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STUDY OF HIGH EFFICIENCY SYSTEMS TO RACTOPMINE DETECTION IN ENVIRONMENTAL SAMPLES

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Abstract

The concern about the effects of growth promoter drugs exposure, such as ractopamine, has mobilized the scientific community in the search for methods to detect this compound in different matrices. The ractopamine is a β-adrenergic agonist largely used in intensive farm. Studies demonstrates that 95% of the quantity of this ingested drug are excreted in the first 3 days in swine and 55% in cattle are excreted in feces, while 10% and 45% respectively are excreted in the urine, being detected until 2 weeks after the treatment. The present study aimed to propose the development of ractopamine extraction, cleaning and detection methodology to environmental samples (water and wastewater) by a biographical evaluation about it determination in different matrices. For the cleaning and extraction/purification different techniques must be tested, for example the immunoaffinity collumn (IAC) to waters and an association of extraction liquid-liquid (LLE) followed by IAC to wastewaters, because they are complex samples. For the detection, a methodology that uses high performance liquid chromatography with detection by fluorescence (HPLC/FLD) was proposed, because it is less expensive when compared to other high efficiency systems, as liquid chromatography/mass spectrometry (LC/MS) and gas chromatography/mass spectrometry (GC/MS). However, the challenge consists in establishing of a method that achieves the validation rules and it is economically feasible to application not just for research, but in industry and government control departments, mainly in developing countries.

Key words: *ractopamine, chromatographic method, environmental samples*

INTRODUCTION

According to data from Abipecs - Brazilian Association of Producers and Exporters of Swine, the world production in 2011 was 101.127 thousand tons in carcass weight equivalent. Brazil is the 4th largest producer (3.227 thousand tons), behind United States, European Union and China, and is also the 4th largest world's exporter [1].

The beneficial effects on the carcass growth and composition, with the use of the β-adrenergic agonists, have been largely proven, resulting in the expansion of the muscle mass, jointly with the reduction of the fatty accumulation.

Due to the new intensive farming ways, the use of veterinarian drugs in animal containment systems has been presented an exponential growth in recent years. The use of growth promoter drugs, as the ractopamine (RAC) raises a range of concerns related to safety and toxicity [3], [14], [29].

These substances are not allowed in many countries, in example of Europe Community members and China, which forbid the use of these substances to zootechnical applications. The concern with the possible effects of prolonged exposure to RAC has been mobilized the scientific community in the search of methodologies development to the search of this compound in foods of animal origin.

The use of highly sensitive methodologies has been indispensable to reach the efficient control of these substances, but there is a lack of analytic methods to detect these compounds [20]. Among the utilized methods to RAC detection, evidence the high performance chromatographic methodologies, usually from the following matrices: animal carcass (meat and viscera), blood and urine [33], [34], [39], [40], [41], [42], [46],[50].

However, methodologies that propose the extraction, cleaning and RAC detection in complex samples, whereby is the case of the environmental samples (water and wastewater) are still scarce. Furthermore, the small concentration of the compounds present in the matrices can conduct alterations in the involved stages of the process [34].

Wherefore, the greatest challenge involves the search of high performance methodologies that presents financial and technical viability, not just for the research, but also to comply with the needs of the industries and governmental organs of control.

The present study revises the utilization of the RAC β_2-adrenergic agonist as animal growth promoter, the regulation of its use as an additive in animal feed, the presence of this compound in environmental samples as well. Ultimately, an evaluation of the methodologies for extraction, cleaning and detection in different matrices was searched, particularly to chromatographic systems, aiming the proposition of a methodology to be applied mainly for emergent countries to the environmental samples.

Ractopamine

The β-adrenergic agonists or β-agonists are substances of analogous structure to hormones called catecholamine (adrenalin and noradrenalin). These compounds are phenyl β-ethanolamine with different substituent in the aromatic ring and in the terminal amine group [4]. The use was previously authorized for therapeutic purposes (humans and veterinarians), mainly as anti-asthmatics, bronchodilators and tocolytics, generalizing as nutrient repartitioning agents from 1998 [34].

They are usually applied in livestock as additives, mostly due to anabolic effects exercised when high doses are administered. They act as animal metabolism modifiers, changing the nutrients partition diverting and promoting the growth and the lean tissue accretion and reduction the fat tenor in termination swine carcass [4], [7]. Among these compounds, the RAC has been studied with more interest for the swine farming.

Among these compounds, the RAC has been studied with more interest for the swine farming. It is a compound that belongs to the phenylethanolamines group, characterized by the presence of aromatic ring, side chain of the ethanolamine and aliphatic nitrogen [4], [13], [14], [43], with solubility in polar solvents. The RAC, (4-hydroxy-α-[[[3-(4-hydroxyphenyl)-1-methylpropyl] amino] methyl] benzenemethanol ($C_{18}H_{23}NO_3$-HCl), that contains two chiral centers, exists as a mixture of four stereoisomers – RR, RS, SR and SS. The chemical structure is represented in the Fig. 1 (A) [43].

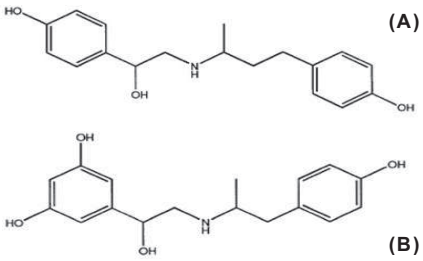

Fig. 1. Chemical structure of Racopamine (A) and Fenoterol (B). Adapted of [43]

The RAC production occurs in an only stage process, where the drug volume is produced as an aqueous solution containing between 3 and 20% of ractopamine hydrochloride. Its solubility in water varies with the pH, so the more the pH is, the more the solubility will be. It is a nonvolatile solid, with low endothermal at 180ºC and exothermal at 188ºC, coinciding with the decomposition [30].

Metabolism

The β-adrenergic agonists are largely utilized by inhalatory administration for human medicine, however, in veterinarian medicine, especially as animal growth promoter, it occurs mainly by oral administration. The low stomach pH favors the formation of a cation in the aliphatic amine, whiles the neutral nature of the duodenum, jejunum and ileum, promotes the reduction of the extension of the ionization and increases the passive absorption through the intestinal mucous [43].

The effects assigned to the RAC look like being related mostly the increase of the lipolytic activity and the inhibition of the lipogenesis, since this compound inhibits the binding of the insulin in the adrenergic receptor of the

adipocytes, and then, antagonizes the insulin action, reducing the synthesis and fat deposition [17].

The elimination of these compounds or their metabolites in the organism depends basically on the administration way. When the administration is realized by parenteral via, the renal excretion will occur, while, by oral via the previous metabolism is present in most cases [35]. Studies demonstrate that 95% of the amount of RAC that is ingested is excreted in the first 3 days: about 90% in swine and 55% in cattle are excreted in feces and between 10% and 45% in urine, respectively [13]. During the treatment, the RAC amounts in the urine vary from 44 to 473 $mg.mL^{-1}$, being detected until two weeks after the terminus of the treatment [45].

Regulation law

The regulation of drugs residues in food derived from animals is an integral component of food security programs worldwide [23]. Nowadays, different β-agonists substances are authorized for growth promoter utilization.

The RAC is allowed for the use as feed additive in 26 countries, such as the United States (where was developed by Eli Lilly®), Canada and Brazil, with the goal to promote de growth of the swine and cattle. Therefore, its use is forbidden in China and other 159 countries, including the ones members of European Union, due to the worries with its safety and toxicity [13], [14]. Its usage as additive in feed is controlled by Food and Agriculture Organization (FAO) by the *Códex Allimentarius* Commission. For the administration in the animals for the food production, the acceptable daily ingestion dosage is 0.1 $\mu g.kg^{-1}$ compared to body weight [51].

There are several documents that describe the side effects of β-adrenergic agonist in human's health, such as food poisoning related to liver residues, cardiovascular and central nervous disease [20], [25], [51].

The prohibition of the use of β-agonist as growth promoters in the European Union is regulated by the Directive 96/22/EC [8] altered by the Directive 2008/97/EC [10]. The control and monitoring must refer to the Directive 96/23/EC [9], in which the β- agonist belongs to the substances of Group A (the forbidden ones and that should be monitored). The performance of the analytical methods of monitoring as well as the interpretation of the results needs to attend the Commission Decision nº 2002/657/EC [11].

Spit of allowed use of ractopamine in Brazil, the Brazilian government with the productive sector has developed the "National Plan for Control of Residues and Contaminants" which includes the Program for Swine Production Free of RAC (Split System) in order to ensure a safe product and free RAC [6]. In the Santa Catarina State, the procedures for official examination of the self control applied in the productive chain for swine ractopamine free is regulated by the Statement Service – Nº 016/2011/GEDSA [36].

Environmental samples

The main sources of pharmaceuticals in aquatic environments are related to chemicals and pharmaceuticals residues from industrial production plants, besides the excretion (in the metabolized and non-metabolized shapes) of human beings and animals [54]. β-adrenergic agonist have been related for causing adverse effects in aquatic systems, as well as hypoactivity in *Daphnias* when exposed to high amount of RAC [12].

The presence of RAC and other β-agonists was investigated in animal feed and its drinking water, both matrices used as vehicle of drugs administration by farmers and veterinarian [20]. In this study, three samples resulted to be positive, two for RAC and one for hydroxymethylclenbuterol, in animal drinking water samples analyzed. Its presence and other veterinarian drugs was also investigated in wastewater originate from farm (swine and cattle) and subjacent underground waters indicating that this ones are inclined to contamination by these drugs in the ponds of residue water treatment [2]. The occurrence and distribution of pharmaceutical products, including RAC, in hospital wastewater and superficial waters before de flush point were investigated by [54]. In this case, although the composed studies have been detected in lower concentrations, in the superficial waters there is

potential risk to the health of human beings and animals.

Shao et al. [37] investigated the occurrence of 76 pharmaceutical drugs, including many β-agonists in slaughterhouse wastewater (influent and effluent) and a receiving river. Clenbuterol was founded at high amounts (~11 $ng.L^{-1}$) in the slaughterhouse wastewater plant located in the Tongzhou District, indicating the clenbuterol use from pig farms in that region. The high concentration of metoprolol (β-blocker) found in the outfall of swine farming suggests that this drug is illicitly used as growth promoter at this farm. The residual concentrations of drugs in the effluent from farms can be used as indicator of veterinary drug use at such venues.

High performance analytical methods for rac analysis

The analytical methods used to monitor veterinarian drugs are essential in the animal and human health protection, control of consumer exposure to drugs, reduction of chemicals impact in environment and support to the execution of laws and regulations that facilitate the international trade of food products of animal origin [23].

The immunochemical screening methods, such as radio and enzyme immunoassays [38], [41], [49] are very sensitive but the cross-reactivity properties of these tests with structural analogs of the controlled residues preclude a non ambiguous identification of the compounds in the complex matrix of a biological sample [48].

The demand of the best possible method has been a constant concern of many research groups. Thereby, a strategy perfectly defined can be observed: in one hand, there is the development of methodologies capable to determine the biggest possible number of multiresidues substances and, particularly in this case, the β-adrenergics [26]; in other hand, there is the utilization of quick detection methodologies, followed by compounds identification and posterior confirmation, always using spectrometric methods [19].

The composition complexity of the matrices and the small quantity of xenobiotics that are normally present ($ng.kg^{-1}$), sometimes modifies the process that conduces the evaluation of residues in general, and the β-adrenergic, specially, in an expensive process. In the area of substances which must have controlled use, like the β-adrenergic agonists, there is a continuous trend to tests development to detect illegal substances residues in trace levels. These methods involve more intensive samples preparation to make the detection of low concentrations residues possible. Sampling stages, pretreatment and, above all, extraction and/or purification have been showing to be of big importance to residues analysis of β-agonists, independent of the chromatographic method to be utilized [34].

Sampling

The sample integrity must be kept, preserving its physical characteristics and chemical composition [52]. The sample has to be representative and big enough to allow an appropriate analysis, the repetition and respective tests confirmation [34]. Due to the complex nature of biological matrices, the stages of sample preparation are the most important part that integrates the bioanalytical methods [27].

Samples for drug screening belonging to Group A [9] have to include matrices like plasma/serum, urine, feces, water, animal food, bile and thyroid gland [21]. When it is investigating the occurrence of β-agonists in the environment, the main points to be considered must include the effluents from farming of animals (swine and cattle), slaughterhouses and hospitals, besides the discharge points of these effluents (rivers) and groundwaters [2], [37], [54].

One of the main problems in biological samples is related to the instability of compounds and metabolites present in this kind of samples. The stability can be affected by the storage temperature, sample pH, anticoagulants and cycles of freezing/defrosting [27]. The time between the collect and analysis is a critical factor to be considered, once the substance instability or the matrix constituents can take it to significant mistakes in the analysis result [11]. The stability to effluent samples, was studied by [24] which were initially filtered with a 1.2 μm filter and divided in six subsamples of 2 L each. Four samples were

spiked with a mixture of β-agonists (including the fenoterol, that shows a structure close to the RAC (figure 1-B) and β-blockers (250 $ng.L^{-1}$ of each compound), storage at 4 ^{o}C in amber glass bottles, which were extracted and analyzed at 24h, 2, 4, and 7 days after the spiking, respectively; the authors observed that there were no alteration in the analytes.

Extraction/Cleanup and Purification

The preparation step of the sample is essential to get trustable results and, maintenance of device performance. Moreover, this procedure is slow and expensive, existing the need of a very efficient cleaning method when a high transference tax is required [40].

The liquid samples (blood, plasma, serum, bile, milk, water) are processed more easily once the present residues are found distributed with more homogeneity [21]. Centrifugation or filtration to eliminate the suspended substances, and eventual dilution are the main stages of pre-treatment to liquid samples. The homogenization is a very important step, mainly about the results accuracy. [34].

Different procedures are necessary to prepare the β2-adrenergic agonists samples to posterior chromatographic determination, the methodologies most commonly employed to extraction and cleaning and/or purification are: liquid-liquid extraction (LLE) and solid phase extraction (SPE) and a variant of this last, immunoaffinity column (IAC).

Liquid-Liquid Extraction

LLE was one of the first techniques used for preparing of biological samples [27], and one of the more classical extraction/purification. For the class of β2-adrenergic agonists are several studies with this process. For RAC, LLE has been used by several authors in different biological matrices, such as animal feed [14], [20], [18], tissue [33], [39], [40], [42] and urine [33], [41].

Solid Phase Extraction

SPE is one of the tools most often used for the extraction and/or pre-concentration of analytes present in complex matrices [32]. This technique has been preferred because it is a rapid method requires low volume of organic solvents, a low risk of contamination and can be used in online system system [52]. The retention and elution are thus faster, and the latter is even possible with a smaller amount of solvent than in the classic SPE. To the analysis of β2-agonists, SPE can be the only method in extraction/purification step or in association [34], such as ELL [33], [42] or IAC [22].

To extract and cleanup veterinary drugs, including β_2-agonists and β-blockers from environmental samples (water and wastewater), has been commonly employed technique of SPE individually [20], [37], [54] by online system [2] and in association LLE [24].

Immunoaffinity Collumn

IAC provides a selective method to isolate and concentrate analytes from complexes matrices. It is particularly advantageous when low detections levels in the $\mu g.kg^{-1}$ to $ng.kg^{-1}$ are required for banned substances, particularly when using less selective HPLC based detection systems [21]. This technique has a wide range of applications for a variety of chemical classes, including β-agonists in different matrices, such as: ractopamine in urine, liver, muscle and kidney of food animals [44]; clenbuterol from bovine pelage [15], urine [16], [31] liver and muscle [22]; salbutamol from human plasma [28].

Detection

The use of GC-MS confirmatory analyzes is recommended for β-agonists. But the methodology for β-agonists requires sample derivation, because of their high polarity and low volatility, which is a time-consuming, tediousness, laborious and expensive process. Also, quantitative results are significantly affected by sample purity when GC–MS is used for this analysis [20]. Therefore, GC methods are not the most suitable for the β-agonists screening in environmental samples [24].

Several studies report the use of LC/MS or LC-MS/MS for the analysis of β-agonists in environmental samples [2], [20], [37], [54]. Spite of the high sensitivity in the confirmation of residues in complex samples, these methods are much expensive yet.

Despite HPLC use to be very widespread, but any research study of RAC by HPLC was

found during this review. It is able to separate highly complex mixtures of compounds with different molecular weights, as well as several matrices with different polarities and acid-base properties. The most used detectors to the RAC determination are shown in Table 1.

Table 1. HPLC methods for RAC analysis

Detection	Column	Mobile phase	Matrice	Clean up	Author
HPLC/EL	C_{18}	Ammonium phosphate buffer 0,05M pH 4,5: acetonitrile (79:21,5)	Serum, plasma	SPE	[46]
HPLC/FL	C_{18}	Acetonitrile: water: acetic acid (280:720:20, v/v/v/) and 1.08 g 1-octanesulfonic acid/L	Urine, liver, muscle, kidney	SPE, IAC	[40]
HPLC/FL	C_{18}	Acetonitrile: water: acetic acid (280:720:20, v/v/v/) and 1.08 g 1-octanesulfonic acid/L	Tissue and urine	SPE	[39]
HPLC/FL	C_{18}	0.005 M sodium octanesulfonate in a mixture of water: acetonitrile: acetic acid (71:27:2).	Urine	SPE	[44]
HPLC/FL	C_{18}	5 mM sodium octanesulfonate in 2% acetic acid in water/acetonitrile (72:28, v/v)	Urine	SPE	[41]
HPLC/FL and LC–MS/MS (to confirmate)	C_{18} C_{16}	Acetonitrile: deionized water: glacial acetic acid (320:680:20, *v/v/v)*and 0.87 g of 1-pentanesulfonic acid, 0mM ammonium acetate buffer, pH 4.5: acetonitrile, gradiente flow	Muscle tissue	LLE, SPE	[42]
HPLC - UV/DAD	C_{18}	0.017 M phosphoric acid brought to pH 2.8 with diethylamine: acetonitrile-water (80:20, v/v), gradient flow	Commercial feed	SPE	[6]
HPLC/FL	C_{18}	Acetonitrile: deionized water: glacial acetic acid (320:680:20, *v/v/v)*and 0.87 g of 1-pentanesulfonic acid	Liver and muscle tissues	LLE SPE	[47]
HPLC/FL	C_{18}	Acetonitrile: deionized water: glacial acetic acid (320:680:20, *v/v/v)*and 0.87 g of 1-pentanesulfonic acid	Animal feed	LLE	[49]
HPLC/FL	C_{18}	Water: acetonitrile (80:20, v/v) with 2 mL of acetic acid and 0,7 g of pentanosulfônic acid/L	Tissue, urine, serum	LLE SPE	[33]
HPLC/FL	-	Water solution (containing 2% acetic acid and 0.087% pentanesulfonste sodium salt): acetonitrile (80:20, v/v)	Tissue	SPE MIPs	[50]
HPLC/UV	C_{8}	Acetonitrile: sodium acetate buffer 0.1 $M.L^{-1}$ pH 5.0 (25:75, v/v)	Ractopamine raw material and Ractosuin® product	SPE	[14]
HPLC/UV	C_{18}	Acetonitrile: monobasic sodium phosphate buffer 0.1M L^{-1} pH 7,6 (26:76, v/v)	Vitamin Mineral Complex and Ractosuin® product	LLE	[14]
HPLC/UV	C_{18}	Acetonitrile: ammonium formiate buffer 0,02 M pH 3,0 (20:80, v/v)	Animal feed	LLE	[14]

CONCLUSIONS

Through this work a general methodology that involves the steps of extraction, cleanup/ purification and detection of RAC for environmental samples is proposed. The care of the pretreatment of the samples are indispensable, the extraction right after the collect and storage becomes impractical and the validity of the analytical results depends totally on the integrity of the interest compounds. After homogenization, the samples must be prepared with the addition of a solution of extraction (LLE). For the extraction of RAC it is believed that there is no necessity of the enzymatic hydrolysis, then the acid hydrolysis should be tested, since RAC has a pKa = 9.4 and compounds similar studies were successful, making the method less expensive in time and economic view point. In order to the steps cleanup and purification filtration is suggested (at least for the samples of wastewater *in nature* and post treatment) followed by application to the immunoaffinity column with bound specific antibodies. The test with commercial RAC-IAC is here suggested, since they provide specificity and are for single use (a procedure that avoids cross-contamination) as well as providing a chromatogram with less interference which shows greater selectivity. The eluent of choice for marketed IAC's is the methane, however, one must carry out a comparative test with buffer solution of glycine, in the study of [40] provided cleaner chromatograms when compared to methanol by coelution of fluorescent compounds.

The HPLC detection is proposed to be the high efficiency equipment easier accessibility. The fluorescence detector was nominated for its performance and for being the most widespread in the literature for RAC. No entanto, cabe aqui ressaltar nenhum estudo com o uso de HPLC para detecção de RAC em amostras ambientais foi encontrado durante a presente revisão.

This review showed itself essential for allowing the proposal of developing a methodology for

fast and high sensitivity for complex matrices such as environmental samples. However, the challenge consists in establishing of a method that achieves the validation rules and it is economically feasible to application not just for applied research, but in industry and government control departments, mainly in developing countries.

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