertSep® MAX cartridge (upper part) and InertSep® MCX cartridge (lower part). The effects of the matrix also improved from 0.75 to 0.95 for rimantadine. The recovery rate was 90% or higher, which was also good.

Table 2. Recovery of antiviral agents from chicken tissues and eggs

Sample	Recovery (%)※1 (Repeatability (%), Matrix effect※2)						
	Amantadine	Rimantadine	Arbidol	Laninamivir	Oseltamivir	Peramivir	Zanamivir
Muscle	94.8 (2.8, 0.94)	93.1 (5.5, 0.92)	92.3 (8.3, 0.96)	88.5 (5.5, 0.94)	97.5 (3.2, 0.99)	84.4 (3.1, 0.92)	88.1 (1.7, 0.94)
Fat	81.1 (7.7, 0.99)	80.8 (7.7, 0.98)	86.7 (6.4, 0.90)	85.1 (6.5, 1.00)	86.2 (7.8, 1.01)	89.7 (7.8, 1.04)	87.6 (6.7, 1.01)
Liver	81.8 (4.7, 0.92)	82.7 (8.7, 0.95)	83.2 (6.4, 0.91)	81.4 (5.3, 0.98)	77.9 (5.9, 0.86)	87.7 (8.7, 0.96)	83.7 (7.6, 0.90)
Gizzard	89.5 (9.2, 0.95)	78.8 (6.1, 0.87)	95.1 (9.0, 0.93)	80.4 (4.5, 0.97)	91.6 (6.9, 0.93)	86.4 (4.9, 0.92)	90.0 (3.8, 0.96)
Heart	86.9 (5.9, 0.92)	89.5 (4.3, 0.96)	91.4 (5.6, 0.99)	86.1 (6.0, 0.96)	80.6 (4.4, 0.96)	86.8 (6.8, 0.96)	79.4 (5.6, 0.84)
Egg	88.8 (3.7, 0.94)	90.9 (3.7, 0.99)	85.9 (6.8, 1.00)	83.6 (3.1, 0.90)	85.7 (5.7, 1.00)	85.2 (7.5, 0.96)	83.3 (4.7, 0.94)

Samples were spiked with 0.01 mg/kg of each antiviral agent (each compound), n=5.
※1 Mean of five replicates.
※2 The matrix effect is expressed as a ratio of the peak area of the matrix standard to the peak area of the standard (0.001 mg/L) in the solvent.

Table 3. Recovery of antiviral agents from chicken products and boiled eggs

Sample	Recovery (%)※1 (Repeatability (%), Matrix effect※2)						
	Amantadine	Rimantadine	Arbidol	Laninamivir	Oseltamivir	Peramivir	Zanamivir
Yakitori (sauce)	82.8 (7.7, 0.85)	78.0 (9.0, 0.78)	99.2 (4.9, 1.00)	88.9 (4.3, 0.88)	89.1 (8.8, 0.86)	89.5 (7.6, 0.88)	72.6 (4.8, 0.81)
Yakitori (salt)	90.3 (9.7, 0.93)	97.8 (8.3, 1.05)	98.5 (3.1, 0.98)	88.5 (5.1, 0.90)	92.4 (7.0, 0.94)	79.1 (9.4, 0.83)	86.3 (8.9, 0.94)
Fried chicken	79.5 (7.3, 0.80)	80.5 (5.1, 0.78)	84.2 (9.7, 0.83)	84.0 (8.3, 0.91)	86.3 (9.5, 0.96)	78.8 (9.1, 0.92)	76.1 (7.6, 0.84)
Steamed chicken	90.4 (8.9, 0.91)	82.9 (7.0, 0.89)	91.5 (8.4, 0.92)	90.5 (5.0, 0.95)	90.4 (6.1, 0.92)	93.5 (8.1, 0.91)	88.4 (6.2, 0.93)
Chicken cutlet	92.5 (8.9, 0.85)	88.5 (10.8, 1.02)	96.7 (4.4, 0.98)	85.3 (3.0, 0.91)	74.0 (4.8, 0.82)	87.7 (7.4, 0.92)	92.1 (3.2, 0.89)
Chicken steak	93.2 (7.9, 0.94)	91.0 (5.3, 1.00)	91.5 (3.8, 1.01)	87.0 (7.0, 0.95)	90.2 (8.2, 0.99)	91.0 (4.1, 0.95)	98.6 (8.1, 0.96)
Roasted duck	91.3 (3.8, 0.88)	95.4 (6.9, 1.04)	97.1 (5.8, 0.97)	82.3 (7.0, 0.86)	88.5 (9.3, 0.96)	97.0 (7.2, 0.99)	93.6 (5.0, 0.92)
Chicken soboro	87.2 (8.7, 0.94)	88.1 (7.5, 0.96)	89.6 (8.2, 1.00)	82.2 (7.3, 0.88)	76.6 (11.2, 0.90)	82.4 (5.6, 0.91)	87.5 (8.0, 0.91)
Boiled egg	80.6 (6.0, 0.90)	75.0 (7.8, 0.88)	85.8 (9.2, 0.91)	78.8 (4.2, 0.86)	73.3 (5.8, 0.84)	78.4 (3.1, 0.90)	75.3 (4.7, 0.88)

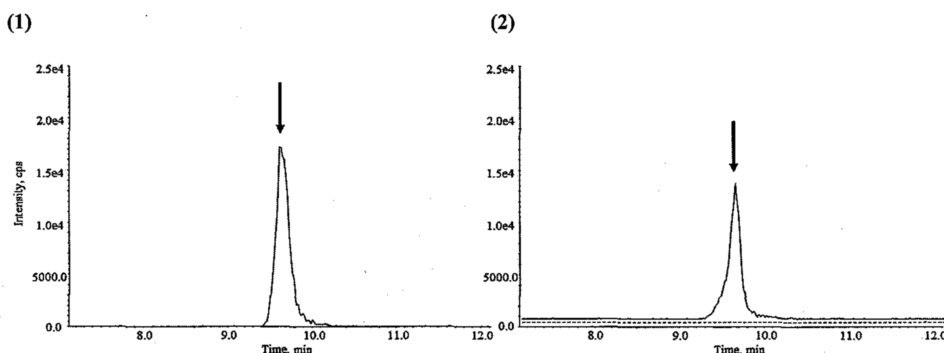
Samples were spiked with 0.01 mg/kg of each antiviral agent (each compound), n=5.
※1 Mean of five replicates.
※2 The matrix effect is expressed as a ratio of the peak area of the matrix standard to the peak area of the standard (0.001 mg/L) in the solvent.

3. Recoveries

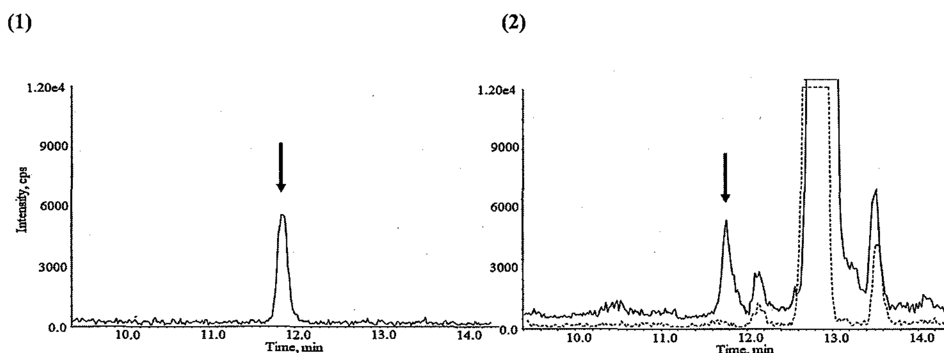
After 6 types of samples, namely, muscle, fat, the liver, heart, gizzards, and eggs, were grinded into pastes, they were spiked with the antiviral agent mixed standard solution corresponding to the default regulatory limit (0.01 mg/kg), left at room temperature for 30 min, and then subjected to extraction. The results obtained are shown in Table 2. The accuracies of the 6 samples ranged between 77.9 and 97.5% (n=5), while repeatability ranged between 1.7 and 9.2%. In addition, recovery tests were conducted on 9 samples of chicken products, such as yakitori, fried chicken, and boiled eggs. As shown in Table 3, accuracies ranged between 72.6 and 99.2%, and repeatability between 3.0 and 11.2%. Figure 5 shows representative SRM chromatograms of each of the antiviral agents and chicken muscle. No peaks that interfered with the analysis were observed in any of the samples analyzed. Although peaks were observed near the retention times of amantadine and rimantadine, they did not affect selectivity.

The limit of quantification (LOQ) for this assay was set at 0.01 mg/kg because the S/N ratio of the chromatograms of samples containing 0.01 mg/kg of each antiviral agent was fortified and ranged between 10 and 830,

①Arbidol



②Rimantadine



③Oseltamivir

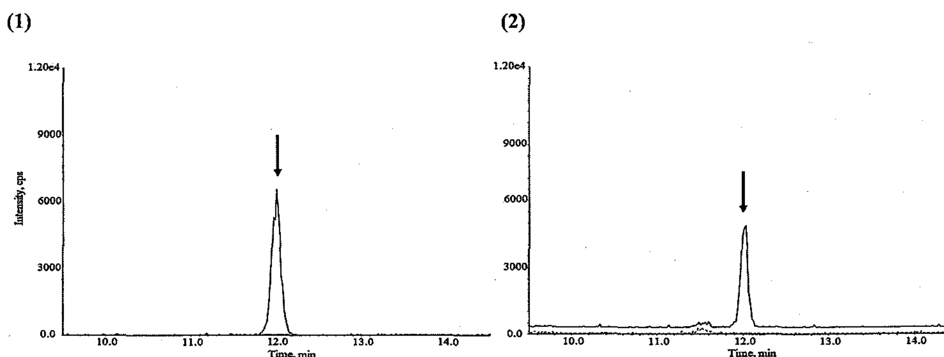
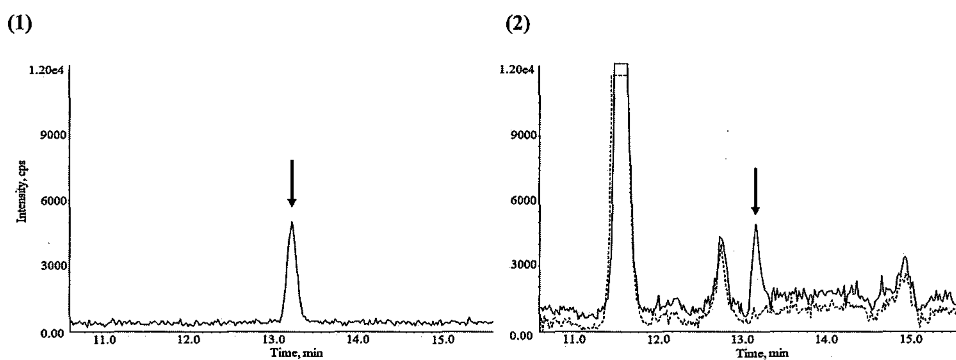


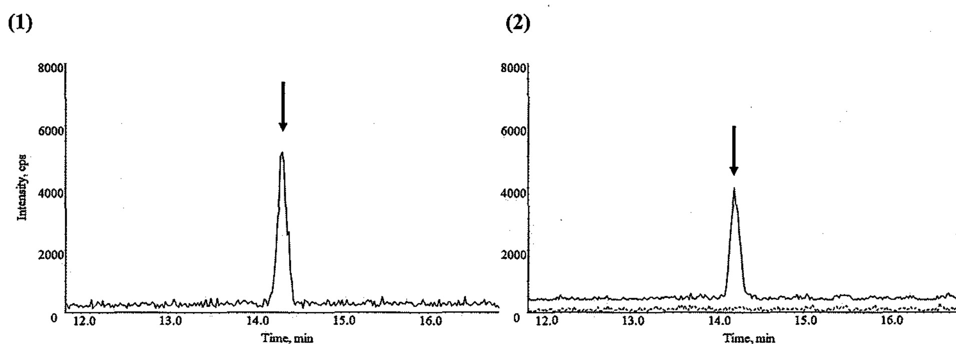
Fig. 5. SRM chromatograms of each antiviral agent

(1) each antiviral agent standard solution (0.001 mg/L).
(2) (--) : test solution prepared from a chicken muscle sample, (——): test solution prepared from a chicken muscle sample spiked with antiviral agents at a concentration of 0.01 mg/kg. ① Arbidol, ② Rimantadine, ③ Oseltamivir, ④ Amantadine, ⑤ Peramivir, ⑥ Laninamivir, ⑦ Zanamivir

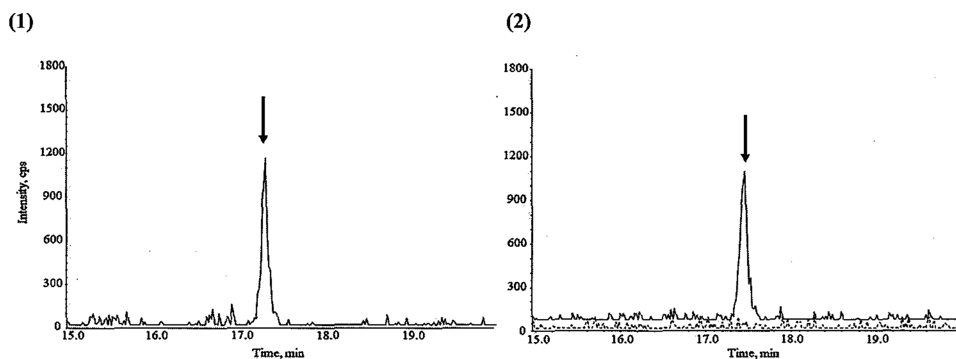
④**Amantadine**



⑤**Peramivir**



⑥**Laninamivir**



⑦**Zanamivir**

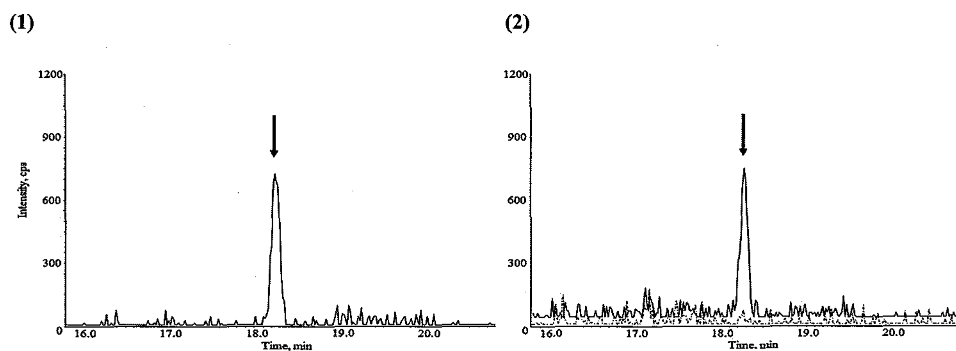


Fig. 5. Continued

which indicated that the S/N ratio satisfied the value of 10.

Matrix effects were evaluated by comparing the peak area of the matrix-matched standard to that of the solvent standard at 0.001 mg/L (equivalent to 0.01 mg/kg samples), and the results obtained ranged between 0.84

and 1.04 in chicken tissue. Matrix effects were slightly greater in oseltamivir and zanamivir in muscle, the liver, and heart. However, in the case of antiviral agents targeted in the present study, since internal standard reagents labeled with stable isotopes, such as deuterium or C13, were available for all antiviral agents, the calibration curve obtained by the internal standard was used.

4. Survey

Seven types of antiviral agents were analyzed by our test method using a total of 42 samples, including 10 samples of commercially available chicken (muscle, fat, liver, heart, and gizzard), 2 samples of chicken egg, and 30 samples of their processed products, consisting of 6 samples of yakitori (seasoned with salt or savory sauce), 4 samples each of fried chicken, salad chicken, smoked chicken, and chicken cutlet, 3 samples of chicken steak, 2 samples each of boiled egg and meat dumplings, and 1 sample of chicken soboro. As a result, no antiviral agent was detected. The samples used in the field survey were subjected to a spike and recovery test spiked with antiviral mixed standard solution with 0.01 mg/kg, demonstrating that the antiviral agents were detected at an S/N of ≥ 3. Therefore, none of the antiviral agents were detected.

Conclusion

In the present study, we developed an LC-MS/MS method for the simultaneous examination of seven antiviral agents that may be used as prophylactic agents against avian influenza. The method consists of the extraction of antivirals from a sample with methanol–water (9 : 1), purification with a tandem column, which is a combination of the InertSep® MAX cartridge (upper part) and InertSep® MCX cartridge (lower part), and measurements by LC-MS/MS. The test method developed was applied to six samples, such as chicken tissue and eggs, and nine processed products, including yakitori, fried chicken, salad chicken, chicken steak, and chicken cutlet, and good results were achieved in terms of trueness and concurrent accuracy. Therefore, this method may be used to monitor various processed chicken products imported from China or other countries, which are commonly consumed in Japan.

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LC-MS/MS による鶏組織およびその加工品中の 7 種の抗ウイルス剤一斉分析法（報文，英文）

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食衛誌 **63**(1)，1～11(2022)

LC-MS/MS を用いた鶏組織およびその加工品中からの 7 種の抗ウイルス剤（アマンタジン，リマンタジン，アルビドール，ラニナミビル，オセルタミビル，ペラミビル，ザナミビル）の分析法を確立した．

試料からメタノール－水（9：1）で抽出し，InertSep MAX ミニカラム（上側）及び InertSep MCX ミニカラム（下側）を連結したタンデム型のミニカラムで精製した後，LC-MS/MS で測定した．

鶏組織および鶏卵など 6 試料に適用した結果，真度 77.9～97.5%，併行精度 1.7～9.2% の良好な結果が得られた．また，焼き鳥，唐揚げなどの加工品 9 試料に適用した結果，真度 72.6～99.2%，併行精度 3.0～11.2% の良好な結果であった．

開発した試験法を鶏の組織と鶏卵の 12 試料および焼き鳥，唐揚げ，サラダチキン，チキンステーキ，チキンカツなど 30 試料の加工品の実態調査を行ったところ，抗ウイルス剤は検出されなかった．

開発した試験法は，鶏組織だけではなく加工品等にも適用できることが確認された．

本分析法における定量限界値は，0.01 mg/kg であった．

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