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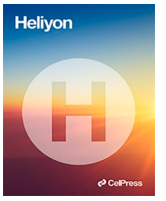

Review article

Antiviral drugs in animal-derived matrices: A review

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ABSTRACT

The ban of antiviral drugs in food-producing animals in several parts of the world, latest by Commission Delegated Regulation (EU) 2022/1644, has increased the need for food control laboratories to develop analytical methods and perform official controls. However, little is known about antiviral drugs, their use, and its analysis in food-producing animals in the EU. This review aims to provide insights into relevant viruses, antiviral drugs, and animal-derived matrices for analytical method development and monitoring purposes to implement in food control laboratories. For years, animal viruses, such as African swine fever and avian influenza, have caused many outbreaks. Besides, they led to large economic losses due to the applied control measures and a lack of available treatments. Considering these losses and the known effectiveness of authorized human antiviral drugs in different organisms, medicines such as amantadine in Chinese poultry have been misused. Various analytical methods, including screening assays and sensors (published 2016–2023), and mass spectrometry methods (published 2012–2023) have been outlined in this review for the monitoring of antiviral drugs in animal-derived matrices. However, pharmacokinetics information on metabolite formation and distribution of these substances in different animal-derived matrices is incomplete. Additionally, apart from a few countries, there is a lack of available data on the potential misuse of different antiviral drugs in animal-derived matrices. Although a handful of important antiviral drugs, such as influenza, broad-spectrum, antiretroviral, and herpes drugs, and animal-derived matrices, such as chicken muscle, are identified, the priority of the scope should be further specified by closing the aforementioned gaps.

1. Introduction

Viral infections have been well-known to the human population since ancient times because viruses can infect a large variety of organisms through different routes, such as direct contact, air, and food [1,2]. Viruses can be classified based on the type of genome and its replication strategy in the host cell, i.e. whether the viral genome consists of DNA or RNA, the genome is single or double-stranded and the sense of the single-stranded genome is positive or negative [3,4]. When viruses are pathogenic, they are harmful and cause health issues to the host, such as flu-like, respiratory, digestive, or skin symptoms. To treat viral infections, antiviral drugs, which are a class of pharmacologically active substances, have been frequently used [5,6].

Antiviral drugs inhibit the development of the virus. Each virus replicates differently and that influences the effectiveness of an

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antiviral towards a specific virus. Although there are several types of antiviral drugs, the main challenge is to develop an antiviral drug that suppresses viral replication without harming the host cell, i.e. it is a compromise between the drug's efficiency and the drug's safeness [5]. These substances are unauthorized in food-producing animals in the European Union (EU) since they are not listed in Table 1 of the Annex of EU regulation No 37/2010 [7]. One of the main concerns is that the use of antiviral drugs in food-producing animals leads to more drug-resistant virus strains in humans [8,9]. As a consequence, the effectiveness of approved antiviral drugs for humans could decrease, resulting in a lack of cures to treat the health issues of the virus's host.

To prevent misuse of antiviral drugs in food-producing animals, it is essential to monitor these substances in animal-derived matrices. For this purpose, antiviral drugs have been recently included in Commission Delegated Regulation (EU) 2022/1644 which specifies the requirements for the performance of such official controls in the Member States [10]. Amongst others, criteria for sampling strategy and selection of specific combinations of substance and commodity groups for the national risk-based control plan have been established, although not yet completely defined for antiviral drugs. Under this scenario, Wageningen Food Safety Research (WFSR) has been appointed as the European Union Reference Laboratory (EURL) and is responsible for substantive technical matters related to the analytical methods to be used for antiviral drugs, quality assurance of the research and knowledge transfer to National Reference Laboratories (NRLs) of the EU member states [11].

Since antiviral drugs are newly added to the national risk-based control plan, a survey was initially carried out among the EURLs and NRLs for residues of veterinary medicines and contaminants in food of animal origin to investigate the current knowledge of antiviral drugs in animal-derived matrices within the EU. A total of 16 laboratories from 16 Member States participated in this survey. The survey determined whether country-specific registration and limits of antiviral drugs apply, and provided insights on the availability and application of methods for the determination of antiviral drugs in animal-derived matrices as well as future perspectives of the laboratories. From the 16 responses, only one NRL performed analysis of antiviral drugs by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) for the determination of several influenza drugs in poultry and porcine muscle. However, they have not detected any antiviral drug in real samples so far. Furthermore, several laboratories indicated that they were willing to develop a method when it is required by the competent authority and if recommendations about the method as well as substances and matrix to be measured are given. Overall, the survey showed a lack of information about antiviral drugs, their use, and analysis among the NRLs as well as a need to focus on the future steps for the monitoring of antiviral drugs in animal-derived matrices.

Accordingly, the present review aims to prioritize the scope of antiviral drugs and animal-derived matrices to develop analytical methodologies and to implement them in food control laboratories. In this context, the study summarizes the relevant findings of animal viruses and their occurrence in the EU, as well as the current and alternative disease control measures and their effectiveness and (mis)use. Furthermore, an overview of available analytical methods, including screening assays and sensors (published 2016–2023) and chromatographic methods (published 2012–2023), as well as their application on real samples to determinate antiviral drugs in animal-derived matrices is presented.

2. Animal viruses and current disease control in Europe

2.1. Important infectious animal viruses

In the framework of the Animal Health Law, important infectious animal diseases have been identified that require disease prevention, control, or trade measures within the EU [12]. These animal diseases are assessed by Article 7 of Regulation (EU) 2016/429

Table 1
Overview of viral diseases in food-producing animals described in the Animal Health Law.

Poultry	Porcine	Cattle	Caprinae	Equine	Aquaculture
Low and high pathogenic avian influenza[a]	Foot-and-mouth disease	Foot-and-mouth disease	Foot-and-mouth disease	African horse sickness	Epizootic haematopoietic necrosis
Newcastle disease[a]	African swine fever	Enzootic bovine leukosis	Sheep and goat pox	Equine infectious anemia	Viral haemorrhagic septicaemia
West Nile fever[a]	Classical swine fever	Rift Valley fever[a]	Rift valley fever[a]	West Nile fever[a]	Infectious haematopoietic necrosis
	Aujeszky's disease	Bluetongue	Bluetongue	Equine viral arteritis	Infectious salmon anaemia
	Porcine reproductive and respiratory syndrome	Infectious bovine rhinotracheitis/infectious pustular vulvovaginitis	Peste des petit ruminants	Equine encephalomyelitis (Eastern and Western)	Koi herpesvirus
	Japanese encephalitis	Epizootic haemorrhagic disease	Epizootic haemorrhagic disease	Venezuelan equine encephalomyelitis	Taura syndrome
		Lumpy skin disease		Japanese encephalitis	Yellowhead disease
		Bovine viral diarrhoea			White spot syndrome
		Rinderpest			

[a] Zoonotic viruses.

and meet certain criteria, such as scientific evidence of disease transmissibility, disease susceptibility to animal species, negative effects on animal or human health, available diagnostic tools and effective risk-migrating measures. Currently, the list consists of 63 animal diseases, 35 of which are caused by viruses [12,13]. Most of these diseases are also listed by the World Organisation for Animal Health (WOAH), which provided the scientific expertise on the Animal Health Law together with the EU Animal Health Reference Laboratories [13–15]. An overview of viral diseases included in the Animal Health Law per food-producing animal species is presented in Table 1.

As can be observed in Table 1, some viral diseases such as foot-and-mouth disease and bluetongue can occur in several animal species. Furthermore, some viral diseases such as avian influenza and Newcastle disease are zoonotic, i.e. transmittable from animals to humans. Moreover, viruses like African swine fever and classical swine fever are symptomatically very similar [16]. To distinguish between these viral diseases, diagnostic multiplex-PCR assays have been developed [17]. In bees, viruses are generally latent and cause little or no damage to bee colonies, which is probably one of the reasons that no bee viruses are included in the Animal Health Law [18]. In the last decades, zoonotic and other infectious diseases have led to significant problems and permanent threats, which is partly due to the increasing human population together with animal production that result in increasing quantities and speed of international transportation of animals [19]. Therefore, with a view to the future, the spread and occurrence of new emerging (zoonotic) diseases could be expected in any part of the world.

2.2. *Occurrence of animal viruses*

Nowadays, the occurrence of animal viruses in the EU is documented through the Animal Disease Information System (ADIS) that is directly linked to the World Animal Health Information System (WAHIS) [20]. ADIS and WAHIS are designed to register and document the number of outbreaks of the most relevant animal diseases listed by the Animal Health Law and WOAH, respectively, per country during the years. These information systems permit the possibility to oversee the development of the situation of important animal diseases while ensuring that authorities are warned and are able to respond quickly in case of a rapid spread of the virus. Fig. 1 presents an overview of the total registered outbreaks, including the five most common viral diseases in food-producing animals, per year through the ADIS system for the last six years (2018–2023) [20].

As observed in Fig. 1, African swine fever, a double-stranded DNA virus, is currently the most common viral disease in animal species in the EU. In fact, among all collected outbreaks within the EU in the last six years, 71.7 % correspond to those from African swine fever. More specifically, African swine fever outbreaks were mainly detected in eastern Europe with 18.6 % of the outbreaks occurring in domestic pigs and 81.4 % in wild boars. Additionally, in Africa and Asia, the virus is responsible for many outbreaks [21]. Apart from African swine fever, avian influenza also poses a significant threat in the EU with 18.1 % of the total outbreaks corresponding to avian influenza in the studied period. More specifically, 32.8 % of the avian influenza outbreaks occurred in poultry and 67.2 % in captive and wild birds. Furthermore, it is worth noticing that the number of avian influenza outbreaks has massively increased since 2020 (Fig. 1), which may be partly caused by climatological factors such as temperature and relative humidity, and the more and more free-range conditions of poultry [22,23]. In fact, this trend has also been observed in other continents, such as Asia, North and South America [21]. Apart from avian influenza infections in wild birds, which are the natural hosts, and poultry, the virus is also able to spread to other organisms, such as humans, pigs, horses, marine mammals, cats, and dogs depending on the subtype of the virus [24]. Currently, H5N1, a highly pathogenic avian influenza strain that has occasionally infected humans, has been mainly detected [25]. In fact, 99.6 % of the avian influenza outbreaks correspond to infections of high pathogenic strains in the studied period. High pathogenic strains cause a contagious and serious illness compared to low pathogenic strains that show fewer signs of disease [26]. Other viral diseases that have been frequently detected are bluetongue with 3.1 %, West Nile fever with 1.7 %, and foot-and-mouth disease with 1.2 % of the total outbreaks in the studied period. Overall, these five viral diseases, namely African swine

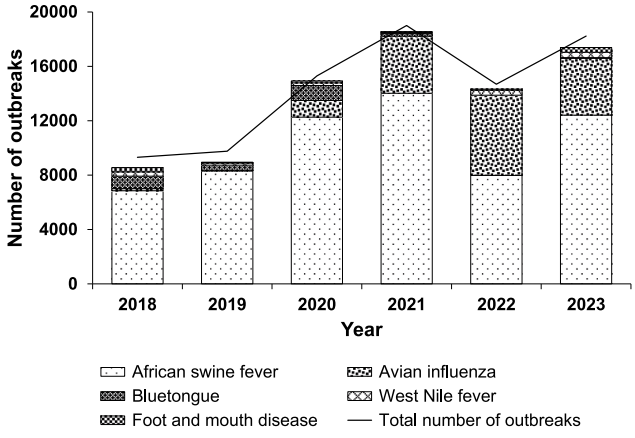

Fig. 1. Total number of outbreaks of viral diseases, including the five most common, occurring in food-producing animals in Europe (2018–2023).

fever, avian influenza, bluetongue, West Nile fever, and foot-and-mouth disease, correspond to 95.8 % of the total number of viral outbreaks in food-producing animals in the studied period (Fig. 1), indicating that the other viral diseases in Table 1 were only occasionally detected in Europe.

2.3. Current disease control measures

For most viral diseases in food-producing animals, no treatment is yet allowed or available. Therefore, disease control is mainly focused on preventive vaccination [27]. The global vaccination program to eradicate rinderpest in cattle has been one of the success stories with no detected cases of the disease worldwide since 2011 [28]. Nevertheless, vaccination is not always the solution and even if a vaccine is available, it might not be safe to use or effective anymore due to mutations of the virus. For example, to date, no high immunoprotective vaccines are available for African swine fever despite infections with the virus result in high mortality rates in pigs and wild boars [29]. Furthermore, there is a short period in which vaccinated animals are susceptible to the virus. Moreover, it is not always possible to distinguish between infected and vaccinated animals, which can cause problems for legally controlled diseases [30]. Vaccination will not always prevent the animal from infection with the virus but will lead to fewer symptoms. Hence, disease control will be more challenging, since the virus can spread unnoticed [31].

Therefore, different prevention and control measures are applied to the different animal diseases listed in the Animal Health Law. For this purpose, five categories (A, B, C, D, and E) based on the potential seriousness of the impact on animals and human health, the economy, the society, and the environment were established, and each animal disease was assigned to one or more categories [12,32]. The obligation to notify regulatory authorities and surveillance measures, as stated for category E diseases, applies to all diseases listed in the Animal Health Law. In other words, when animals show symptoms of a viral infection (e.g. mortality) or in case of any other suspicious situation, it should be determined whether a possible infection of a specific virus is present. If a viral infection of a category A disease occurs, such as highly pathogenic avian influenza and African swine fever, immediate eradication measures should be applied, such as the culling of animals, since these diseases do not normally occur in the EU. For category B diseases, eradication measures are compulsory to control the disease in all Member States, while for category C diseases, such as bluetongue, eradication measures are optional in order to prevent it from spreading to disease-free environments. For category D diseases, additional measures related to animal transportation are required to avoid the disease from spreading through movements between Member States or as a consequence of entry into the EU [32]. These extensive measures have a huge economic impact in terms of loss of valuable animals and animal by-products, costs of disposing of the animals, and restrictions to sell animals to other farms or to slaughter [33].

3. Antiviral drugs to control animal disease outbreaks

An alternative approach to the measures for the prevention and control of viral diseases in animals is the application of antiviral drugs. Indeed, some antiviral drugs are allowed in companion animals under specific circumstances due to the essence of certain antiviral drugs to treat a viral disease. However, in other cases, the use of antiviral drugs is not allowed in food-producing animals. The following sections will focus on the available antiviral drugs for human application as well as the effectiveness and pharmacokinetics of these substances in food-producing animals. Furthermore, the known misuse of antiviral drugs in food-producing animals and the cascade principle that applies to companion animals will be further discussed.

3.1. Available antiviral drugs for human application

Thousands of antiviral drugs have been proposed for humans, but only a small number are available on the market due to various challenges in antiviral drug development, such as toxic side effects, development of drug resistance, and a narrow therapeutic spectrum [34]. To date, more than 100 antiviral drugs have been approved in humans for the treatment of a handful of viruses, namely human immunodeficiency virus (HIV), hepatitis virus, herpes virus, influenza virus, cytomegalovirus, varicella-zoster virus, respiratory syncytial virus, papillomavirus infections and coronavirus disease 2019 (COVID-19) [34,35]. Although it is very difficult to cover the entire spectrum of antiviral drugs, De Clercq et al. have presented an overview of antiviral drugs approved per virus until April 2016 worldwide [35]. Additionally, Tompa et al. have listed the antiviral drugs per virus approved by the Food and Drug Administration (FDA) agency until 2020 [6]. Both reviews from DeClercq et al. and Tompa et al. also cover comprehensive information about individual antiviral drugs and the classification of antiviral drugs per type of inhibitor and virus [6,35]. Antiviral drugs can also be classified by the so-called Anatomical Therapeutic Chemical (ATC) System from the World Health Organisation (WHO) which takes into account the organ or system on which the drugs function and their therapeutic, pharmacological, and chemical properties [36]. For instance, antiviral drugs for systematic use have the ATC code J05, antiviral drugs for dermatological use have the ATC code D06BB, and antiviral drugs for ophthalmological use have the ATC code S01A. Particularly, the number of approved antiviral drugs to treat HIV infections has been a remarkable success, although COVID-19 drugs, including molnupiravir, remdesivir, and nirmatrelvir, have mainly pointed the attention in recent years due to the severe symptoms and the high number of infections worldwide [37]. Preferably, the focus should be on the development of broad-spectrum antiviral drugs that show activity against different viruses and that can be directly applied in cases when a new emerging viral disease is detected [34]. Amongst others, ribavirin, arbidol, favipiravir, and molnupiravir have demonstrated broad-spectrum antiviral activity in humans [38,39]. Specifically, ribavirin was examined in clinical models for the treatment of hepatitis C and respiratory syncytial virus and is approved for both conditions; arbidol for influenza and COVID-19; favipiravir for influenza virus and COVID-19; and molnupiravir for COVID-19 and respiratory syncytial virus [38]. The broad-spectrum antiviral drugs ribavirin, arbidol, favipiravir, molnupiravir, galidesivir, triazavirin, nitazoxanide, and its metabolite

tizoxanide, the influenza drugs amantadine, rimantadine, laninamivir, peramivir, oseltamivir, zanamivir, and baloxavir marboxil, the hepatitis C drug celgosivir as well as the drugs lactimidomycin and methisazone are specifically reserved for the treatment of certain infections in humans and are prohibited for use in animals in the EU [40]. All in all, the approved antiviral drugs have played a crucial role in the treatment of viral diseases and have together saved tens of millions of human lives over the last decades.

3.2. Effectiveness of antiviral drugs in food-producing animals

Antiviral drugs, especially influenza drugs, intended for humans were shown to reduce symptoms and mortality in animal models, but in several cases, transmission of the virus still occurred [41]. Specifically, Webster et al. have demonstrated that the influenza drugs amantadine and rimantadine, both inhibitors of the viral membrane fusion, were effective prophylactically and therapeutically in chickens when administrated via drinking water [42]. Lee et al. have shown that orally administrated oseltamivir, a neuraminidase inhibitor, significantly reduces viral replication of low pathogenic avian influenza strains in a chicken model and shows complete suppression in the duck model [43]. Meijer et al. have demonstrated that orally administrated oseltamivir reduces transmission, morbidity, and mortality of highly pathogenic avian influenza strains in chicken, however, authors highlighted that locally active zanamivir was not effective [44]. Furthermore, Yamanaka et al. have found that an intravenous dose of peramivir reduced and led to a shorter duration of clinical signs in horses infected with equine influenza [45]. Additionally, Twabela et al. evaluated baloxavir marboxil and peramivir to treat high pathogenicity avian influenza in chickens and observed effectiveness in simultaneous treatment [46]. However, the authors indicated that early administration is essential since the effectiveness was limited for the delayed treatment of chickens which reflects a more real-life situation.

Apart from influenza drugs, it has been shown that anti-herpes drugs, such as acyclovir and ganciclovir, reduce clinical signs of equine herpesvirus and lead to the survival of horses [47]. In addition, there is evidence of effective antiviral drugs for foot-and-mouth disease and classical swine fever. For instance, imidazo [4,5-c]pyridines, particularly BPIP, inhibit the in vitro replication of classical swine fever by targeting the viral polymerase [48], whereas T-1105, an analog of favipiravir, fully protects pigs from foot-and-mouth disease infection [49].

3.3. Pharmacokinetics of antiviral drugs in food-producing animals

To the best of our knowledge, relevant studies to investigate the absorption, distribution, metabolism, and/or excretion of antiviral drugs in food-producing animals were only conducted for amantadine and oseltamivir in poultry species. You et al. performed an animal study to evaluate the target tissues for monitoring amantadine abuse in broiler chickens [50]. The authors mixed amantadine with chicken feed at different concentrations (10, 20, and 40 μg kg^{-1}) and fed to broiler chickens for five consecutive days. After amantadine withdrawal, plasma, liver, and breast samples were collected. The results showed that the highest concentrations of amantadine were found in the liver and that it also took the longest time to eliminate amantadine residues after withdrawal. Chicken breast and plasma showed similar results. Furthermore, Wu et al. performed an animal study to assess the applicability of the developed method to monitor antiviral residues in feces [51]. Ducks were orally administrated with amantadine and oseltamivir, followed by the collection of feces samples over approximately four weeks. In the first week, the residue concentrations in feces were the highest and peaked at day 4 after administration of the drug. Furthermore, it was observed that oseltamivir was highly metabolized to oseltamivir acid. Between days 2 and 4, the concentration of oseltamivir acid was almost two times higher than oseltamivir. Residues of amantadine, oseltamivir, and oseltamivir acid could be detected in feces within approximately four weeks after oral administration.

3.4. Misuse of antiviral drugs in food-producing animals and cascade principle

When antiviral drugs are used illegally in food-producing animals, these medicines can land in different animal-derived matrices that are consumed by humans (e.g. meat) or be spread in the environment (e.g. feces). In the case of feces, antiviral drugs can be further present in fertilizer, water, and food crops and ultimately also reach humans via this route. Intake of high concentrations of these antiviral drugs in humans and repeated exposure are associated with toxic health effects and the development of antiviral drug resistance. The consequences of drug resistance and lack of effective alternative treatments can range from the use of second-line antiviral drugs with higher toxicity to severe disease or death.

Generally, the misuse of antiviral drugs in food-producing animals has been sporadically reported at a global level [8,9]. In the 2000s, the widespread misuse of amantadine in Chinese poultry has led to large-scale resistance problems [52–54]. As a consequence, China banned the use of amantadine in poultry farms in 2005 [55]. In the United States, the FDA prohibited the extralabel use of adamantanes and neuraminidase inhibitors in chickens, turkeys, and ducks since 2006 [56,57]. Neuraminidase inhibitors, especially laninamivir, have shown lower resistance to influenza subtypes compared to adamantanes since they are relatively new anti-influenza drugs and are more expensive to use [24]. Newer antiviral drugs are often expensive in the first few years after approval in humans and might not be the first choice for misuse in food-producing animals by farmers. Apart from the misuse of amantadine in Chinese poultry, there is evidence that several antiretroviral drugs, including saquinavir, lopinavir, efavirenz, and nevirapine, have been applied as immune boosters or to control diseases, such as African swine fever and Newcastle disease in Ugandan pigs and chickens, respectively [58,59]. Although the effectiveness of antiretroviral drugs has not been shown for these viruses, the easy access and low cost of these antiviral drugs could have played a significant role in the misusage. In addition, acyclovir and idoxuridine have been used as they are on the list of essential antiviral drugs for the treatment of eye ulcers in horses [60]. These antiviral drugs could therefore be applied

using the cascade principle of EU regulation No. 2019/6 under the condition of a withdrawal period of six months [61]. Also in companion animals, such as cats, antiviral drugs are administered using this same cascade principle, e.g. acyclovir, famciclovir, trifluridine, and idoxuridine for the treatment of ocular feline herpesvirus 1 [33,61,62].

4. Analytical strategies for detection of antiviral drugs in animal-derived matrices

Since antiviral drugs have recently been unauthorized as veterinary medicinal products and feed additives, official controls are required by EU regulation. Therefore, there is an immediate need to develop analytical methods for their determination. In the last decade, antiviral drugs have been included in several methods for the analysis of animal-derived matrices including screening assays and sensors as well as chromatographic methods [58,63,64]. To the best of our knowledge, there are no available reviews in the literature on this topic. Hence, this section aims to provide an overview of the available methods for the determination of antiviral drugs in animal-derived matrices and share insights on the application of these methods to real samples.

4.1. Screening assays and sensors

In the last decade, the development of screening assays and sensors for antiviral drugs has ever increased due to their simplicity, rapidness, limited need for chemical solvents, cost-effective analysis, and on-site detection capabilities [65]. Table 2 summarizes the publications about the developed screening assays and sensors for antiviral drugs in animal-derived matrices of the last eight years. Most of these screening assays and sensors are immunological methods, i.e. based on specific antibodies, such as enzyme-linked immunosorbent assay (ELISA) and immunochromatographic assay (ICA), while a few are molecularly imprinted polymer (MIP) based methods or spectroscopic methods, such as surface-enhanced Raman spectroscopy (SERS).

As shown in Table 2, test developers of the screening assays and sensors have mainly focused on the detection of influenza drugs, for which 86 % selected amantadine as a target compound [74,83–85] followed by ribavirin (14 %) [67,99–101], rimantadine (11 %) [68–70], and oseltamivir (3 %) [97]. Most screening assays and sensors are developed for chicken muscle and sometimes for other poultry muscle, porcine muscle, and beef [79,94]. Although animal muscle is often the matrix of choice [73], some methods have been developed for eggs [63,97], liver [86,96], milk [90,91,95], and feed [81]. The traditional indirect-competitive ELISA has been widely used to detect different antiviral drugs in animal-derived matrices and is still being used [70,72,100]. As screening assays and sensors are aimed at obtaining a rapid result, the required sample treatment is often a simple and quick solid-liquid extraction (SLE) [71,101]. However, in some cases, a more extensive sample treatment is needed, where a simple SLE is combined with a liquid-liquid extraction (LLE) or even an Oasis mixed-mode cation-exchange (MCX) solid-phase extraction (SPE) clean up [68,93,94,96]. This hampers the simplicity, speed, and applicability of the on-site use of the method. Thus, although traditional ELISAs are cost-effective, eco-friendly, high-throughput, and quantitative methods, the procedures are still time-consuming, labor-intensive, and require specific instruments for signal readout [102]. Another drawback of the developed screening assays and sensors for antiviral drugs is that the detection is mostly restricted to a single analyte or a group of similarly structured analytes, which gives specificity but limits the scope of the method.

Although the scope of the method about the target compound, matrix, and sample treatment is very straightforward and often based on antibodies, the eventual type of readout of the screening assays and sensors used is very versatile. This is because the focal point in the development of many immunological methods in recent years has been the exploration of new labels and screening approaches in order to improve the detection performance as well as the simplicity, speed, and on-site applicability of the method. For instance, novel fluorescence and plasmonic ELISA have been explored using carbon dots or silver-gold nanorings as a label increasing both the simplicity of label measurement and the sensitivity of the methods compared to the traditional ELISAs based on colorimetric detection [75–77]. In addition, multiplex immunoassays have been developed that allow the simultaneous detection of several target analytes, e.g. antibiotics, anthelminthics, mycotoxins, pesticides, and allergens [103,104]. Moreover, multi-mode platforms that allow both fluorescence and colorimetric detection by a multifunctional microplate reader and smartphone reader have been developed [63,90]. While the multiplex immunoassays have shown to be sensitive and are validated in our laboratory to detect antibiotics and anthelminthics in swipe samples taken from animals, the multi-mode platform (e.g. smartphone reader) is currently less sensitive than the conventional reader.

Apart from ELISA, ICAs and lateral flow immunoassays (LFIAs) have been developed for antiviral drugs with mostly gold nanoparticles as labels [66,80,82,86]. As this type of assay combines immunolabeling and chromatography, it is possible to develop an assay to detect several target analytes [102]. LFIAs, like a COVID-19 home test, are quick and can easily be performed on-site. Although detection is often still through visual examination that results in a qualitative and semi-arbitrary outcome [102], nowadays many LFIAs are not only visually inspected, but can be measured on a simple and inexpensive reader, resulting in objective and (semi-) quantitative outcomes [85,97]. Unlike the previously mentioned heterogeneous immunoassays, such as ELISA and ICA, homogeneous immunoassays do not require analyte-antibody complex separation or washing steps and thus benefit in facile operation and speed of analysis [105]. In fact, Dong et al. and Guo et al. have developed homogeneous immunoassays based on fluorescence resonance energy transfer and fluorescence polarization, respectively, to detect amantadine in chicken muscle [87,88]. Another technique that offers unique spectral fingerprints is SERS [91,92]. The SERS sensor has several advantages for on-site analysis including limited need for sample preparation, portable device, potential in non-destructive detection, strong resistance to background water and air, and applicability to different states of matter [106]. However, there are still challenges in terms of poor reproducibility, specificity, and sensitivity. The aforementioned screening techniques utilize labels, such as enzymes, fluorescent agents, or silver and gold nanoparticles to enhance the signal and obtain higher sensitivity [89]. Nevertheless, label-free detection methods, such as a

Table 2
Screening assays and sensors for the detection of antiviral drugs in animal-derived matrices and feed (publications 2016–2023).

Analytes	Matrix	Sample treatment	Method	Signal indicator	LOD (μg kg^{-1})	Ref.
Adamantane methanol, 1-adamantyl methyl ketone, amantadine, rimantadine, somantadine	Chicken muscle	SLE[a]	LFIA[b]	Gold NPs[c]	0.1–10	[66]
Amantadine, ribavirin	Chicken muscle	SLE	MWFPIA[d]	AEDA-AF647	1.0–1.7	[67]
Amantadine, rimantadine	Chicken muscle	SLE, LLE[e]	MIP[f]-based fluorescence assay	DC[g], FITC[h], TAMRA[i]	-	[68]
Amantadine, rimantadine	Chicken and porcine muscle	SLE, LLE	MIP-based chemiluminescence sensor	HRP[j]	-	[69]
Amantadine, rimantadine	Chicken muscle and liver	SLE	icELISA[k]	HRP	5.0–5.4	[70]
Amantadine	Chicken muscle	SLE	icELISA	HRP	1	[71]
Amantadine	Chicken muscle	SLE, LLE	icELISA	HRP	-	[72]
Amantadine	Chicken muscle	–	icELISA	HRP	0.64	[73]
Amantadine	Chicken muscle	SLE	icELISA	Gold NPs	-	[74]
Amantadine	Chicken muscle	SLE	Fluorescence ELISA	CDs[l]	-	[75]
Amantadine	Chicken muscle	SLE	Fluorescence ELISA	CDs@manganese dioxide nanosheets	-	[76]
Amantadine	Chicken muscle and egg, duck and porcine muscle	SLE, defatting	Plasmonic ELISA	Silver-Gold NRs[m]	-	[77]
Amantadine	Chicken muscle	SLE	AIE[n]-icELISA	GOx[o]	0.06	[78]
Amantadine	Chicken muscle and egg, duck muscle, beef	SLE	icELISA, ICA[p]	HRP, Gold NPs	-	[79]
Amantadine	Chicken muscle	SLE	ICA	Gold NPs	–	[80]
Amantadine	Suckling pig, piglet, sow and laying duck feed	SLE	ICA	QD[q]	0.08–0.19	[81]
Amantadine	Chicken muscle	SLE	ICA	Gold NPs	–	[82]
Amantadine	Chicken muscle	SLE	bFQ[r]ICA, TRF[s]ICA	Gold NPs, Europium NPs	0.29–0.62	[83]
Amantadine	Chicken muscle	SLE	FQICTS[t]	Gold NPs, Gold NCs[u]	0.45	[84]
Amantadine	Chicken muscle	SLE	LFIA	CdSe/ZnS[v] QD	0.18	[85]
Amantadine	Chicken muscle and egg, porcine muscle and liver	SLE	Dipstick LFIA	Gold NPs	-	[86]
Amantadine	Chicken muscle	SLE	FPIA[w]	FITC, DTAF[x], EDF[y], BDF[z], HDF[aa]	0.9	[87]
Amantadine	Chicken muscle	SLE	FRET[ab]-based immunoassay	CDs, tungsten disulfide nanosheets	0.1	[88]
Amantadine	Chicken muscle	SLE	SF-T-SN[ac] colorimetric immunoassay	Silver NPs	-	[89]
Amantadine	Chicken muscle and egg, duck muscle	SLE	Dual-mode immunoassay	Carbon NPs	0.05–0.06	[63]
Amantadine	Chicken muscle and egg, beef, milk	SLE	Smartphone-assisted dual-mode immunosensing microarray	NH2-UiO-66 PtNPs[ad]	-	[90]
Amantadine	Chicken muscle and egg, milk	SLE	SERS[ae]	Gold nanorots@Silver	-	[91]
Amantadine	Chicken muscle	SLE	SERS	Gold NPs	-	[92]
Amantadine	Chicken muscle, liver and egg	SLE, Oasis-MCX[af] SPE	MIP-QCM[ag] sensor	rGO[ah]-Gold NPs	-	[93]
Amantadine	Chicken muscle and egg, duck and porcine muscle	SLE, Oasis-MCX SPE	QCM piezoelectric immunosensor	-	-	[94]
Amantadine	Chicken muscle and egg, duck muscle, milk	Pure milk: protein and lipid removal Others: SLE	SPR[ai] immunochip	-	-	[95]
Amantadine	Chicken muscle and liver, porcine muscle, beef, mutton	SLE, Oasis-MCX SPE	MIP-electrochemical sensor	-	-	[96]
Oseltamivir phosphate	Chicken muscle and egg	Homogenisation, SLE	LFIA	Gold NPs	0.42–0.43	[97]

(*continued on next page*)

Table 2 (*continued*)

Analytes	Matrix	Sample treatment	Method	Signal indicator	LOD (μg kg^{-1})	Ref.
Ribavirin	Chicken muscle, milk	SLE, LLE	QCM piezoelectric immunosensor	–	–	[98]
Ribavirin	Chicken muscle	SLE	icELISA, ICA	Gold NPs	–	[99]
Ribavirin	Chicken muscle	SLE	icELISA	HRP	4.2	[100]
Ribavirin	Chicken muscle and egg, duck muscle	SLE	icELISA	HRP	1.0–1.2	[101]

[a] Solid-Liquid Extraction; [b] Lateral Flow Immunoassay; [c] Nanoparticles; [d] Multi-Wavelength Fluorescence Polarization Immunoassay; [e] Liquid-Liquid Extraction; [f] Molecularly Imprinted Polymers; [g] Dansyl Chloride; [h] Fluorescein Isothiocyanate; [i] 5-Carboxytetramethylrhodamine; [j] Horseradish Peroxidase; [k] indirect competitive Enzyme-Linked Immunosorbent Assay; [l] Carbon Dots; [m] Nanorings; [n] Aggregation-Induced Emission; [o] Glucose Oxidase; [p] Immunochromatographic Assay; [q] Quantum Dots; [r] background Fluorescence Quenching; [s] Time-Resolved Fluorescent; [t] Fluorescence Quenching Immunochromatographic Test Strip; [u] Nanoclusters; [v] Cadmium Selenide Zinc Sulfide; [w] Fluorescence Polarization Immunoassay; [x] Dichlorotriazine Aminofluorescein; [y] Fluoresceinthiocarbamyl Ethanediamine; [z] Fluoresceinthiocarbamyl Butanediamine; [aa] Fluoresceinthiocarbamyl Hexamethylenediamine; [ab] Fluorescence Resonance Energy Transfer; [ac] Signal-off tuned Signal-on; [ad] Zr-based Metal-Organic Framework Platinum Nanoparticles; [ae] Surface-Enhanced Raman Spectroscopy; [af] Mixed-Mode Cation-Exchange; [ag] Quartz Crystal Microbalance; [ah] reduced Graphene Oxide; [ai] Surface Plasmon Resonance.

surface-plasmon resonance immunochip [95], a quartz crystal microbalance piezoelectric immunosensor [94,98], and a MIP electrochemical sensor [96], have also been developed for the detection of antiviral drugs. Label-free methods are more simple and do not hamper experimental errors induced specifically by the label, e.g. fluorescence quenching by the matrix [107]. Additionally, it is worth noticing that immunological methods use antibodies as recognition reagents and these antibodies can most often not be re-used. For this reason, several test developers have explored MIPs as specific recognition material to prepare chemiluminescence, quartz crystal microbalance, and electrochemical sensors as MIPs can be reused up to a hundred times or more and thereby lead to reduced costs [69,93,96].

The limit of detection (LOD) of the different screening assays for antiviral drugs is based on either an instrumental signal, such as color, fluorescence, chemiluminescence, or visual examination. However, although the assays are purposely developed for real samples, many authors only determined the LOD with pure standards. When the LOD was determined in an animal-derived matrix, the lowest value was achieved by Xie et al. and Yu et al. for amantadine using a carbon nanoparticle-based dual-mode immunoassay and an aggregation-induced emission-based indirect-competitive ELISA, respectively [63,78]. Besides, none of these screening assays and sensors are validated according to a specific regulation, such as Commission Implementing Regulation (EU) 2021/808 [108]. According to this regulation, the detection capability for screening (CCβ), selectivity/specificity, stability, and ruggedness should be examined. Furthermore, depending on whether it is a qualitative, semi-quantitative, or quantitative screening method, additional performance characteristics must be examined, such as trueness, precision, relative matrix effect, and absolute recovery. Only when validated on a relevant concentration level these screening assays and sensors are suited to separate compliant (negative) samples from non-compliant samples (suspect) but are not able to confirm the presence of specific analytes according to Commission Implementing Regulation (EU) 2021/808. Therefore, the suspect samples need to be further analyzed. Chromatographic techniques in combination with mass spectrometry are required for such confirmations and thus they are eventually needed for the official controls in food laboratories to monitor antiviral drugs in animal-derived matrices [108,109].

4.2. Chromatographic methods

Apart from screening assays and sensors, chromatographic methods in combination with different detectors, mainly mass spectrometry, have been described in the literature to determine antiviral drugs in animal-derived matrices. Table 3 summarizes the publications on this topic in the last twelve years.

As can be observed in Table 3, most of the available methods focus on the detection of influenza drugs [117,120,121], followed by broad-spectrum drugs [126,127] and herpes drugs [64,110], whereas only a few methods include HIV drugs [58,115]. Amantadine, rimantadine, oseltamivir, arbidol, ribavirin, moroxydine, and acyclovir are the most commonly included analytes of these categories of antiviral drugs. Nevertheless, the approval of new antiviral drugs is continuous and subsequently causes ongoing shifts in the substances considered important to monitor. For example, favipiravir, laninamivir, and peramivir are relatively new antiviral drugs included in the most recent methods [64]. Other substances included in some studies are metabolites of oseltamivir [51] and arbidol [64], namely oseltamivir acid, and arbidol sulfone and sulfoxide, respectively. However, the latter metabolites are not known to be formed in food-producing animals and are only based on findings in rat or human studies [128,129]. Therefore, pharmacokinetics studies are essential for antiviral drugs in food-producing animals to determine whether the marker for analysis should be the parent drug or one of the possibly formed metabolites. Although some methods are developed for a single analyte [124], most methods contain up to fifteen analytes of several types of inhibitors [64]. These multi-residue methods are preferred, since they allow the food safety monitoring of a large number of antiviral drugs in animal-derived matrices within a single analytical run and thereby reduce the time and cost of analyses. The preference for influenza drugs in both screening assays and sensors, as well as chromatographic methods, is not outstanding, since avian influenza is one of the viral diseases with the most outbreaks in the world. Also, due to the

Table 3
Chromatographic methods for the detection of antiviral drugs in animal-derived matrices and feed (publications 2012–2023).

Analytes	Matrix	Sample treatment	Chromatographic separation	Detection system	LOD (μg kg^{-1})	Validation; CCα (μg kg^{-1})	Ref.
Acyclovir, amantadine, arbidol, arbidol sulfone, arbidol sulfoxide, favipiravir, ganciclovir, laninamivir, peramivir, oseltamivir, oseltamivir acid, ribavirin, rimantadine, viramidine, zanamivir	Chicken, duck, quail and turkey muscle	Protein precipitation	LC[a], BEH[b] Amide HILIC[c] (2.1 × 100 mm, 2.7 μm)	QqQ[d] (ESI + [e], MRM[f])	0.01–3.1	(EU) 2021/808; 0.12–34.7	[64]
Acyclovir, amantadine, arbidol, famciclovir, ganciclovir, imiquimod, memantine, moroxydine, oseltamivir, oseltamivir acid, penciclovir, ribavirin, rimantadine, triazole carboxamide	Chicken muscle	QuEChERS[g]	LC, SB-aq (3.0 × 100 mm, 1.8 μm)	QTRAP[h] (ESI+, MRM)	0.02–1.0	-	[110]
Acyclovir, amantadine, arbidol, ganciclovir, imiquimod, memantine, moroxydine, oseltamivir, oseltamivir acid, rimantadine, somantadine	Livestock and poultry feces	In-cell clean-up PLE[i]	LC, BEH-HILIC (2.1 × 100 mm, 1.7 μm)	QTRAP (ESI+, MRM)	0.6–1.4	-	[51]
Acyclovir, amantadine, imiquimod, memantine, moroxydine, oseltamivir, rimantadine	Chicken muscle, liver and egg	SLE[j], Chromabond HR-XC SPE[k]	LC, BEH Amide (2.1 × 100 mm, 1.7 μm)	QqQ (ESI+, MRM)	-	CD 2002/657/EC; 0.04–0.64	[111]
Amantadine, arbidol, oseltamivir, oseltamivir acid, ribavirin, rimantadine, zanamivir	Chicken and turkey muscle	SLE, Strata-X C and PBA[l] SPE	LC, Symmetry C18 (3.0 × 150 mm, 5 μm) and Thermo Fisher Hypercarb (3.0 × 100 mm, 5 μm)	QqQ (ESI+, SRM)	-	CD 2002/657/EC; 0.1–8.0	[112]
Acyclovir, amantadine, moroxydine, ribavirin, rimantadine	Chicken muscle	Modified QuEChERS	LC, BEH Amide (2.1 × 100 mm, 1.7 μm)	QqQ (ESI+, SRM)	0.3–2.2	-	[113]
Amantadine, memantine, moroxydine, ribavirin, rimantadine	Honey	SLE, PBA SPE	LC, Poroshell 120 SB-Aq (2.1 × 100 mm, 2.7 μm)	QqQ (ESI+, MRM)	0.1–2.0	International Conference on Harmonization guidelines	[114]
Efavirenz, nevirapine, tenofovir	Porcine plasma	Protein precipitation	Efavirenz: LC, Eclipse (7.5 cm × 4.6 mm 3 μm) Nevirapine: Zorbax eclipse XBD[m]-phenyl (4.6 × 150 mm, 5 μm) Tenofovir: Atlantis C18 (150 × 3.0 mm, 3 μm)	Ultraviolet	-	-	[58]
Indinavir, ritonavir, saquinavir	Yellow catfish	QuEChERS, G/KCC-1[n] pipette tip SPE	LC, Alltima C8 (4.6 × 150 mm, 3 μm)	QTRAP (ESI+, MRM)	0.4–0.8	Partly	[115]
Amantadine, memantine, rimantadine	Fish, meat, blood and feather meal	SLE, Oasis PRiME HLB[o] SPE	LC, BEH C18 (3.0 × 150 mm, 1.7 μm)	QqQ (ESI+, MRM)	0.15–0.31	-	[116]

(*continued on next page*)

Table 3 (*continued*)

Analytes	Matrix	Sample treatment	Chromatographic separation	Detection system	LOD (μg kg^{-1})	Validation; CCα (μg kg^{-1})	Ref.
Amantadine, memantine, rimantadine	Chicken muscle, liver, gizzard and egg, (deep) fried and grilled chicken, fried quail egg	Modified QuEChERS, Oasis MCX[p] SPE	LC, Kinetex® XB-C18 (2.1 × 100 mm, 2.6 μm)	QqQ (ESI+, SRM[q])	-	Ministry of Health, Labour and Welfare 2010, Director Notice, Syoku-An No. 1224-1	[117]
Amantadine, memantine, rimantadine	Chicken muscle	SLE, PCX/Fe_3O_4 dispersive micro SPE	LC, HSS[r] T3 (2.1 × 100 mm, 1.8 μm)	Q-Orbitrap (ESI+, tSIM/dd-MS2 (Top N)[s])	0.03	–	[118]
Amantadine, memantine, rimantadine	Chicken muscle	SLE, MWCNTs[t] dispersive SPE	LC, BEH C18 (2.1 × 100 mm, 1.7 μm)	QqQ (ESI+, MRM)	-	CD 2002/657/EC; 0.15–0.20	[119]
Amantadine, rimantadine	Formula and conc. feed pig, premix feed chicken	SLE, MCX SPE	LC, XDB-C18 (2.1 × 150 mm, 3.5 μm)	QTRAP (ESI+, MRM-IDA-EPI[u])	0.2–0.5	-	[120]
Amantadine, rimantadine	Chicken muscle, liver and egg, duck muscle, porcine muscle, liver and kidney	Multifunctional filter based on QuEChERS	LC, XDB-C18 (2.1 mm × 150 mm, 3.5 μm)	QqQ (ESI+, MRM)	0.5	-	[121]
Amantadine, rimantadine	Chicken muscle	Modified QuEChERS	LC, HSS T3 (2.1 × 150 mm, 1.8 μm)	LTQ[v]-Orbitrap (ESI+)	0.67–1.0	-	[122]
Amantadine	Chicken muscle	SLE, derivatization	GC[w], DB-5MS Ultra Inert fused-silica capillary (30 m × 0.25 mm, 0.25 μm)	QqQ (CI-[x])	0.020	-	[123]
Moroxydine	Chicken muscle, liver, kidney and egg, porcine muscle, liver, lung and kidney	SLE, LLE[y], SCX[z] SPE	LC, Cortecs HILIC (2.1 mm × 50 mm, 1.6 μm)	QqQ (ESI+, MRM)	0.3	–	[124]
Oseltamivir	Carp, Shrimp, Yellow croaker	Protein and fat precipitation, magnetic SPE with MCOF/GO[aa]	LC, C18 column (250 × 4.6 mm, 5 μm)	Q-Orbitrap (ESI+)	0.035	–	[125]
Ribavirin	Chicken muscle	Modified QuEChERS	LC, Hypercarb (4.6 × 100 mm, 7.0 μm)	QqQ (ESI+, MRM)	–	CD 2002/657/EC; 1.1	[126]
Ribavirin	Chicken muscle	SLE, Hypercarb SPE	LC, Hypercarb (2.1 × 100 mm, 5 μm)	QqQ (ESI+, MRM)	–	Partly	[127]

[a] Liquid Chromatography; [b] Ethylene Bridged Hybrid; [c] Hydrophilic Interaction Liquid Chromatography; [d] Triple Quadrupole; [e] positive Electrospray Ionization; [f] Multi-Reaction Monitoring; [g] Quick Easy Cheap Effective Rugged Safe; [h] Triple Quadrupole Ion Trap; [i] Pressurized Liquid Extraction; [j] Solid-Liquid Extraction; [k] Solid-Phase Extraction; [l] Phenylboric acid; [m] Extra-Dense bonding; [n] Graphene/Silica Nanospheres; [o] Hydrophilic-Lipophilic Balanced; [p] Mixed-mode Cation-Exchange; [q] Selective Reaction Monitoring; [r] High Strength Silica; [s] Targeted Single Ion Monitoring/Data-Dependent MSMS; [t] Multi-Walled Carbon Nanotubes; [u] Multi-Reaction Monitoring Information Dependent Acquisition Enhanced Production Ion; [v] Linear Ion Trap; [w] Gas Chromatography; [x] negative Chemical Ionization; [y] Liquid-Liquid Extraction; [z] Strong Cation Exchange; [aa] Magnetic Covalent Organic Framework/Graphene Oxide Composite.

zoonotic character of the disease, the effectiveness of these antiviral drugs has been well-known in animals [42–44]. Since avian influenza mainly spreads between wild birds and poultry, mostly chicken has been selected as an animal of choice [24,110] followed by pigs because influenza and other viruses can also occur in this species [24,124]. The matrix of choice for these animal species is muscle which is from a food safety point of view explainable as it is an edible matrix [122]. In some cases, liver [111], eggs [121], kidney [124], and processed food [117] have been included in addition to muscle. Only a few methods are developed for honey, fish, and crustaceans, which might be related to the lack of information available on the use of antiviral drugs in bees and aquaculture [114,115,125]. Nevertheless, antiviral drugs can also enter the aquatic environment through wastewater and eventually accumulate in aquaculture.

The sample treatment for antiviral drugs is mainly dependent on the analytes included in the method. When the analytical method only contains a single analyte or a few analytes, a specific sample treatment, such as SLE in combination with SPE, is applied. For

instance, amantadine (pKa = 10.7), rimantadine (pKa = 10.1), and memantine (pKa = 10.7) are usually cleaned up using Oasis PRiME hydrophilic-lipophilic balance (HLB) SPE or Oasis MCX SPE, relying on a reversed-phase mechanism or a reversed-phase and cation-exchange mechanism, respectively [116,117,120]. Furthermore, Li et al. have used a strong cation-exchange (SCX) SPE to clean up moroxydine (pKa = 11.9) [124]. Several analytes shown in Table 3 have good affinity to an SCX SPE, including the above-mentioned analytes, oseltamivir (pKa = 9.3), oseltamivir acid (pKa = 4.2), arbidol (pKa = 6.0), arbidol sulfoxide (pKa = 6.0), arbidol sulfone (pKa = 6.0), zanamivir (pKa = 3.8), peramivir (pKa = 4.1), viramidine (pKa = 5.3) and imiquimod (pKa = 5.0). However, ribavirin and favipiravir are one of the few analytes that have no interaction with the aforementioned mechanism as they are neutral at low pH and when included in the methods either clean up is performed with phenylboronic acid (PBA) SPE cartridge that retains ribavirin through a reversible covalent bond or a more general sample preparation method is applied for both analytes [64,114,126]. Nevertheless, Berendsen et al. have shown that antiviral drugs with very different properties in terms of polarity and pKa values can be cleaned up well by using two SPE cartridges with different interaction mechanisms such as strong cation exchange (Strata-X C) and strong covalent bonding (PBA) [112]. However, due to the lab-extensive sample treatment procedure, it can be genuinely observed that the more analytes are included in a method, the more general sample treatment is applied. For instance, Mu et al. and Douillet et al. have included the most antiviral drugs into a single method and used the Quick Easy Cheap Effective Rugged Safe (QuEChERS) method and protein precipitation method, respectively [64,110]. Although these methods are easy and cost-effective, the drawbacks are lower selectivity and more matrix effects compared to an extensive sample treatment procedure. Especially the lower selectivity is a challenge for low molecular weight antiviral drugs, such as amantadine, rimantadine, memantine, and favipiravir, which can lead easily to interferences at the retention time of the analytes.

Liquid chromatography (LC) is the method of choice for the chromatographic separation of antiviral drugs. Only Ho et al. have used gas chromatography (GC) for the determination of amantadine in chicken muscle [123]. However, this requires derivatization of the analyte, which is an additional step in the sample treatment, and therefore LC is the preferred chromatographic technique. When adamantanes are the analytes of interest, mostly reversed-phase columns, such as XDB-C18 and BEH C18, are used in combination with a mobile phase of 0.1 % formic acid in water and methanol [119,121]. As soon as the number of analytes increases, the choice for column switches to normal phase columns, such as BEH Amide or BEH-HILIC, and reversed-phase columns, such as SB-aq, that can maximize the retention of hydrophilic analytes [51,110,111,113,114]. However, the latter one is not used when zanamivir and laninamivir are included in the method, since these analytes are probably too polar to be retained by the reversed-phase mechanism. For BEH Amide and BEH-HILIC columns, typically a mobile phase of acetonitrile and 0.1 % formic acid, 2–5 mM ammonium acetate in water is selected [51,113], whereas SB-aq columns use a mobile phase of 0.1 % formic acid in water and methanol [110]. Mostly low-resolution mass spectrometry (LRMS), either quadrupole ion trap (QTRAP) or triple quadrupole (QqQ), is used to detect the antiviral drugs. In all cases, the ionization technique is electrospray ionization (ESI), and the acquisition mode is multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) mode. Chen et al. and Yan et al. have applied high-resolution mass spectrometry (HRMS), using an Orbitrap mass analyzer, to detect adamantanes [118,122]. In comparison to LRMS, HRMS has the advantage that, especially for low-molecular weight antiviral drugs, higher selectivity is obtained due to available information on the exact masses, and also, untargeted data is generated to search for other antiviral drugs outside the targeted scope. However, the main disadvantage, especially for antiviral drugs that are unauthorized in several parts of the world, is the lower sensitivity in HRMS. Furthermore, Nakato et al. are the only authors who used ultraviolet (UV) as a detection technique for antiviral drugs in an animal-derived matrix [58]. However, the main disadvantage compared to mass spectrometric methods is that, according to (EU) 2021/808, UV is not suitable on its own for use as a confirmatory method [108].

Many authors have evaluated their analytical methods, but only seven of them have been fully validated according to either (EU) 2021/808 [108], CD 2002/657/EC [130], International Conference on Harmonization guidelines or Syoku-An No. 1224-1 [131]. (EU) 2021/808 is the follow-up to CD 2002/657/EC in the EU and it has changed in several aspects, such as validation levels, criteria for performance characteristics, and calculations for decision limit (CCα) for forbidden substances. Thus, a direct comparison of the CCα levels between these two regulations is not possible for antiviral drugs. Nevertheless, it can be observed that generally, the multi-residue methods have the highest LOD up to 3.1 μg kg^{-1}. Naturally, the more analytes included in the analytical method, the more compromises are needed in terms of sample preparation, as well as dwell time in MS analyses.

4.3. Application to real samples

Most of the authors have applied their developed chromatographic methods to real samples, although for screening assays and sensors, this has been done to a limited extent. As mentioned before, most available methods (Tables 2 and 3) target amantadine in the applied samples. Among them, 670 poultry muscle, 107 chicken eggs, 10 chicken liver, and 563 honey samples were analyzed. Amantadine was detected in 0.02 % of the poultry muscles [68,69,110,113,119,121,122], 13.1 % of the chicken eggs [111,121], 50 % of the chicken livers [111] and 0.4 % of the honey [114]. Among all samples analyzed (Tables 2 and 3), amantadine concentrations were found in the range of 0.53–100 μg kg^{-1}. The high percentage of positive results for amantadine in chicken liver is not representative due to the limited number of samples analyzed. Generally, it can be observed that the more samples were analyzed for amantadine for a specific animal-derived matrix, the lower the positive rate. However, many other factors may contribute to the varying detection rate of amantadine in the animal-derived matrix, such as the time of sampling, geographical region of sampling, storage of sample, sensitivity of method and detection criteria, etc. Ribavirin was found by Xu et al. in 6 % of the 50 analyzed chicken muscles [127], while Zhang et al. have detected acyclovir in 1.7 % of the 60 analyzed chicken muscles [113], and Mu et al. detected ganciclovir and imiquimod in 4 % and 1 % of the 100 analyzed chicken muscles, respectively [110]. Additionally, rimantadine was detected by Decheng et al. in 1.1 % of the 90 analyzed animal-derived feedstuffs [116] and by Wang et al. in 0.18 % of the 563 analyzed

honey [114]. Besides, moroxydine, memantine, and ribavirin were found in honey by Wang et al. in percentages of 1.2 %, 0.89 %, and 0.26 %, respectively. Nevertheless, memantine was only detected at very low concentrations in the range of 0.21–0.28 μg kg^{-1}, whereas the concentrations of ribavirin and moroxydine were in the range of 5.7–14.9 μg kg^{-1} and 0.60–28.3 μg kg^{-1}. Moreover, saquinavir was found by Shen et al. in 16.7 % of the 12 analyzed yellow catfishes, at low concentrations ranging from 0.90 to 3.2 μg kg^{-1} [115]. It must be mentioned that amongst the samples analyzed by authors, the abovementioned positive results were based on samples originating from the Chinese (local) supermarkets or farms. However, a few studies performed on samples from Ugandan farms also resulted in positive results of antiviral drugs, specifically HIV drugs [58,59]. In fact, efavirenz and nevirapine were found in 13.6 % and 13.8 % of the 361 analyzed porcine plasmas, respectively [58], and saquinavir and lopinavir were detected in the range of 3.0%–17 % and 1.5%–6.9 % in the analyzed chicken- and pig-derived matrices, respectively [59].

Unfortunately, none of the authors of the detected antiviral drugs had analyzed different matrices from the same animal that could lead to useful information on the most suitable matrix to detect antiviral drugs. Antiviral drugs that were not detected in the listed samples include oseltamivir, oseltamivir acid, arbidol, arbidol sulfone, arbidol sulfoxide, zanamivir, peramivir, laninamivir, favipiravir, viramidine, ritonavir, indinavir, tenofovir, somantadine, famciclovir, penciclovir and triazole carboxamide. Apart from the antiviral drugs found in the Chinese and Ugandan markets, and the non-found antiviral drugs in the Irish and Japanese markets [64,117], there is a lack of knowledge on the application of antiviral drugs in food-producing animals in other parts of the world including Europe. No Rapid Alert System for Food and Feed (RASFF) notifications have been found for antiviral drugs in Europe, which is likely due to the lack of available methods and control of these substances in animal-derived matrices.

5. Conclusions and future perspectives

This review provides a comprehensive outlook on the current state of animal viruses and the disease control measures; the effectiveness, pharmacokinetics, and misuse of antiviral drugs; the screening assays and sensors (published 2016–2023) and mass spectrometry methods (published 2012–2023) for the determination of antiviral drugs in animal-derived matrices as well as their application. Based on this review, the scope for analytical method development to implement in food control laboratories could be prioritized to some extent although a lack of information has also been observed in certain areas.

The suggested prioritization is based on the information that is available from the veterinary field perspective and takes into account 1) common animal viruses and information on what antiviral drugs could be used for these specific diseases, 2) detected antiviral drugs in animal-derived matrices through the application of the methods and misusage, 3) the scope of published analytical methods, 4) costs of antiviral drugs and 5) availability of antiviral drugs. Bearing in mind these criteria and the available information to date, the following classes and specific antiviral drugs are within the targeted scope: influenza drugs (amantadine, rimantadine, memantine, oseltamivir, zanamivir), broad-spectrum drugs (ribavirin, arbidol, moroxydine), herpes drugs (acyclovir, ganciclovir), immunomodulator (imiquimod) and antiretroviral drugs (saquinavir, lopinavir, efavirenz, and nevirapine). The analysis of these substances is currently recommended in chicken and porcine muscle.

However, to further prioritize antiviral drugs and animal-derived matrices, it is essential to perform animal exposure studies to have more pharmacokinetics information, such as the metabolite ratio to be formed in different food-producing animals, when applicable, as well as the distribution ratio of antiviral drugs in different animal-derived matrices. Furthermore, a higher number of antiviral drugs should be included in analytical methods since there are many more authorized antiviral drugs in humans of which a lack of information on their use in food-producing animals is notable. However, to extend the scope, advances in sample preparation and chromatographic methods are required, especially when including different classes of antiviral drugs with other properties, such as polarity and pKa values. Also, the importance of measuring antiviral drugs in honey and aquaculture should not be underestimated despite the limited studies that have been focused on these animal species. Finally, the monitoring of antiviral drugs should be extended to countries other than China, Japan, Uganda, and Ireland to observe if they pose a food safety risk in the rest of the world. These research gaps should serve as the next point of attention by research institutes and food control laboratories.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Samantha Sasse: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Ane Arrizabalaga-Larrañaga:** Writing – review & editing, Supervision, Investigation, Conceptualization. **Saskia S. Sterk:** Writing – review & editing, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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