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Ractopamine: analytical method validation; and the detection in loin, tissues and urine of pigs fed meat and bone meal containing this growth promoter

Carolina Naves Aroeira

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

Piracicaba
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Veterinary Medicine

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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To my parents Nádia and Hélio, for their love, support and education

To my sister Ana Paula and my niece "Mel", for their love and support

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I'm finishing my big dream and I wish to begin a lot of others!

CONTENTS

RESUMO

Ractopamina: validação do método cromatográfico, detecção em lombo, tecidos e urina de suínos alimentados com farinha de carne e ossos contendo este promotor de crescimento

O cloridrato de ractopamina (RAC) é um aditivo β-agonista que tem sido usado em muitos países para redirecionar nutrientes a fim de aumentar a deposição de tecido muscular e diminuição de lipídios em suínos. Países da União Européia e Ásia questionam sua segurança, enquanto os países da América e a Austrália permitem seu uso controlado como um aditivo adicionado à ração na fase de terminação em suínos. No Brasil, o Ministério da Agricultura, em conjunto com o setor produtivo nacional, desenvolveu um programa chamado "SplitSystem" para garantir um produto seguro sem RAC, a fim de atender aos requisitos sanitários internacionais. No entanto, os co-produtos utilizados na ração animal podem conter RAC, como farinha de carne e ossos (FCO), um dos principais ingredientes utilizados em muitos países para substituir parcialmente o farelo de soja, a fim de reduzir os custos de produção. Como o nível de RAC nessa fonte de proteína não foi estabelecido, um experimento foi conduzido para examinar o impacto sobre os tecidos suínos que receberam níveis crescentes de FCO, divididos em quatro grupos: 0, 7, 14 e 21% de FCO contendo 53,5 µg kg^{-1} de RAC, na dieta dos animais. O objetivo foi verificar se resíduos de RAC permanecem nos tecidos suínos (lombo, fígado, rim e pulmão) e o quanto é eliminado através da urina. Para atender a essas preocupações, as leitoas foram alimentadas com RAC via FCO diariamente, desde o desmame até o abate. A RAC foi determinada em lombos, rins, fígados e pulmões com um limite de detecção (LOD) = 0,15; 0,5; 0,5 e 1,0 µg kg^{-1}, respectivamente, e nenhum resíduo de RAC foi quantificado acima do limite de quantificação (LOQ) = 0,5; 2,5; 2,5 e 2,5 µg kg^{-1}, respectivamente. Na urina, a concentração de RAC permaneceu abaixo de 1,35 µg L^{-1}. Estes valores são inferiores aos limites máximos residuais (LMRs) estabelecidos pela legislação. Concluindo que a FCO (53.5 µg kg^{-1} de RAC) pode ser utilizada com até 21% em rações para suínos, entretanto, ao considerar mercados restritivos, recomenda-se não usar a FCO.

Palavras-chave: β-agonistas; Segurança dos alimentos; Suinocultura; Coprodutos; Metodologia de detecção; LC-MS/MS

ABSTRACT

Ractopamine: analytical method validation; and the detection in loin, tissues and urine of pigs fed meat and bone meal containing this growth promoter

Ractopamine hydrochloride (RAC) is a β-agonist additive that has been used in many countries as a repartitioning agent, redirecting nutrients in order to increase leanness and decrease lipid deposition in pigs. Countries from the European Union and Asia question their safety, while American countries and Australia allow their controlled use as an additive added to the feed of pigsin the finishing phase. In Brazil, the Ministry of Agriculture, Livestock and Food Supply together with the national production sector, developed a Program called "SplitSystem" to ensure a safe product without RAC in order to meet international sanitary requirements. However, co-products used in animal feed may contain RAC, such as meat and bone meal (MBM), one of the main feed ingredients used in many countries which can partially replace soybean meal to lower costs. As the level of RAC in this protein source has not been established an experiment was under taken to examine the impact on pig tissues of increasing amounts of meat and bone meal (MBM) in four dietary groups: 0, 7, 14 and 21% w/w of MBM-containing RAC (53.5 µg kg^{-1}) in the diet. The purpose was to verify if ractopamine residues remain in pig tissues (muscle, liver, kidneys, and lungs) and how much is eliminated through urine. To addrress these concerns, gilts were fed RAC via MBM daily, from weaning until slaughter. RAC was determined in muscle, liver, kidneys, and lungs with a limit of detection (LOD) = 0.15, 0.5, 0.5 and 1.0 µg kg^{-1}, respectively), and no RAC residues were quantified above the limit of quantification (LOQ) = 0.5, 2.5, 2.5 and 2.5 µg kg^{-1}, respectively). In urine, RAC concentration remained below 1.35 µg L^{-1}. These values are below the maximum residue limits (MRLs) established by legislation. Therefore, MBM (53.5 µg kg^{-1} of RAC) can be used up to 21% in pig diets, however when considering restrictive markets, it is recommended not to use MBM.

Keywords: β-agonists; Food safety; Pork production; Co-products; Methods of detection; LC-MS/MS

1. INTRODUCTION

The progress of a country is measured partly by the economy, which has a direct consequence on the welfare of the population. The gross domestic product (GDP) denotes the aggregate value of all services and goods produced within a country and it's an important indicator of a country's economic power. In this context, Brazilian livestock and agricultural production is very important. These were the best performing in 2017 among the national sectors, representing 13% (Brasil 2018).

Brazil has a high level of representation and growth in agriculture and livestock; the country has very important production in several crops, beef, broilers and pork. Expectations for further growth are driving a major expansion in pork production across countries as outlined by the United States Department of Agriculture (USDA), of which Brazil is the 4th largest pork producer, behind China, the European Union and the United States of America (USDA 2018). National pork consumption in Brazil rose by nearly 16% from 2007 to 2015 (Brazilian Association of Animal Protein [Associação Brasileira de Proteína Animal; portuguese acronym: ABPA, 2016). Worldwide the consumption of pork is greatest with 110 million metric tonnes (mmt); for chicken and beef this value corresponds to 104 and 67 mmt, respectively (McGlone 2013).

According to Bridi et al. (2006), Brazil is a country with good forecasts for increased pork production, mainly because it’s one of the largest producers of soybeans and corn, among other important crops. In most countries, these ingredients contribute up to 70% of pig diets. However, the high cost of commodities has encouraged pork producers to replace protein sources with more economical options. One of the alternatives is meat and bone meal (MBM) to partially replace soybean meal in animal diets. MBM is a major abattoir co-product, and it’s an excellent source of protein (crude protein content between 35 and 55%) and minerals, such as calcium and phosphorus (Vieites et al. 2000). According to Brasil (1962), the MBM must contain at least 40% protein, at maximum 10% moisture and 10% fat. Among the different types of meal, MBM is the dominant type, totaling 60% of the meals produced in Brazil (ABRA 2016).

This high cost of commodities also has encouraged pork producers to using a growth promoter in the pig diets such as ractopamine (RAC) which is a commercially available β-agonist that is supplied as an additive for pig diets. RAC acts as a repartitioning agent that redistributes the nutrients that would be destined to the synthesis and deposition of lipids to favor protein synthesis due to an alteration of cell metabolism (Peterla and Scanes 1990) providing lower adipose tissue deposition in carcasses and a higher percentage of lean meat (Bohrer et al. 2013). In a meta-analysis by Apple et al. (2007) it was shown that pigs fed RAC had an improved feed efficiency and the average daily feed intake was numerically reduced when compared with the untreated controls.

RAC demonstrated some advantages in the pig production, for instance: increased weigh gain, improved feed efficiency, reduced fat thickness, increased depth the loin eye area and meat: fat ratio (Marinho et al. 2007; Cantarelli et al. 2009). However, the use of RAC is controversial among the countries. For instance, in Brazil the use of RAC was approved (Brasil 2015), also by USA, Australia,

Canada and Mexico as a commercial feed additive (Valese et al. 2016), while China, Russia and European Union have banned their use (Pacelle, 2014).

RAC was approved in the United States by the Food and Drug Administration (FDA), in 1999 (Vulic´ et al. 2012). The Codex Alimentarius Committee (Joint FAO/WHO Expert Committee on Food Additives 2012) established maximum residue limits (MRLs) of RAC in pig muscle (10 ppb); fat (10 ppb); liver (40 ppb); kidney (90 ppb) and urine (90 ppb), after an extensive debate. Brazil follows these recommended MRLs. In Brazil, RAC has been added to diets in the last 28 days before slaughter without a withdrawal period, and is approved at up to 20 ppm in pig diets (Brasil 2015).

Thereby, there is a concern about the use of RAC in animal feeds in order to meet these restrictive countries. Furthermore, the use of MBM in animal feeds may lead to RAC residues. Study by Gressler et al. (2016) showed the presence of RAC in MBM at the concentration of 3.87 to 81.25 $\mu g\ kg^{-1}$, collected at four renderers in the South of Brazil. There are no reports on the presence or absence of RAC in feeding pigs with MBM containing RAC residue. Livestock production in Brazil is important to global trade in food (Ferraz and Felício 2010) and in order to maintain export markets for pork and its derivatives, it's necessary to assure the absence of RAC. Thus, the objective of this study were develop an analytical method and then validate it and apply it in pig tissues by determining RAC residues, and evaluate the safety of MBM containing RAC in pig feed, quantifying the RAC residue in loin, urine, kidneys, liver and lungs of pigs.

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2. LITERATURE REVIEW

2.1. Pork production in Brazil

Brazilian agribusiness has always been a strategic sector and as such has played relevant roles in the structural configuration of Brazilian society as well as in the evolution and performance of the economy (Buainain and Garcia 2015). In 2017, Brazilian Agribusiness GDP volume, reached 7.2%, and it has largely contributed to the Brazilian economy due the high availability of food, fiber and energy, guaranteeing the domestic supply and an increasing volume to export, as reported by the Center for Advanced Studies on Applied Economics (Gilio and Rennó 2018).

Consumption of meat increased globally by 230% between 1995 and 2008 (FAO 2010) and pork demand and other meats increased worldwide. In Brazil, pork production is 3,755 million metric tonnes (mmt) and the total domestic consumption is 2,927 mmt making Brazil the 4th largest producer, and the 5th largest pork consumer in the world (USDA 2018). In 2016, Brazil exported 19.6% of its pork production to several countries like China (ABPA 2017).

China is the biggest pork-consuming accounting for 56,115 mmt in the own country (USDA 2018). McOrist et al. (2011) affirms that for Han Chinese pork is the meat of cultural choice which corresponds to 70% or more of meat products consumed in that country. China has been a good market to support Brazilian pork exports (Wilkins 2018). One good alternative for the export of pork will be countries which have a high consumption of this protein and some studies have suggested countries such as South Korea, Mexico and Japan. Brazil has good scope to continue to grow in the global pork market once it has advanced productive supply chains, the pork exporters embrace high-technology processing and total control in processes for the production of high-quality meat. High-quality, industrial production (as opposed to subsistence production) has increased and accounts for 90% of overall Brazilian pork production (Barcellos et al. 2011).

The Southern region of Brazil is composed of Rio Grande do Sul, Santa Catarina and Parana States, where the majority of production takes place representing nearly 70% of the pig slaughter and 80% of pork exports according to estimates by ABPA (2017). There are no official statistics, but it can be estimated that 88% of the technologically advanced pig farms are vertically connected to contracts or prize programs from agri-food industries and cooperatives (Barcellos et al. 2011). This is dominant in the Southern region, but is also growing in the Southeast and Central-West regions, which have recently developed as pork production regions.

In the Southern region there is a predominance of small integrated pig farms and in the Southeast region there is a predominance of full cycle production, and each of these systems has been gaining efficiency and competitiveness, maintaining a constant growth of the national production, according to Brazilian Association of Pig Breeders [Associação Brasileira dos Criadores de Suínos (portuguese acronym: ABCS). ABCS has estimated that pork production companies employed around

126,000 people in 2015, and in this same year more than 29 million pigs were slaughtered, such that the GDP for pork production in Brazil was approximately US$ 18 billion (ABCS, 2016). The Free-Range Pig Production Intensive System (Sistema intensivo de produção de suínos criados ao ar livre, or SISCAL in Portuguese) is practiced among several producers in Brazil which is the main national free-range commercial housing system used. Compared to confinement systems, the SISCAL has a high technical performance, provides a reduction in building costs to house the pigs and easiness in expanding facilities (Humane Society International, no date).

Production has changed from a small, subsistence model to larger concentrated animal feeding operations. According to McGlone (2013) developing countries are rapidly moving pork production to the industrialized model. The number of piglet/sow/year has been increasing every year due to a parallel increase in the number of piglets born and weaned by each sow during each production cycle (Rocadembosch et al. 2016). Simultaneously, consumer demand has also changed and there is a preference for leaner pigs (i.e. pigs with low fat content) or fattening homogenous batches of pigs (Støier et al. 2016). According to Almeida et al. (2012) the demand for leaner and healthier pork products has risen, and some improvements especially in nutritional technologies, like the use of β-adrenergic agonists have become important in helping pork producers to meet this demand.

During the pig finishing phase RAC can be used in Brazil (Brasil 2015). The use of RAC in the pig finishing phase has resulted in some production advantages (i.e. improvements in average daily gain and gain: feed) (Armstrong et al. 2004). The fattening period is one of the most important stages in the pig production chain, because this phase impacts on the end product and its duration depends on several factors, such as starting weight, batch homogeneity, feeding system, growth curve, reward system and chain management (Rodríguez-Sanchez et al. 2018).

2.2. Co-products: meat and bone meal and ractopamine in Brazilian pig production

Commodities like cereals are the most important food source for human consumption currently 2.3 billion tonnes produced per annum. From this amount, roughly 1 billion tonnes are destined for food, 750 million tonnes are utilized as animal feed, and the remaining 500 million tonnes are processed for industrial purposes (FAO, no date).

The pork industry is facing lower profit margins per pig, or negative profits with prices lower than marginal production costs, from time to time. Therefore, economically rational decisions should be based on an assessment of costs and benefits at the producer level (Rocadembosch et al. 2016). Describing the values for production parameters and pig production costs from 2010 to 2014, they reported that the production performance continuously improved in terms of piglet production and the fattening phase, but despite this no reduction happened in pig production costs due to high

feed prices. Gottlob et al. (2004) reported that due the high soybean-meal prices meat and bone meal (MBM) can be a potentially important alternative protein source.

MBM is composed of > 50% protein and 20–30% ash, which contains inorganic materials such as minerals (Johnson et al. 2011). To be used in animal diets, MBM should have specified the species from which it came from. According to a Ministry of Agriculture, Livestock and Supply (MAPA) directive number 34 from May 2008, if species separation is not possible during MBM processing, the final product must be called mixed meal with the animal species identified in the label (Brasil 2008).

There is no exact database about the amount of meal used for pork production in Brazil, because according to the Brazilian Association of Animal Recycling (ABRA) there is no official control and monitoring of this use (ABRA 2016). Some of the MBM produced nationally is subjected to the federal inspection system carried out by MAPA, but this does not cover all establishments.

The distribution of factories that produce MBM by region in Brazil indicates that the largest numbers are in the South and Southeast regions. In addition, the South region accounted for 65.4% of the national slaughter of pigs in 2016 followed by the Southeast with 19.0%, Central-West with 14.3%, Northeast with 1.1% and North with 0.2%, (IBGE 2016).

There are no data about the level of RAC consumption by pig produced in Brazil. However, there are data about the amount of growth promoters used which was estimated as 4,400 tonnes in 2017 (Sindirações 2016). The use of RAC in pork production is regulated by MAPA and the maximum residue limits (MRLs) have been monitored since 2007 by the National Program for the Control of Residues and Contaminants (Brasil 2018). This Program comprises veterinary drug residues, inorganic contaminants, pesticides and biological contaminants in samples of animal origin.

This monitoring by MAPA occurs mainly in establishments that are reported as "ractopamine free", and which provide pig diets without RAC, and they are included in the Official Program for Verification of Non-Consumption of Ractopamine by Pigs. Based on the last update in March 2017, it was reported that there are currently 71 establishments in Brazil that guarantee the supply of pig diets without RAC residues, and of these approximately 60% are located in the Southern region of Brazil (Brasil 2017). As well as RAC used in animals diets the MBM must be traceable otherwise it may become a barrier for the export of pork products. However, this restriction is stronger for ruminants mainly due to bovine spongiform encephalopathy (BSE), as according to Johnson et al. (2011) MBM can be responsible for the spread of BSE, and as such bovine MBM, is banned for consumption by ruminants in Canada, the United States, and most European Union countries, but is still consumed by monogastric livestock (i.e. pigs) in many countries, and the ruminant consumption of MBM derived from nonruminant species remains permitted in some countries. The national market follows the rules of the International Zoosanitary Certificate (CZI) administered by MAPA, and must follow the specific standards for the importer’s country.

2.3. Ractopamine properties, carcass improvement and meat quality

A wide range of studies have been undertaken in which β-agonists including: cimaterol, clenbuterol, fenoterol, isoprenaline, mabuterol, salbutamol, terbutaline, zilpaterol and ractopamine (RAC) have been studied. They are derivatives of catecholamines, epinephrine and norepinephrine hormones; in veterinary medicine β-agonists have an important use for therapeutic purposes as bronchodilators and tocolytics agents (Pleadin et al. 2012). In animal production systems, these substances are used to enhance growth efficiency by stimulating the β-adrenergic receptors on cell surfaces. According to Dunshea et al. (2005) they are absorbed into the blood and bind to these specific β-receptors on the muscle cell membrane and the primary response is to increase the muscle fibre size. They act as repartitioning agents to modify carcass composition by altering nutrient partitioning to increase muscle protein content up to 40% and lower fat deposition up to 40% (Herago and Agonafir 2017) due the inhibition of proteolysis and enhanced lipolysis, respectively (Divari et al. 2017).

The actions of β-agonist occur in cells which express their specific receptors, called β-adrenergic receptors (β-ARs). The distribution of β-ARs in many tissues is species-specific and the activation of the different types of β-AR depends specifically on the target tissue. The β-ARs are divided into 3 subtypes: β_1, β_2 and β_3. There are studies reporting a subtype β_4, but it is not fully elucidated, according to Almeida et al. (2012). Mills and Mersmann (1995); Mersmann (1998) categorized β-AR and its antagonists in adipose tissue of many species and, observed that in pigs, RAC binds mainly to the β_1 subtype and partially to β_2. Moody et al. (2000) tested the interaction with the different β-agonists and their receptors and confirmed that RAC binds mainly to the β_1 subtype supporting the claim of Mills and Mersmann (1995), and Mersmann (1998).

Due to this, it is suggested that RAC has higher concentrations in tissues that have greater β-AR of subtype β_1. Mills and Mersmann (1995); Mersmann (1998) demonstrated the following distributions in pig tissues: the heart had more than 65% of β_1; the lung 67% of β_1 and adipose tissue 73% of β_1, 20% of β_2 and 7% of β_3. Sillence et al. (1991) showed that in bovine *longissumus* muscle there were predominantly β_2-type receptors and, they did not detect the presence of the β_1 subtype in the sarcolemma. These results were corroborated by Johnson et al. (2014) who reported the presence of subtype β_1 and β_3 in bovine muscle, but declared that their presence was very small more than 90% of the β-AR present in the muscles was subtype β_2.

Smith and Paulson (1997) reported that cattle supplemented with clenbuterol (β-agonist) had the highest residual levels in the lungs, livers and kidneys, in relation to other tissues evaluated, among them the skeletal muscle. The lung has been one tissue of interest to verify the presence/absence of RAC, and the β-AR concentration is higher in this tissue, according to Antignac et al. (2002), which can be attributed to receptor-ligand interaction.

There is a lack of consensus in the literature about the action of RAC in lipid metabolism. According to Mersmann (1998) the administration of RAC increases muscle mass with minimal effects on adipose tissue. While, Ferreira et al. (2013) reviewed several studies about RAC and its action on lipid metabolism, and they concluded that there was evidence that RAC reduced the amount of lipids in the carcass through a greater inhibition of lipogenesis than an increase in lipolysis.

Pleadin et al. (2013) reported that the interaction of the β-agonists with their receptors which are located in the cell membranes, results in biochemical signals and some reactions. First there are G proteins and adenylate cyclase (AC) activation, which produces cyclic adenosine monophosphate (cAMP). One of the major intracellular signalling molecules, cAMP produces effects to bind to the regulatory subunit of protein kinase A to release the catalytic subunit that then phosphorylates a number of intracellular proteins. Some of these are enzymes that are activated when phosphorylated such as hormone sensitive lipase, the rate-limiting enzyme for adipocyte triacylglycerol degradation (Mersmann 1998), promoting some cellular responses such as the stimulation of lipolysis (Moody et al. 2000).

Dunshea et al. (1992) observed that the biosynthesis of fatty acids from glucose is the predominant source in 74% of fatty acids deposited in triglycerides in adipose tissue. As for the mechanism of lipogenesis, it is important to point out that this reaction will cause fatty acid synthesis: acetyl-CoA is carboxylate in malonyl-CoA by acetyl-CoA carboxylase, which is then polymerized in fatty acids. These two latter enzymes are often used as markers of the lipogenic state in adipose tissue (Harris et al. 1993) and studies by Mills et al. (1990) observed a reduction in malonyl enzyme activity with the use of RAC, suggesting an effect on lipogenesis. Moura et al. (2011) indicated that there is efficiency in the process of lipogenesis in pig adipose tissue by the action of RAC, but it becomes more pronounced in situations where the diets have a higher energetic content, mainly in the amount of lipid.

Dunshea and D'Souza (2003) reported that due to the use of β-agonists in pig diets an increase in lipolysis, and decrease in lipogenesis is expected, with a consequent decrease in fat deposition. They reported that pig adipose tissue has a high specificity in stimulating lipolysis and inhibiting lipogenesis. They also indicated that some researchers may not have detected the two mechanisms together, since the preparation of pig adipocytes for *in vitro* analysis is not simple and also because of the difficulty in interpreting the data in these systems due to the accumulation of adenosine in the solution. When adenosine is inactivated, the lipolytic and lipogenic activity in adipose tissue isolated from pigs can be demonstrated. Ricks et al. (1984) had already proposed in their study that the effect on adipose tissue related to β-adrenergic effects was to inhibit fatty acid biosynthesis and stimulate the hydrolysis of triglycerides, simultaneously.

According to Herago and Agonafir (2017), the most effective use of a repartitioning agent is in the finishing period, from one to two months prior to slaughter. Specifically RAC is permitted to be used for 28 days before slaughter (in the finishing phase) in Brazil at a maximum of 20 ppm added in

pig diets (Brasil 2015). RAC is classified as a feed additive or growth promoter and according to Brazilian legislation (Brasil 2004), feed additives are any substance, micro-organism or any product included in the diet of the animals which may or not have a nutritional value, but they are able to improve the characteristics of the diets and/or the performance of the animals.

Feeding RAC to finishing pig resulted in improvements in growth performance and carcass characteristics (See et al. 2004). According to Dikeman (2007) there was a RAC response in finishing pig, by improving the growth rate. Bark et al. (1992) studied the addition of 20 ppm of RAC in pig's diet and they observed that RAC increased the average daily gain (ADG), improved the feed conversion ratio (FCR) increased muscle and decreased fat deposition. These finding corroborated with other reports in the literature: (See et al. 2004; Dikeman 2007; Cantarelli et al. 2009).

In terms of meat yield, according to Stites et al. (1991), when RAC was added in the finishing diets of pigs (5, 10 and 20 ppm), the weight and percentage yield of hams and loins was greater, and hams from pigs treated with RAC had improved ham processing yields. A level of 20 ppm of RAC was observed to give a greater numeric advantage in lean cut yield and carcass cutting yield by approximately 2%. This translates to a potential economic benefit for pig producers and the processing industry.

Currently, some parameters have been used as a commercial strategy to encourage the production of pig carcasses with more meat and less fat and premiums and bonuses are paid out by some abattoirs (Cantarelli et al. 2009). This can stimulate producers to modify their production and increase returns by delivering what the consumer requires. Schinckel (1999) reported that the implementation of lean value carcass pricing systems has led to the selection of pigs with increased lean growth rates, increased carcass lean percentages, and improved lean feed conversion. According to Smits and Cadogan (2003), it's necessary to calculate the cost-benefit of the use of growth promoters which varies according to the market pigs are sold to, the diet's cost and the management of the production facility.

In animals that receive RAC in their diets, Schinckel et al. (2001) reported no effect on attributes like water holding capacity (WHC) and ultimate pH and Apple et al. (2007) reported no detrimental effects on fresh pork color, firmness, WHC, marbling and intramuscular fat content. Bridi et al. (2006) demonstrated that RAC (10 ppm) had no effect on the ultimate pH, WHC, color and tenderness. In some studies less tenderness in the meat as judged by the Warner-Bratzler shear force (WBSF) method has been reported (Aalhus et al. 1990). Xiong et al. (2006) reported the WBSF of *longissimus* muscle from RAC-fed pigs was 36.1 N on day 2 post-mortem, a value of 20% greater than that of muscle from control pigs (30.0 N) and the tenderness difference between RAC-treated and control pig muscles was related to the proteolysis rate, and could be diminished with adequate *post-mortem* ageing

2.4. Divergent ractopamine utilization among countries

The use of RAC depends on of the laws of different countries and it has been a subject of concern lately. It is banned for use in livestock in countries like Taiwan, Russia, European Union, China, and Japan (Valese et al. 2016). China and Japan represent respectively the first and second largest pork importers in the commercial trade (USDA 2018) so this impacts on the use of RAC. The European Union has banned the use of β-agonists since 1996 by Directive nº 96/23/CE. In December 2017, Russia restricted imports of all Brazilian pork after RAC residues were found in imported meat (USDA 2018). Otherwise, RAC as a commercial feed additive is used in the USA, Australia, Canada, Mexico and Brazil (Valese et al. 2016).

In 2010, a pilot program of segregated production "Split-System" was implemented in Santa Catarina state Brazil to ensure that RAC is absent in specific pork production systems. According to Nino et al. (2015) the split system ensures that animals not treated with RAC at any stage in the pork production system are kept separate from those that are in order to allow exports of meat products to the EU. If RAC residues are found in concentrations above the minimum performance required level (MPRL) and level of action (RPA), by the Rapid Alert System for Foods and Feeds (RASFF) the meat products may undergo rejection, recall, destruction and/or protective measures.

In Brazil, the Institution that controls the use of products destined for use in animal feed is the Ministry of Agriculture, Livestock, and Food Supply [Ministério da Agricultura, Pecuária e Abastecimento (portuguese acronym: MAPA). According to the list of antimicrobials, anticoccidials and agonists authorized by this agency and last updated in 2008, RAC can be used exclusively in the finishing phase of pig life at concentration varying from 5 to 20 ppm (MAPA 2008). The levels of veterinary drug residues in food of animal origin are dependent on the countries' laws, but most countries follow the guidelines recommended by *Codex Alimentarius* (Food and Agriculture Organization).

Asian countries and the European Union prohibit the use of RAC in pork production, even if residues are found below the MRLs. Moreover, these countries are concerned with other tissues such as lungs, which usually present a high concentration of RAC residues (Quiang et al. 2007; Dong et al. 2011; Feddern et al. 2018).

According to the "Sanitary and phytosanitary requirements for Brazilian products exported to Russia", pork and processed pork cannot been subjected to the action of pesticides or natural or synthetic estrogen, hormonal and thyrostatic substances, antibiotics as well as drugs given prior to slaughter that had the withdrawn period less than recommended by the instructions for its use" (Brasil 2014).

Despite the divergences about the use of RAC across countries is interesting to consider that the challenge of food security will require an ability to deal with increasing food shortages for an ever

expanding world population, with more and more pressure on finite resources used to produce our food (McCarthy et al. 2018). In this case RAC use could be seen as a way to improve efficiency.

2.5. The improvement and validation of a method of ractopamine detection

Concerns surrounding food safety and international trade have necessitated the development of analytical methods for determination of ractopamine (RAC). Dong et al. (2011) developed a sensitive and reliable analytical method using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and validated this for the determination of RAC in pig tissues, using a deconjugation step during sample preparation, a liquid-liquid extraction, and a further solid phase extraction (SPE) clean-up cartridge. There are a number of some studies which have been undertaken to determine RAC in tissues, in most cases using gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS), in tissues, such as muscle and liver (Antignac et al. 2002), kidney (Antignac et al. 2002; Shao et al. 2009), lung (Antignac et al. 2002; Quiang et al. 2007; Dong et al. 2011) and urine (Antignac et al. 2002; Thompson et al. 2008).

Gressler et al. (2016) developed a simple, less time-consuming and reliable method using QuEChERS followed by LC–MS/MS for RAC determination. The QuEChERS extraction is a fast, easy, cheap, effective, robust and safe method defined by some authors as a successful technique for the extraction of residues of veterinary drugs (Zhang et al. 2013; Gressler et al. 2016). Further, liquid chromatography coupled to mass spectrometry (LC-MS/MS) has been used to quantify RAC residues.

Suo et al. (2014) argue that precise quantification and confirmation of RAC presence in samples requires the sensitivity and specificity of this technique. LC-MS/MS is considered the most appropriate technique for the detection of different veterinary drugs in foods (Shao et al. 2009). Valese et al. (2016) reported that the QuEChERS extraction followed by LC-MS/MS showed satisfactory results when fully validated to quantify RAC in pork products such as tenderloin, loin, ham, bacon, coppa, fresh sausage, Parma ham and cooked ham.

Sample extraction is important to the efficiency of the analytical method, and many techniques are costly and labour intensive, and in addition use large sample volumes adding to increased environmental risk due to the disposal requirements for organic solvents (Du et al. 2014). Farré et al. (2010) reported that there is a growing concern about environmental issues which is leading to the development of rapid and non-polluting methods of sample preparation. Therefore, analytical methods based on QuEChERS extraction are an attractive alternative.

The QuEChERS method is among the most suitable extraction methods that enable the detection and identification of veterinary drugs with detection capability (CCβ) lower or equal than the recommended values established worldwide (León et al. 2012). Lin et al. (2017) recommended the QuEChERS extraction method for β-agonist monitoring and LC-MS/MS for detection and

quantification. Valese et al. (2016) evaluated QuEChERS extraction as an easy handling step, which also favored less organic solvents.

The matrix effect is an important issue that must be evaluated and discussed in the context of method development before studying its performance characteristics (León et al. 2012). Due to the complexity of the biological matrices and the trace levels in real samples, some methods cannot fully remove salts and endogenous compounds, such as fat, phospholipids and aliphatic acid, from the sample, leading to possible matrix effects (Xiong et al. 2015). These authors reported that compared with the traditional methods, the method for the determination of the level of β-agonists using QuEChERS pre-treatment followed by LC–MS/MS exhibits high sensitivity and accuracy, reduced interference and good repeatability and selectivity. Signal suppression is commonly encountered in LC electrospray mass spectrometry analysis in complex matrices, high amount of endogenous compounds may potentially co-elute with target analytes and significantly affect the efficiency of the ionization process (Dong et al. 2011).

2.6. Strategies in pig improvement: an applied and short perspective

Continuous improvement is of great importance to apply a validated combination of screening and confirmatory methods to deal with relatively large number of samples and to guarantee high reliability of results. Some relevant studies have demonstrated the deposition of RAC residues in a variety of pig tissues (Quiang et al. 2007; Dong et al. 2011), but there are no studies that quantify the levels of MBM-containing RAC in pig diets which can lead to serious consequences for the national and international market. Currently, significant advances in pig nutrition have been made. However, there is still no substitute for RAC in order to maintain the same efficiency of protein utilization in the diet for muscle growth in pigs (Silva et al. 2014). Jacela et al. (2009) reported that the compounds commonly used as carcass modifiers and available for use in pigs include chromium, betaine, conjugated linoleic acid, carnitine and RAC.

Many studies have examined the effects of supplementing pigs with the vitamin-like substance L-carnitine which has been attributed to the increased ability of the pig to more efficiently use fat for energy, divert carbon toward amino-acid synthesis, and spare branched-chain amino acids for protein synthesis (Jacela et al. 2009). The role of carnitine in long-chain fatty acid oxidation is well defined, and recent evidence supports a role in the voltage-dependent anion channel in the transport of acyl-CoAs through the mitochondrial outer membrane (Steiber et al. 2004). L-carnitine modification of metabolism provides greater amounts of lipids that can be used for energy, such as carbon to amino acid synthesis, and replaces branched-chain amino acids for protein synthesis. These factors contribute to higher rates of weight gain, higher feed efficiency, and higher carcass yield in pigs (Owen et al. 1996; Owen et al. 2001).

Irekhore et al. (2016) showed that supplementation with L-carnitine in weaned pigs increased feed intake and daily weight gain, however, pigs on the control diet had superior feed conversion ratio, and they observed that the L-carnitine supplementation improved serum biochemical parameters with the best combined effect being at a level of 225mg. At this level total cholesterol, triglycerides and low density lipoproteins were reduced while achieving optimal growth performance and haematological parameters.

Betaine is a nontoxic amino acid derivative distributed widely in nature. Cadogan et al. (1993) reported a 14.8% decrease in backfat thickness of the pigs fed betaine-supplemented diets. Betaine can induce hormone changes and inhibit lipid synthesis by reducing the activities of lipogenic enzymes in addition to promoting lipid degradation by increasing the lipase sensitive hormone with a consequent decrease in adipose tissue and improvements in carcass traits (Huang et al. 2006). Data presented by Matthews et al. (2001) supported these findings where they demonstrated an increase in the percentage of lean meat and Dunshea et al. (2008), also reported a decrease of fat percentage in the carcass and backfat thickness.

Other substances used in pork production to improve the animal performance can be the amino acids that are essential precursors for the synthesis of several nitrogenous substances of great biological importance. Amino acids in the industrial form are absorbed more rapidly than those derived from the digestion of feed ingredient proteins (Yen et al. 2004). Kim et al. (2004) observed that supplementation with L-arginine in pig diets reduced circulating ammonia levels, contributed to higher animal weight gain, and increased lean meat deposition. Wu et al. (2007) reported that a dietary supplementation with glutamine to early-weaned piglets prevented intestinal atrophy and improved growth performance.

And also, the exogenous porcine somatotropin (PST) which leads to increased weight gain, improved feed efficiency, reduced backfat thickness, increased loin eye area, and increased protein yield carcass (Holden 1994). According to Campbell et al. (1991) the action of PST is due to the stimulation of protein deposition and inhibition of lipogenesis, resulting in improvements in animal performance and reduction in carcass fat content.

2.7. Main Goals

The main goals of the work reported in this thesis are outlined below. For the first phase of the study, an analytical method was developed to evaluate ractopamine (RAC) residues in pig tissues, which was validated in commercial samples in order to ensure the accuracy of the measurements. Due the importance of this topic, one research article was published entitled “Ractopamine analysis in pig kidney, liver and lungs: A validation of the method scope extension using QuEChERS as a sample preparation step” presented in Chapter 1, with the purpose of demonstrating a validated method to

guarantee high reliability of results to ensure the maintenance of national and international markets, as well as provide scientific information to help exportation in those countries where RAC is restricted or has zero-tolerance policy.

This method was based on the study developed by Gressler et al. (2016) which consisted in a simple, less time-consuming and reliable method using QuEChERS followed by LC–MS/MS for RAC determination in MBM. The performance of the modified method was evaluated through specificity, recovery, linearity, reproducibility, repeatability, decision limit (CCα), and detection capability (CCβ), in accordance to the Commission Decision 2002/657/EC. The results showed that this method was capable of detecting RAC residues in all evaluated matrices.

Furthermore, once the analytical method was stablished during the first phase, the second step consisted of verifying if RAC residues remain in pig tissues (muscle, liver, kidneys, and lungs) and how much is eliminated through urine in gilts that received meat and bone meal (MBM) in four dietary groups: 0, 7, 14 and 21% (m/m) of MBM-containing RAC (53.5 μg kg^{-1}) in the diet. More details are presented in Chapter 2.

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3. RACTOPAMINE ANALYSIS IN PIG KIDNEY, LIVER AND LUNGS: A VALIDATION OF THE METHOD SCOPE EXTENSION USING QuEChERS AS A SAMPLE PREPARATION STEP

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ABSTRACT

Ractopamine has been allowed by some countries as a repartitioning additive in pig diet, since it promotes protein synthesis and fat lipolysis. Most regulatory agencies only propose the ractopamine assessment in meat, kidney, liver and fat. Aiming at contributing to the scarcity data regarding this analyte in pig lungs, we extended the scope of a LC–MS method to evaluate pig offals. Homogenized tissue samples were extracted by a QuEChERS procedure; following by clean up steps and further tandem mass spectrometry determination. Method performance was evaluated through specificity, recovery, linearity, reproducibility, repeatability, decision limit (CC_α), and detection capability (CC_β), in accordance to the Commission Decision 2002/657/EC. Regression coefficients (R^2) between 0.994 and 0.999 were achieved for kidney, liver and lungs. Recoveries ranged from 92.0 to 127%. CC_α and CC_β values ranged from 3.65 to 4.86 μg kg^{-1}, and from 6.27 to 7.21 μg kg^{-1}, respectively. These values were under the maximum residue limits suggested by Codex Alimentarius, which are 90 and 40 μg kg^{-1} for kidney and liver, respectively. When applied to real samples up to 22.5, 92 and 1003 μg kg^{-1} of ractopamine residues were detected in pig liver, kidney and lungs, respectively. The results allowed concluding that the proposed analytical method is capable to detect ractopamine residues in all evaluated matrices. Therefore, it can be successfully applied and used as a routine method in laboratories of residue analysis.

Keywords: Food safety; β-Agonists; Hydrolysis; LC-MS; Maximum residue limit; Matrix effect

3.1. Introduction

Ractopamine (RAC) has been used in many countries to redirect nutrients to favor leanness and decrease fat in pork and other meat animals [1–3]. It is commercially authorized as RAC hydrochloride in concentrations ranging from 5 to 20 mg kg^{-1} exclusively in finishing pig diets 28 days prior to slaughter [4,5]. The effect of RAC in improving growth performance is well documented [6]. The Codex Alimentarius has established 90, 40, 10 and 10 μg kg^{-1} as RAC maximum residue limits (MRLs) for kidney, liver, fat, and meat, respectively [7], as recommended by the Joint Expert Committee on Food Additives (JECFA). Although Codex set these limits, each country has its own standards and may follow or not Codex criteria due to concerns surround food safety [8]. Furthermore, China, Taiwan, and even the European Union have zero tolerance policies for RAC residues in pork products. In the production chain, non-utilization or underutilization of byproducts leads to loss of potential revenues, besides the increasing cost of disposal of these products [9].

However, by-products such as kidney, liver, and lungs have good nutritive value [10] and therefore they are highly prized as foods in many parts of the world, particularly Southeast Asia. Considering the high potential use of by-products, some studies have shown the presence of RAC residues in pig offals. Among the evaluated tissues, lungs presented the highest concentration, indicating the high affinity of the analyte due to their large number of β-agonist receptors [11, 12]. Thus, lungs are an important tissue for RAC residue control. However, to our knowledge, only these studies evaluated RAC residues in swine lungs and therefore literature is scarce in order to provide data to establish a MRL for lungs. Due to its high selectivity and reliability of results, liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) has played an important role in the monitoring of veterinary drug residues in foods and feeds [13–15]. However, sample preparation method shall be carefully chosen or developed to ensure suitability. Recently, QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction and clean-up has been successfully applied before LC–MS/MS analysis to determine RAC and showed high tendency to be the most used sample preparation technique due to its simplicity, speed, lower reagent costs and higher sample throughput [13–15]. In our lab, we first developed a practical method [15] to detect and quantify RAC residues in meat and bone meal (MBM), which is used in many countries to partially replace soybean meal in animal diets, for economic reasons. However, RAC residues are not fully eliminated by the animal organism and deposition in different organs may occur. Thus, the need of measuring RAC residues in other tissues than the ones covered by regulatory agencies with fast response has arisen in order to contribute to food security. In this context, the main objectives of this study were: to extend the scope of the previously developed method [15] and validate it to pig offals (kidney, liver and lungs) using the QuEChERS approach followed by LC–MS/MS analysis.

3.2. Material and methods

3.2.1. Standards, reagents and samples

All analytical standards were purchased from Sigma-Aldrich (St. Louis, USA), including RAC (95.5% purity), isoxsuprine hydrochloride (ISOX, 99% purity) used as internal standard (IS), protease (lyophilized powder, for use in total dietary fiber assay, TDF-100A) and β-glucuronidase (from Helix pomatia, Type HP-2, aqueous solution, ≥100.000 units mL−1). All solvents were in chromatographic grade and acquired from Panreac (Darmstadt, Germany), Sigma-Aldrich (USA), and J.T. Baker Chemical Co. (Phillipsburg, USA). An ultrapure water purification system provided by Millipore (Advantage A10) was used to obtain ultrapure water. Other reagents were purchased from different companies: formic acid (eluent additive for LC–MS) from Sigma-Aldrich (USA), acetic acid (glacial,>99.7% purity), ammonium acetate (98% purity) and sodium hydroxide (> 97% purity) from

Vetec (Brazil), tris (> 99.8% purity) from Promega (USA), and hydrochloric acid (36.5–38.0% purity) from Panreac (Spain). Phenomenex® provided QuEChERS kits (KS0-8909 and KS0-8921).

The blank samples from pig kidney, liver and lungs, were obtained from federally inspected Brazilian slaughterhouses and stored at −20 °C prior to use. RAC stock standard solution of 0.5 mg mL^{-1} was prepared in methanol and stored at −20 °C. Stock and working solutions were prepared weekly. Working solution was prepared by diluting the stock solution with methanol up to 0.5 μgmL^{-1} and stored at −20 °C. Internal standard (IS) was prepared similarly, although the final concentration was 5.0 μgmL^{-1}.

3.2.2. RAC extraction

The extraction method was based on QuEChERS extraction proposed by Gressler et al. [15] for feed ingredient (meat and bone meal) commonly used in poultry and pig diets. Swine matrices (liver, kidney and lungs) were first chopped into small pieces and homogenized, then an accurate amount of sample (5.00 ± 0.05 g) was weighed into 50 mL polypropylene centrifuge tube. The internal standard (IS) was added in a concentration of 10 μg kg^{-1}. Afterwards, 5 mL of 1 mol L^{-1} Tris buffer (pH 9.5) and 5.00 ± 0.05 mg of protease was added. The samples were manually stirred for 1 min and digested overnight at 60 °C. After cooling to room temperature, 3 mL of 2 mol L^{-1} ammonium acetate buffer (pH 5.2) were added and the pH adjusted to 4.5–5.0 with 4 mol L^{-1} HCl. The enzyme β-glucuronidase (100 μL) was added in order to break RAC glucuronide metabolites leading to free RAC. The samples were allowed to water bath for 2 h at 65 °C, followed by adjustment to pH > 12 with 10 mol L^{-1} NaOH prior to QuEChERS extraction. Organic solvent (10 mL of acetonitrile) was added to the sample and the mixture was stirred manually for 1 min and then the salts content of roQ™ QuEChERS extraction package (4.0 g of $MgSO_4$, 1.0 g of NaCl, 1.0 g of sodium citrate tribasic dihydrate and 0.5 g of sodium citrate dibasic sesquihydrate, KS0–8909) were added. The samples were stirred by hand for 1 min and centrifuged at 1500×g for 10 min. An aliquot of 6 mL of the upper phase (acetonitrile) was transferred to the 15 mL dSPE centrifuge tube containing roQ™ QuEChERS KS0–8921 kit (900 mg $MgSO_4$, 150 mg PSA and 150 mg C18E) for cleaning up. The tube was stirred in a vortex for 1 min and centrifuged at 1500×g for 10 min. From the upper layer 1 mL was transferred to a vial for LC–MS/MS analysis.

3.2.3. LC–MS/MS analysis

A 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Sciex, Framingham, MA, USA), equipped with a Turbo IonSpray electrospray ionization (ESI) source, set in positive mode (ESI+) and multiple-reaction monitoring (MRM) mode was coupled to a 1290 Infinity

high-performance liquid chromatography system (HPLC) from Agilent Technologies, Inc. (Waldbronn, Germany). The chromatographic separation was performed on a C18 Symmetry column (50mm×2.1 mm, 3.5 μm) from Waters (USA), with a C18 AJ0-4287 guard column (4.0mm×3.0 mm) from Phenomenex (Torrance, USA). Mobile phase was composed by solvent A (aqueous solution with 0.1% formic acid) and solvent B (ACN with 0.1% formic acid). Gradient elution started at 15% of eluent B for 0.5 min and then it was linearly increased up to 90% of eluent B in 2.5 min, held constant for 4 min, and returned to the initial conditions in 0.5 min (15% of B) and held for 1.5 min. The injection volume was 10 μL. Both RAC and ISOX were separated at a flow rate of 0.3 mL min−1 at 40 ± 1.0 °C. The diverter valve was used (2 min) to help in the elimination of sample interferences and to avoid ESI contamination.

Direct infusion of standard solution was performed to optimize MRM+ transitions and associated acquisition parameters. For RAC, the m/z 302.1 ion was selected as the monitor ion for MRM and the product ions m/z 164.2 (21 V as collision energy) for quantification and m/z 121.2 (29 V) for confirmation. As IS, the m/z 302.0 ion was selected as the monitor ion and the product ions m/z 284.1 (19 V) for quantification.

For the mass spectrometric analysis, a turbo V™ source, operating in positive ionization mode, was set with the following parameters: ion spray voltage: 4500 V; curtain gas: 20 psi; nebulizer gas: 50 psi; auxiliary gas: 45 psi; source temperature: 500 °C. Nitrogen was used as a nebulizer and collision gas. The Analyst and MultiQuant softwares (Sciex, Framingham, MA, USA) performed all system control, data acquisition and data processing.

3.2.4. Method performance evaluation

The optimized method followed the criteria established by the European Commission Decision to validate the procedures [16]. Analytical characteristics evaluated were specificity, linearity (calibration curve), trueness through recovery studies, repeatability (intra-day precision) and reproducibility (inter-day precision, within-laboratory) and analytical limits as decision limit (CCα) and detection capability (CCβ). We also evaluated matrix effect and sensitivity in terms of limits of detection (LOD) and quantification (LOQ) according to the Brazilian Ministry of Agriculture, Livestock and Food Supply [17]. The outliers were removed by Grubb's test at 95% confidence level, and systematic analytical errors were eliminated using dispersion graph and control charts. The moving range control chart was applied considering the center line (trend) as the average of the moving range. The upper control limit (UCL) and lower control limit (LCL) were defined by multiplying the mean range by the constants 3.27 and 0, respectively, as a function of the number of replicates.

3.2.5. Selectivity/specificity

Selectivity/specificity was evaluated by the analysis of at least 20 samples for each category studied. The results were evaluated by the presence of interfering peaks around the analyte retention time. For this assay, the retention times were compared with and without analyte and IS fortification. The maximum permitted tolerances for relative ion intensities was at least 20%.

The selectivity/specificity test was performed in order to verify identification and quantification of the target analyte and avoid the elution of interfering compounds at the same time.

3.2.6. Linearity, sensitivity and matrix effect

The linearity of the method was evaluated through the reproducibility of calibration curves prepared in matrices (lungs, kidney, and liver) by spiking 0, 2.5, 5.0, 10.0, 25.0 and 50.0 μg kg^{-1} of RAC in liver and kidney, while lungs calibration curve had an additional concentration point of 100 μg kg^{-1}. From the IS working solution (5.0 μgmL^{-1}), 10 μL were also added. The calibration curves were built by plotting RAC concentration (x-axis) versus RAC/IS peak area ratios (y-axis). All curves points were performed in triplicate. The acceptance criterion was the mean of the regression coefficients (R^2) which should be above 0.99 [17].

The LOQ was established as the first point of the analytical curve with proven recovery (CV < 30%) and precision. LOD was established as the lowest response value for the analyte with acceptable reproducibility (n=10; CV < 30%) according to Mologni et al. [18].

The matrix effect (ME) was evaluated in the matrix presence to verify the signal suppression or enhancement. The procedure was based on the analysis of two types of calibration curves. Curve I was built in extracted blank sample with further fortification with standards, while curve II was performed in solvent [19]. The evaluation was performed comparing the mean slope values of the standard curves by means of the t-test (in pair for averages) at 5% significant level, and categorized according to Ferrer et al. [20], where percentage between −20% and 20% was considered as no ME; a medium ME when the values were between −50% and −20% or 20% and 50%; and a strong ME when below −50% or above +50%.

3.2.7. Recovery and precision

Recovery and precision were determined by spiking blank samples with 2.5, 5.0 and 10.0 μg kg^{-1} RAC (five replicates per level). Repeatability and within-laboratory reproducibility were assessed in terms of intra-day and inter-day precision, respectively, considering two different analysts and two

days of analysis for each matrix. These parameters were determined by processing independently the five spiked samples for each matrix at two different days.

Recovery was accepted if recovery rate was among −30% and +10%. These criteria were used to evaluate the method precision and results with coefficient of variation (CV) lower than 20% were considered satisfactory. Moreover, analysis of variance was carried out through ANOVA (unique factor) at 95% confidence level (p=0.05), to determine if significant differences were detected between days and analysts.

3.2.8. Analytical limits

CCα was obtained by multiplying the standard deviation of the mean of the fortifications during the validation, by the factor 1.64 and weighted in the general average. The CCβ was obtained by multiplying the standard deviation by the factor 1.64 and adding to the CCα value. These parameters were calculated considering the minimum required performance levels (MRPL) and fortification levels. The errors generated by the residues of the analytical curves were also considered in the calculations.

3.2.9. Method applicability

The effectiveness of the proposed method was verified by analyzing 17 samples of kidney, liver and lungs, provided from accredited farms. The pigs were fed RAC up to 20 mg kg^{-1} in their feed in the last 28 days before slaughter.

3.3. Results and discussion

The scope extension is the inclusion of more analytes, other matrices and/or minor changes made in previously existing extraction procedures, without necessarily perform the full validation protocol which is time-consuming, laborious and expansive [21]. We compared in our lab two methods: SPE and QuEChERS, which were able to extract RAC from MBM samples and we verified that the time was reduced from 4 days to 2,5 days when using QuEChERS [22]. The full validation of this QuEChERS-LC–MS/MS method to quantify RAC residue in MBM was then published [15] and in the present study, it was successfully validated to new matrices (kidney, liver, and lungs). Minor but important changes, in relation to the original method in the sample pre-treatment procedure and chromatographic conditions will be further detailed. The effectiveness of the scope extension can be demonstrated by parameters as linearity, recovery rate, precision, CCα, CCβ, and matrix effect, which were here well succeeded for liver, kidney, and lungs. The evaluation of these parameters

demonstrates the analytical quality of the measurements by the comparability, traceability, and reliability, and they affirm that the method is suitable for routine monitoring of RAC residues in edible tissues.

3.3.1. Extraction method and LC–MS/MS analysis

The use of QuEChERS instead of SPE (solid phase extraction) was chosen because it showed good applicability to extract RAC from muscle, feed, and MBM matrices [14,15,23,24]. The QuEChERS method was originally developed for pesticides extraction [25], although other compounds such as pharmaceuticals, mycotoxins, β-agonists and polycyclic aromatic hydrocarbons have also been extracted by this method in a wide variety of complex matrices [26–28]. Therefore, QuEChERS extraction method needs to be adapted to different analytes or matrices. Originally, QuEChERS has targeted application in high water content (~90%) matrices and, considering that swine liver and lung contain approximately 71% and 78% of water, respectively [29], it was necessary to liquefy the sample with 5 mL of Tris buffer, in order to favor RAC extraction.

Some studies do not use the step of hydrolysis with β-glucuronidase enzyme in the determination of RAC in tissues [14,30–32]. In this work, we still reinforce the use of it, because RAC glucuronide metabolites A–F (Fig. 1) are present in significant concentrations in pig liver and kidney. Parent RAC and its metabolites A, B, C, D, E, and F represented 28.7, 7.9, 10.4, 4.6, 5.0, 2.7, and 2.8% of total extractable RAC-related radioactivity in the liver, and 23.4, 11.0, 13.2, 22.3, 6.1, 1.4, and 1.9% in the kidneys [33]. The use of a β-glucuronidase hydrolysis step has also been shown to enhance RAC concentration extracted in pig urine and MBM, showing to be indispensable in the analysis of β-agonists [15,34,35].

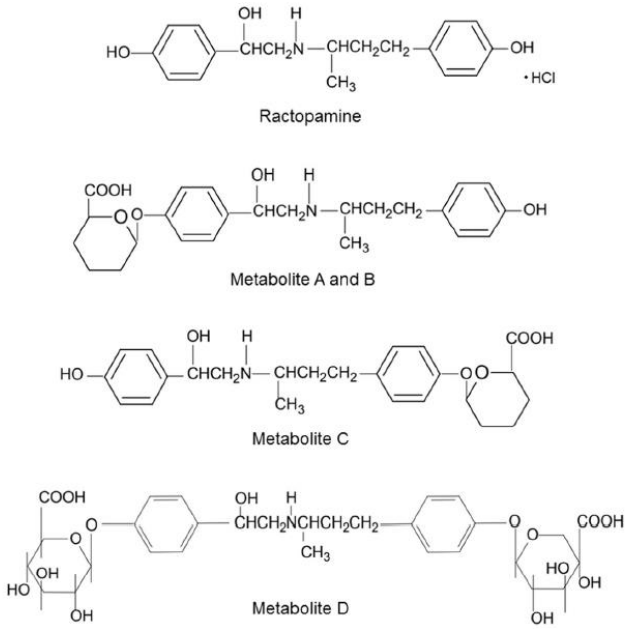

Fig. 1. Structural formulas of ractopamine and its metabolites A–C, and the probable structure of metabolite D. Source: [33] The chemical structures of metabolites E and F may not be fully elucidated.

Chromatographic separation and MS parameters are important factors to be optimized in different instruments [36]. RAC and ISOX are basic compounds, which are better analyzed by positive electrospray ionization (ESI+) in LC–MS/MS as their corresponding pseudo-molecular ions [M+H]+ of m/z 302. First, for improving MRM–MS/MS sensitivity, an appropriate tuning of the instrument parameters such as collision energy and cone voltage were studied, in order to generate optimal fragmentation and best response of the desired product ions. The optimized conditions of each parameter were described in Section 2.3. The chromatographic separation was achieved in a run time of 9 min (Fig. 2).

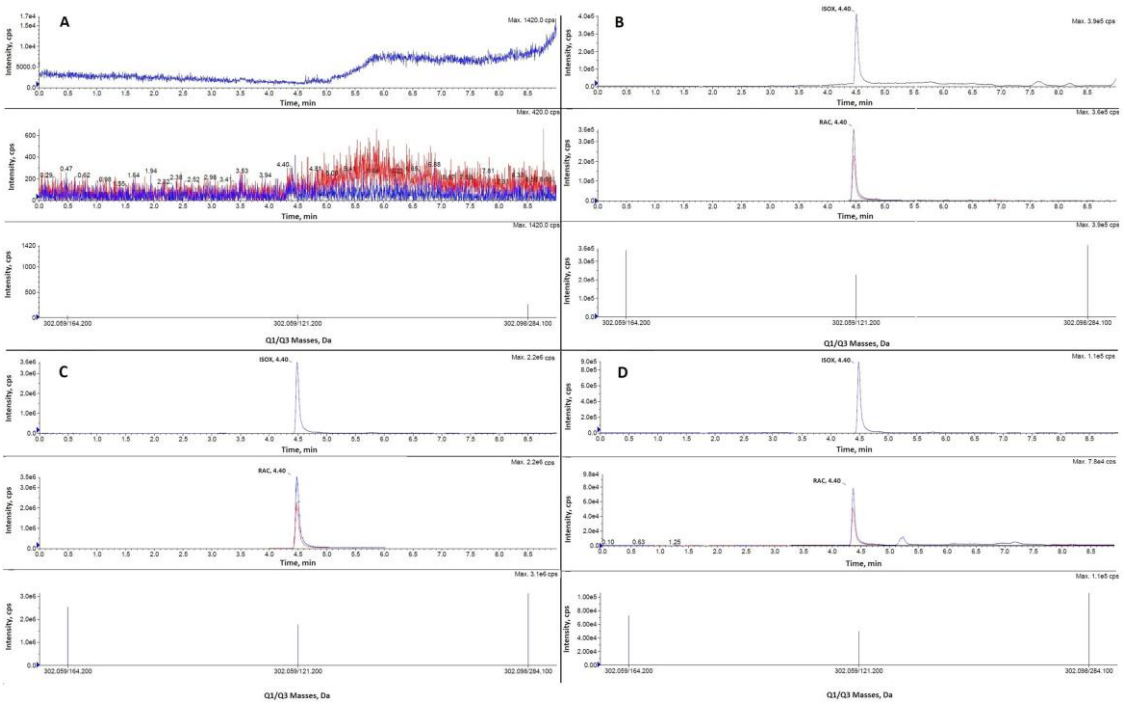

Fig. 2. Chromatograms and mass spectra of blank samples (A), blank kidney fortified with ractopamine spiked at 25 μg kg^{-1} (B), blank lungs fortified with ractopamine spiked at 25 μg kg^{-1} (C), blank liver fortified with ractopamine spiked at 25 μg kg^{-1} (D).

3.3.2. Method performance evaluation

Method validation is a necessary tool for residue analysis due to their great importance in the statutory programs involved in the international trade of commodities [21]. Following Qiang et al. [34] conclusions regarding the determination of validation parameters, no β-glucuronidase enzyme was added in the extraction procedure, because blank sample with no supposed presence of RAC-glucuronides metabolites was used. Besides of that, the samples remained in water bath to ensure the same sample conditions.

The method selectivity was proven for all analytes. No interfering transitions from matrix endogenous compounds (Fig. 2) were observed around the retention time of the analyte fragments and nor of the IS. The relative intensities of the transitions were all above 20%, which avoid false positive results during analysis. This parameter also evaluates system contamination, once HPLC column can trap particles, precipitated proteins, and other organic contaminants. In addition, the equilibration period was enough to clean the system between each chromatographic run. The Supplemental Figs. (S1, S2 and S3) show the chromatograms and spectra of real offal samples.

The regression equations and the coefficient of determination for kidney, liver and lungs showed adequate linearity in the range from 0 to 50 μg kg^{-1} of RAC in kidney and liver and from 0 to 100 μg kg^{-1} of RAC in lungs. The equations and the coefficients of determination obtained for all

standard calibration curves considering ISOX as IS were y=0.0528x−0.0010 (R^2=0.996) for kidney, y=0.0323x+0.0027 (R^2=0.999) for liver, and y=0.0544x−0.0107 (R^2=0.994) for lung.

In terms of sensitivity, the LOD and LOQ obtained for liver, kidney and lungs are summarized in Table 1. For liver and kidney, our results were below the MRL established by Codex, showing that the method is sensitive for RAC residues monitoring in these matrices. For lungs, no MRL had already been established by regulatory agencies, however, as this tissue has been reported in higher concentration of RAC than in other tissues [11,12], the analytical limits found are highly satisfactory.

Table 1. Analytical limits for the determination of ractopamine in three swine matrices by QuEChERS-LC–MS/MS.

	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	CC_α ($\mu g\ kg^{-1}$)	CC_β ($\mu g\ kg^{-1}$)
Lungs	1.0	2.5	3.7	7.1
Kidney	0.5	2.5	4.4	6.3
Liver	0.5	2.5	4.9	7.2

When dealing with real complex samples such as edible tissues from animals, these matrices usually require an appropriate sample preparation in order to decrease interfering compounds [37]. Coeluting components may be nebulized together with the analyte in the ionization source, what results in signal suppression or enhancement in the mass spectrometer [38] and might exert a detrimental impact on important method parameters such as linearity, recovery and precision [39].

With respect to clean-up of the biological matrices analyzed herein, the QuEChERS method was effective. Liver, kidney and lungs showed −54.4%, −57.9%, and −69.9%, respectively, what is considered a strong suppression, according to the classification adopted by Ferrer et al. [20]. Thus, the ME of RAC should not be ignored. In practice, in the final extraction stage, a clear solution was observed for kidney and liver, whereas for lungs the solution became darker. The dark color of the lung extracts may be due to the higher ME. In this case, use of higher amounts of C18 and/or primary/secondary amine (PSA) or even a different sorbent in the clean-up process (QuEChERS step) could help minimize the high ME observed.

Dong et al. [40] reported a 20% decrease in signal intensity comparing the matrix-matched standards with the standard solution. However, the values of slope of matrix-match standard calibration curve versus that of the standard calibration curve (both using IS) ranged from 96% to 102%. This work did not specify ME results for all swine matrices studied, then we can deduce that they did not find differences between them [40]. Another study showed 11% of ion suppression in the analysis of β-agonists in animal liver [41]. Ion suppression of 44, 49 and 40% were found for pig muscle, kidney and liver, respectively [42]. However, signal enhancement (111–114% of ME) in porcine liver has also been reported [43]. This author also compares their results with the literature and

shows higher ME when compared to porcine muscle. Ho et al. [44] studied ME in rat organs and found higher ion suppression for kidney (59.5%) when compared to liver and lung (97.5 and 112.6%).

The range of ME found for swine muscle (obtained by different types of sample preparation but analyzed by mass spectrometry) was 8.6–18.3% of ion suppression [13,45,46]. Comparing to our findings for liver, kidney, and lungs, ME are within the expected values. Despite not being clear, Xiong et al. [47] reported higher ME in beef and goat liver when compared to beef and goat muscle and attribute it to large amounts of glycogen, fats, proteins, carbohydrates and vitamins in liver whereas the main component of muscle is protein. This may also be one of the reasons of higher ME found herein for liver, kidney and lungs, since optimization of sample preparation may reduce the level of matrix-induced interference but hardly eliminate it.

As shown in Table 2, RAC was satisfactorily extracted from liver, kidney and lungs, with mean recoveries ranging from 92.0 to 127%. The precision of this method is also demonstrated in Table 2 as repeatability (intra-day precision), and reproducibility (inter-day precision), expressed as CV (%).

Table 2. Recovery, intra-day (repeatability) and inter-day precision (within-laboratory reproducibility) in swine matrices by spiking ractopamine in three levels.

Tissue	Fortification level ($\mu g\ kg^{-1}$)	Recovery (%)	Intra-day CV (%), $n = 5$	Inter-day CV (%), $n = 5$
Liver	2.5	98.0	14.2	16.4
	5.0	98.0	5.7	9.7
	10.0	96.0	8.3	7.8
Kidney	2.5	92.0	15.8	9.8
	5.0	98.0	7.4	17.9
	10.0	92.0	7.7	5.0
Lungs	2.5	127	16.5	19.6
	5.0	102	24.1	6.7
	10.0	94.0	18.7	22.3

Liver and kidney recovery rates remained between the limits established (70 to 110%) and the literature data corroborates our results. QuEChERS was also used as RAC extraction procedure by other authors and recovery ranges of 89.8–100.7% [14], 77–82% [13] and 80.4–113% [36] for swine muscle were described. Hu et al. [48] found recoveries of 102–104% and 98.7–108% for pork and liver respectively, and Xu et al. [49] when analyzing β_2-agonists (using molecularly imprinted stir bar sorptive extraction), observed recoveries of 85.1–92.3% and 82.6–90.2% for pork and liver respectively. Recoveries from 86.6–88.7 and 80.2–87.9% (data adapted) were found for swine kidney and liver respectively [50]. For pig kidney, liver, and lungs, Dong et al. [40] reported recovery values of 96.6–99.8, 99.8–101 and 93.3–96.0%, respectively. Results obtained from studies with other animal species can be helpful in RAC recovery understanding in different tissues. Pleadin et al. [51] studied RAC residues in guinea pig tissues and found recoveries of 80.5–82.3, 72.4–78.6, 77.4–82.3% for kidney, liver and lungs, and Ho et al. [44] reported mean recoveries of 88.0, 89.3 and 97.2%,

respectively, in rat. Studies referenced here obtained lower recoveries for kidney, liver and lungs when compared with muscle, showing that independent of sample pre-treatment and/or analysis technique, muscle usually is easier to handle.

The value of CV regarding the repeatability and within-laboratory reproducibility were below the maximum acceptable value (CV < 20%) for kidney and liver. Although some recoveries and precision for lungs were in disagreement with the validation protocol adopted, all results were considered acceptable for the purpose, since due to the strong ME observed, matrix-matched analytical curves were used. Moreover, the evaluation of method precision by using a limited number of observations is usually difficult in terms of validation. However, these values represent the method starting point and over time, in most cases, routine method repetition reduces the uncertainties based on random effects obtained in the validation, what allows the method to be more precise as the number of measures increases (degrees of freedom) [18].

Decision limit (CC_α) and detection capability (CC_β) takes into account the method variability and the statistical risk of making wrong decisions. In our study, CC_α and CC_β values ranged from 3.65 to 4.86 μg kg^{-1} and from 6.27 to 7.21 μg kg^{-1}, respectively. Both limits were established for all matrices and they were considered low enough for the control of RAC residues in swine kidney, liver, and lungs (Table 1).

The use of QuEChERS as extraction method for RAC in kidney, liver, and lungs was not reported yet. Thus, it is difficult to compare our analytical limits and affirm if we achieved better results or not. Comparison with analytical limits reported by the literature using SPE shows that our values are a bit higher [52], but still suitable, once MRL set by control agencies for liver and kidney are higher and no MRL for lungs has already been determined.

It has been reported that CCα and CCβ found for kidney and liver are higher [42] in comparison to muscle tissue and this could help explaining the less ME in muscle. For instance, the high complexity of kidney and liver matrix contribute to these higher analytical limits and ME (−57.9 and −54.4%) were similar for these tissues. However, the literature is scarce regarding lung tissue data, which diverted from kidney and liver.

3.4. Method applicability

In this research, the method initially developed by Gressler et al. [15] for the analysis of meat and bone meal was satisfactorily adapted to new matrices. The extended method was applied in 17 samples for each matrix (kidney, liver, and lungs), as demonstrated in Fig. 3A, B, C. The results (μg kg^{-1} ± SD) of RAC residues in kidneys ranged from 2.9 ± 0.2 to 92 ± 2, in liver ranged from 0.9 ± 0.1 to 22.5 ± 0.1, and lungs ranged between < CCβ and 1003 ± 45. Thus, the higher RAC concentration in the evaluated tissues was in the following order: lungs > kidneys > liver.

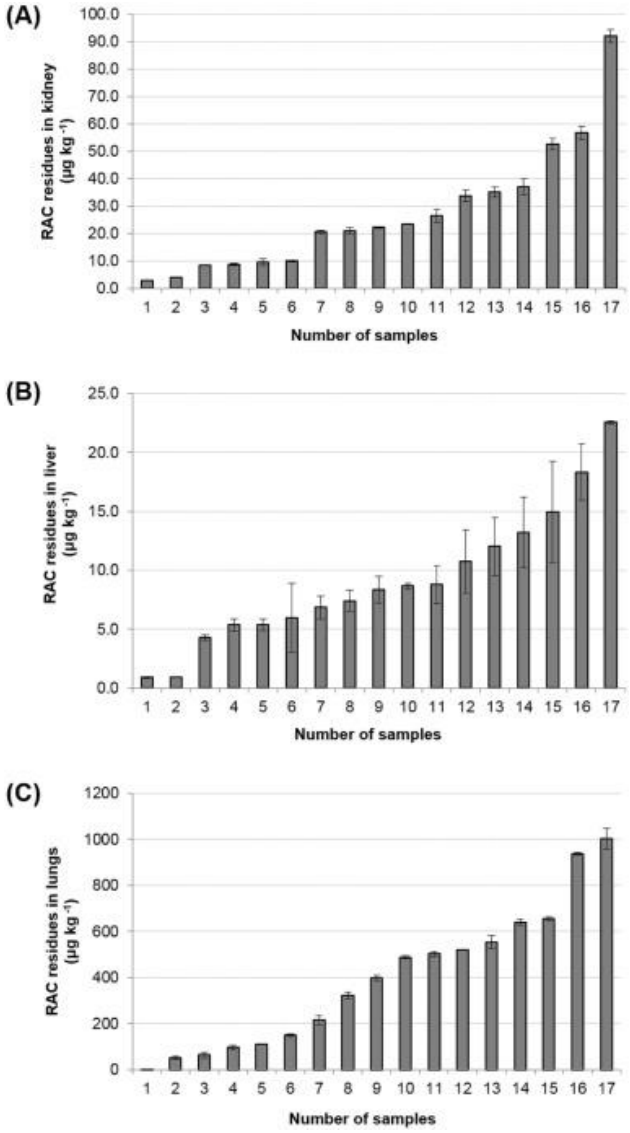

Fig. 3. Residues of ractopamine (µg kg^{-1} ± SD) in swine offals: kidney (A), liver (B), and lungs (C).

Dong et al. [40] evaluated RAC depletion after 12 and 24 h withdrawal time. They observed that RAC values decreased, on average, from 6.54 to 3.46 µg kg^{-1} in muscle, 36.78 to 18.6 µg kg^{-1} in liver and 128.48 to 67.32 µg kg^{-1} in kidney, while RAC concentration in lungs remained high (332 to 343.9 µg kg^{-1}). These authors also found concentrations of RAC in swine in the following order: lung > kidney > small intestine > large intestine > stomach > liver > hearth > muscle. Antignac et al. [53] found the order of lung > kidney > retina > hearth > muscle in swine and Pleadin et al. [54] reported the following concentration order: hair > lung > kidney > liver > muscle in guinea pigs.

These results and the ones demonstrated herein highly suggest that swine lungs showed the highest RAC concentration. This probably occurs because lungs have a larger number of β-agonists receptors [53] together with pigs behavior, for example, their snout is a very effective organ for foraging as well as exploring the environment by rooting, sniffing, chewing and manipulating; thus inhaling RAC particles from feed when it is not pelleted [55]. Therefore, we confirm that the method

is suitable for the purpose and may be used in the control of ractopamine residues in the evaluated pig matrices.

3.5. Conclusion

The main highlight of this study was the possibility of method application for three new matrices (kidney, liver, and lungs) through scope extension using QuEChERS as a sample preparation step. Besides, the use of QTRAP system improved sensitivity, and other parameters such as LOD and LOQ. The concentration of ractopamine residue in the evaluated tissues were in the following order lung > kidney > liver.

This is a very useful and practical method by considering easy handling, the use of small amounts of organic solvents and popular laboratory instruments during sample preparation. This method is suitable for application by surveillance programs, which take into account veterinary drug residue analysis. In addition, the validation parameters evaluated were satisfactory. The method was successfully tested on real samples, being suitable for routine analysis and is likely to be adaptable to other swine tissues too.

This research is helpful for countries that have established strict surveillance programs for official control purposes to check compliance with regulatory limits, for both domestic and imported production. It may also be useful as a reference for lung analysis, since it is an edible tissue of high interest in some countries.

Declarations of interest

None.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2018.05.033

Supplemental Figures

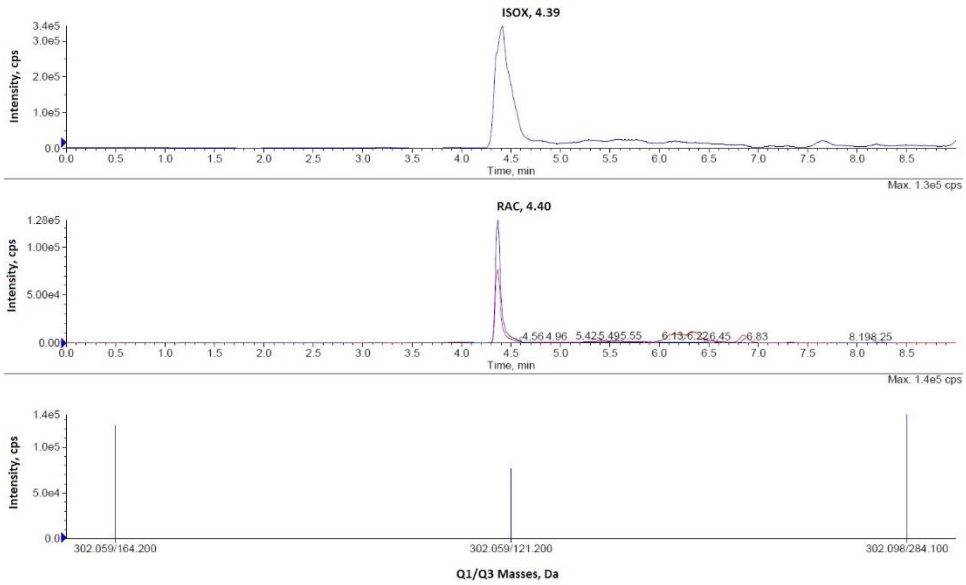

Figure S1. Chromatogram and spectra of real kidney sample.

RAC = Ractopamine transitions 302.1/164.2 (blue) and 302.1/121.2 (red)

ISOX = isoxsuprine, as internal standard, transition 302.1/284.1

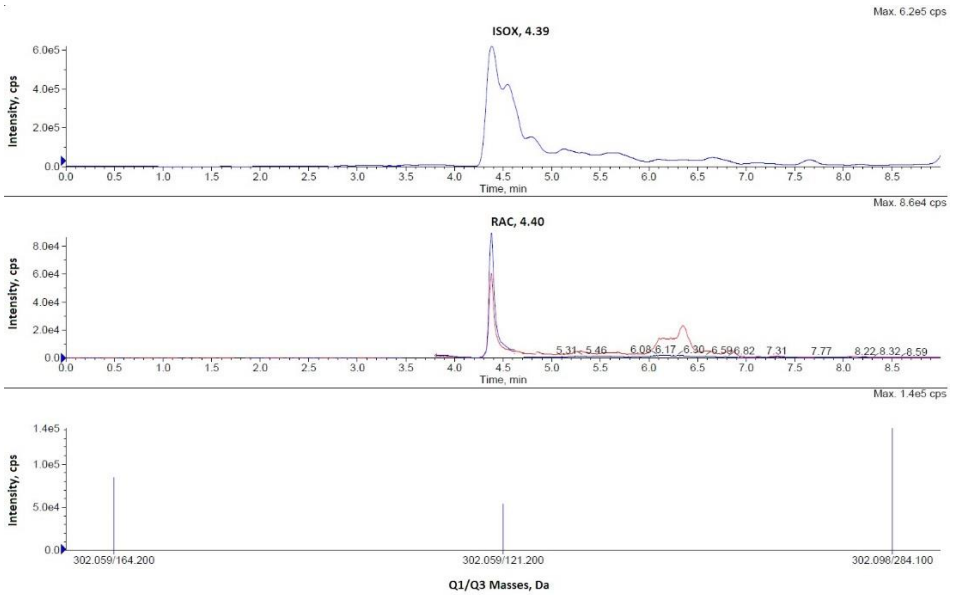

Figure S2. Chromatogram and spectra of real liver sample.

RAC = Ractopamine transitions 302.1/164.2 (blue) and 302.1/121.2 (red)

ISOX = isoxsuprine, as internal standard, transition 302.1/284.1

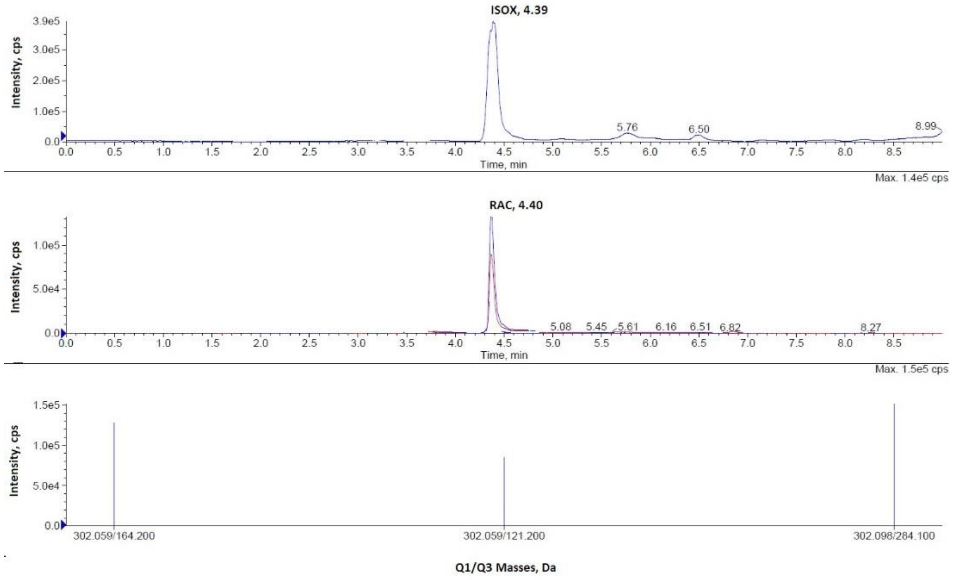

Figure S3. Chromatogram and spectra of real lungs sample.

RAC = Ractopamine transitions 302.1/164.2 (blue) and 302.1/121.2 (red)

ISOX = isoxsuprine, as internal standard, transition 302.1/284.1

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4. DETERMINATION OF RACTOPAMINE RESIDUE IN TISSUES AND URINE FROM PIG FED MEAT AND BONE MEAL

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ABSTRACT

In many countries, ractopamine hydrochloride (RAC) is allowed to be used in animal production as a beta-agonist, which is an energy repartitioning agent able to offer economic benefits such as increased muscle and decreased fat deposition, feed conversion improvement and average daily gain increase. However, some countries ban its use and establish strict traceability programmes because of pharmacological implications of β-agonist residues in meat products. In Brazil, commercial RAC is controlled (5 – 20 mg kg^{-1}) and added only to pig diet during the last 28 days before slaughter. However, the control is more difficult when co-products, like meat and bone meal (MBM), which can be produced from RAC treated animals, is added to feed composition. Therefore, a study was undertaken to evaluate the presence of RAC residue concentration in urine and tissues of gilts (n = 40) in four dietary groups: 0%, 7%, 14% and 21% (w/w) of MBM-containing RAC (53.5 µg kg^{-1}). The concentration of RAC residues in MBM, pig tissues and urine was determined by liquid chromatography coupled to mass spectrometry. Low RAC concentrations were detected in muscle, kidney, liver and lungs (limit of detection (LOD) = 0.15, 0.5, 0.5 and 1.0 µg kg^{-1}, respectively) however, no RAC residues were quantified above the limit of quantification (LOQ) (0.5, 2.5, 2.5 and 2.5 µg kg^{-1}, respectively). In urine, RAC concentration remained below 1.35 µg L^{-1}. These data suggest that MBM (containing 53.5 µg kg^{-1} RAC) added to diet up to 21% (w/w) could hamper the trade where RAC is restricted or has zero-tolerance policy.

Keywords: Feed additives; Pork production; Food safety; Analytical methods

4.1. Introduction

Ractopamine hydrochloride (RAC) is an additive from the class of β_2-adrenergic agonists with structural and pharmacological properties very close to catecholamine. RAC acts as a repartitioning agent by diverting nutrients, by increasing the rate of protein synthesis and/or by decreasing the rate of protein degradation, inducing muscle hypertrophy (Burnett et al. 2012; Bohrer et al. 2013; Beermann 2014).

Many studies have demonstrated advantages for pig production by adding RAC to animal feed such as increased weight gain and improved feed efficiency, increased loin-eye area, meat percentage, meat: fat ratio and reduced back fat thickness (Mimbs et al. 2005; Rikard-Bell et al. 2009; Athayde et al. 2012; Ho et al. 2014; Ritter et al. 2017; Dalla Costa et al. 2018). This improvement in carcass yield is directly related to meat quality, which will result in a higher financial profit to the slaughterhouse. For these reasons, RAC has been used as animal feed additive in many countries.

Despite the fact that the Codex Alimentarius Commission (Codex) has established 90, 40, 10 and 10 µg kg^{-1} as RAC maximum residue limits (MRLs) for kidney, liver, fat and meat, respectively

(FAO 2012), the use of RAC in pig feed is controversial. Countries such as the USA, Australia, Canada, Japan, Mexico and Brazil follow the Codex guidelines and allow the use of RAC in pig diets. As residues of β-agonist remains in tissues and have known toxicological and pharmacological implications, many jurisdictions like the EU, China and Taiwan have banned their use and have in place strong traceability programmes (Alemanno and Capodieci 2012; Freire et al. 2013; Ulrey et al. 2013; Brasil 2016). The EU and China account for 70% of world pork production and have zero tolerance policies for RAC residues (Alemanno and Capodieci 2012; ABPA 2017). This policy affects Brazilian meat exports, for instance. This legal division between countries puts RAC as a potential object of trade disputes.

In Brazil, the use of RAC was approved by the Ministry of Agriculture, Livestock, and Food Supply (MAPA) from 5 to 20 mg kg^{-1} in pig feed during 28 days prior to slaughter, without a withdrawal period (Brasil 2015). In the EU, additives belonging to the β-agonist class were banned through Directive 96/23/EC (European Commision 1996). Although Brazil allows RAC in pig production, MAPA developed the proposal of the Pig Production Program without RAC (Split System), in cooperation with the production sector, to ensure a safe product in order to meet international requirements.

Meat and bone meal (MBM) is considered an important co-product from the animal industry once it is composed by a wide variety of animal organs, which have high nutritional value. Because of its available essential amino acids, minerals and vitamin B_{12} content (Jayathilakan et al. 2012), MBM is used in pig feeding as a protein source which can partially replace amino acid-rich feeds (e.g. soybean meal), decreasing feed costs and increasing competitiveness. According to Gottlob et al. (2004), due to the high soybean-meal costs in the USA, the producers and feed manufacturers were encouraged to search for alternative protein sources to keep feed costs low, therefore interest in MBM has raised.

MBM is also considered a good source of calcium and phosphorus, which is superior than all plant feed ingredients (NRC 2012). In many countries, MBM is consumed by pig, horses, pets, fish and poultry (Johnson et al. 2011) and its production varies from country to country. According to Li et al. (2018), porcine MBM is a widely available animal protein source used in animal feeds in the USA. Karakas et al. (2001) also reported that MBM is widely used in pig and poultry nutrition.

Research results from our laboratory (Gressler et al. 2016) demonstrated the presence of RAC residues at concentrations varying from 3.87 to 81.25 μg kg^{-1} in MBM collected in four meat rendering industries in Southern Brazil. In Brazil, the MBM production is around 2 million tons, 70% of which is destined for animal production, 16.5% to pet food and 3.5% for export (Brazilian Renderers 2016). Therefore, the utilisation of MBM together with the possibility of RAC residues in this co-product make food chain control more complex.

According to Montes Nino et al. (2017), the need to guarantee high-quality products to the consumer, such as the β-agonist zero tolerance adopted by many countries, demonstrates the

importance of residue detection and traceability in animal production. Although there are several reports about RAC residues in tissues from pig fed known concentrations of RAC (Qiang et al. 2007; Thompson et al. 2008; Dong et al. 2011; Pleadin et al. 2012; Chang et al. 2018), there are no reports about pig being fed MBM containing traces of RAC.

Edible tissues such as muscle, lungs, kidneys and livers, among others, have been evaluated for RAC residue detection (Qiang et al. 2007; Dong et al. 2011; Valese et al. 2016; Feddern et al. 2018). The importance of this analysis in pig tissues is because they are exported for *in natura* or processed meat consumption by different countries. According to Alemanno and Capodieci (2012), countries such as China and Taiwan, with zero tolerance for RAC residues in meat products, consume more internal organs compared to USA and European consumers. There is also concern about the consumer health, because animal offals are also destined for the production of emulsified products.

Previous research quantified RAC in meat from pigs fed at a constant concentration of this additive in their feed during a set period. However, there are no reports on the presence or absence of RAC in feeding pigs with increasing concentrations of MBM containing RAC residue. Therefore, the objective of this study is to evaluate the safety of MBM containing 53.5 μg kg^{-1} of RAC by administering increasing doses in pig feed from weaning to slaughter, evaluating whether there are RAC residue at any given dose, in pork and offal, such as kidneys, liver and lungs.

4.2. Materials and methods

4.2.1. Meat and Bone Meal (MBM)

RAC residues in MBM were previously determined in various commercial samples (Gressler et al. 2016) where concentrations from 3.87 to 81.25 μg kg^{-1} were found. Considering that 85% of the samples had less than 55 μg kg^{-1} of RAC, a MBM containing RAC concentration > 50 μg kg^{-1} was chosen as more likely to be commercially found. Therefore, MBM containing 53.5 ± 1.50 μg kg^{-1} of RAC was used in this experiment.

Approximately 2.5 t of MBM was separated and well mixed before sampling. Next, 10 subsamples (~ 100 g each) from different points were collected and final sample was formed and sent to the laboratory. The sample was homogenised again before RAC residue quantification by LC-MS/MS. Then, the samples were extracted and analysed in triplicate. To avoid oxidation, 600 mg kg^{-1} of a mixture of butylated hydroxytoluene and butylated hydroxyanysol was added to MBM. The batch was then kept at -20 ºC during all the experiment once RAC in MBM shows good stability (Gressler et al. 2016).

4.2.2. Animals, housing and handling

The experimental protocol was approved by the Ethics Committee on the Use of Experimental Animals of Embrapa Swine and Poultry, Concórdia, SC, Brazil (protocol no.: 007/2014). The experiment was carried out in Concórdia/SC, South of Brazil. We used gilts as animal model, once urine from females is easier to collect compared to males. Forty gilts from the same genetic line with average age of 63 days and initial weight of 23 ± 2 kg were used in this study. The animals were reared along a total period of 112 days, at an average weight of 94 ± 10 kg.

Animals were housed in 4 compact floor pens of 10 gilts each, with an individual space of 1.15 m^2/animal. Feed allowance was adjusted to average appetite to minimise waste in the trough. Only water was provided *ad libitum*. The MBM batch, composed of only pig offal, was chosen after been identified as the one which showed the highest amount of analysed RAC residues, according to the methodology developed by Gressler et al. (2016). After RAC quantification at 53.5 µg kg^{-1} in the MBM, diets were formulated to contain 0% (control), 7%, 14% and 21% (w/w) of MBM according to Table 1. MBM was added to partially replace soybean meal. MBM was provided to the animals throughout the experiment (112 days). The feeding programme was composed of four phases (growing 1 and 2, finishing 1 and 2) in order to meet or exceed nutrient requirements of pig, as recommended by NRC (2012). Two growth promoters (colistin and lincomycin) were added to all diets, as can be seen in Table 1.

Table 1. Dietary formulas and nutritional compositions by phase, given to gilts along the experiment.

Ingredient/nutrient	Unit *	Growing 1 (23 - 50 kg)				Growing 2 (50 - 76 kg)				Finishing 1 (76 - 98 kg)				Finishing 2 (98 - 120 kg)			
Meat and bone meal	%	0	7	14	21	0	7	14	21	0	7	14	21	0	7	14	21
Corn	%	63.72	65.79	65.49	65.21	66.92	68.26	68.16	67.86	74.03	74.77	74.47	73.97	73.61	74.32	74.12	74.02
Soybean meal	%	29.40	21.80	14.60	7.40	27.40	19.90	12.70	5.50	21.60	14.20	7.00	0.00	22.40	15.10	7.80	0.60
Soybean oil	%	3.20	3.00	3.60	4.20	3.00	3.00	3.40	4.00	2.00	2.20	2.80	3.40	1.60	1.80	2.40	2.80
Mineral-vitamin premix	%	2.00	2.00	2.00	2.00	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Salt	%	0.46	0.36	0.26	0.14	0.40	0.30	0.2	0.1	0.38	0.28	0.18	0.08	0.38	0.28	0.18	0.08
Limestone	%	0.33	-	-	-	0.21	-	-	-	0.12	-	-	-	0.14	-	-	-
Dicalcium phosphate	%	0.84	-	-	-	0.53	-	-	-	0.32	-	-	-	0.37	-	-	-
Colistin sulphate	%	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	-	-	-	-
Lincomycin	%	0.02	0.02	0.02	0.02	0.01	0.01	0.01	0.01	0.02	0.02	0.02	0.02	-	-	-	-
Enramycin	%	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.01	0.01	0.01
Total	%	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Crude protein	%	19.75	19.75	19.75	19.75	18.75	18.75	18.75	18.75	16.55	16.55	16.55	16.64	16.9	16.9	16.9	16.9
Total calcium	%	0.78	1.00	1.54	2.09	0.66	1.00	1.54	2.09	0.56	0.99	1.54	2.08	0.59	0.99	1.54	2.08
Total phosphorus	%	0.51	0.59	0.83	1.07	0.44	0.59	0.82	1.06	0.38	0.56	0.8	1.04	0.4	0.57	0.81	1.05
Available phosphorus	%	0.40	0.49	0.73	0.97	0.34	0.48	0.72	0.96	0.29	0.47	0.71	0.95	0.3	0.47	0.71	0.95
Metabolisable energy	kcal kg^{-1}	3,350	3,350	3,350	3,350	3,350	3,350	3,350	3,350	3,325	3,325	3,325	3.325	3,300	3,300	3,300	3,300
Ether extract	%	6.25	6.88	8.20	9.53	5.97	6.85	8.18	9.50	5.24	6.30	7.63	8.96	4.78	5.80	7.13	8.46
Digestible lysine	%	1.20	1.19	1.17	1.16	1.08	1.07	1.05	1.04	0.95	0.93	0.92	0.92	0.97	0.95	0.94	0.93
Digestible methionine	%	0.40	0.43	0.45	0.48	0.34	0.39	0.39	0.41	0.29	0.32	0.34	0.36	0.30	0.32	0.34	0.37
Digestible threonine	%	0.78	0.78	0.78	0.78	0.70	0.70	0.70	0.70	0.63	0.63	0.63	0.64	0.65	0.65	0.64	0.64
Digestible tryptophan	%	0.22	0.20	0.19	0.17	0.19	0.18	0.16	0.15	0.17	0.15	0.14	0.12	0.17	0.16	0.14	0.13
Sodium	%	0.20	0.20	0.20	0.20	0.18	0.18	0.18	0.18	0.17	0.17	0.17	0.17	0.17	0.17	0.17	0.17
Copper	mg kg^{-1}	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00
Zinc	mg kg^{-1}	90.00	90.00	90.00	90.00	67.50	67.50	67.50	67.50	45.00	45.00	45.00	45.00	45.00	45.00	45.00	45.00
Choline	mg kg^{-1}	125	125	125	125	125	125	125	125	100	100	100	100	100	100	100	100

* The units in % mean w/w.

4.2.3. Urine and tissues sampling

Urine samples were collected after a rest period of the animals early in the morning during 112 days, with an interval of 28 days each. Samples from the same pen (treatment) were pooled and kept frozen (-20°C) until analysis.

Before slaughtered, animals were fasted for about 16 h and then transported to the slaughterhouse in trucks with metallic cages, with a transport density of 233 kg m^{-2}. In the slaughterhouse, animals were kept for 3 h for a rest period. Pre-slaughter management procedures

were carried out following welfare guidelines. Slaughter occurred under the Federal Inspection Service supervision following the standards recommended by MAPA. Half of the animals were sacrificed in the middle of the experiment (56 days) and the other half in the end, after 112 days of experiment in order to verify if animals deposit more residues when they are fed RAC throughout life commercial slaughter age. After slaughtering, *post-mortem* inspection was followed in all carcasses. Approximately 300 g of muscle (n=40) and the whole organs such as lungs (n=40), liver (n=40) and kidneys (n=40) were collected. Before analysis, the sampling procedure was followed in this sequence: (a) the samples were allowed to thaw and kept chilled for around 15 h at 4°C prior to grinding; (b) the loin adipose tissue, the lungs bronchi, the liver gallbladder and the kidney pelvis were removed; afterwards the whole organ was minced in small pieces for further homogenisation in a processor (RI7625, Philips Walita); (c) these samples were weighed (5.00 ± 0.05 g), in triplicate, in 50-mL polypropylene tubes and frozen at -20°C until RAC extraction by QuEChERS, followed by LC-MS/MS analysis.

4.2.4. Analytical procedures

4.2.4.1. Reagents and materials

The analytical standards RAC (95.5% purity) and isoxsuprine hydrochloride (ISOX, 99% purity) used as internal standard (IS), as well as the enzymes protease (lyophilised powder, for use in Total Dietary Fibre assay, TDF-100A) and β-glucuronidase (from Helix pomatia, Type HP-2, aqueous solution, ≥ 100,000 units mL^{-1}), were all purchased from Sigma-Aldrich (St. Louis, USA). All solvents were HPLC grade and Milli-Q water was from Millipore (Advantage A10). Other reagents were purchased from different companies: formic acid from Sigma-Aldrich, Brazil; acetic acid (glacial, > 99.7% purity), ammonium acetate (98% purity) and sodium hydroxide (> 97% purity) from Vetec, Brazil; Tris (> 99.8% purity) from Promega, USA; and hydrochloric acid (36.5–38.0% purity) from Panreac, Spain. Phenomenex® provided QuEChERS kits (KS0-8909 and KS0-8921).

4.2.4.2. RAC analysis in urine

Urine samples were first hydrolysed with β-glucuronidase, then RAC was extracted and pre-concentrated by solid phase extraction. The extracted samples were analysed by LC-ESI^+-MS/MS. A summary of the procedure used for urine sample pre-treatment and subsequent extraction and clean-up is illustrated in Supplementary Figure S1, as well as a typical urine chromatogram (Supplementary Figure S2) showing RAC and IS retention times. In addition, LC-MS/MS analysis and validation

parameters such as specificity, linearity, recovery, repeatability, reproducibility, limit of detection (LOD) and limit of quantification (LOQ) are reported in Supplementary material.

4.2.4.3. RAC analysis in MBM, pig muscle, liver, kidneys and lungs

MBM and tissue samples were extracted and analysed according to our previous validated work (Gressler et al. 2016, 2018; Feddern et al. 2018). Briefly, homogenised samples (5 g) were digested with protease followed by β-glucuronidase hydrolysis.

After pH adjustment, QuEChERS extraction was performed with 10 mL acetonitrile followed by adding an extraction roQ™ QuEChERS KS0-8909 kit (4.0 g $MgSO_4$, 1.0 g NaCl, 1.0 g of SCTD and 0.5 g SCDS). After centrifugation, an aliquot of 6 mL of the upper phase was transferred to the roQ™ QuEChERS dSPE kit KS0-8921 (900 mg $MgSO_4$, 150 mg PSA and 150 mg C18E) for cleaning up. After centrifugation, 1 mL from the upper layer was transferred to a vial for LC–MS/MS analysis. Chromatographic separation was done in a C18 column with gradient elution of methanol (0.1% formic acid) and water (0.1% formic acid). A triple quadrupole mass spectrometer detector was equipped with electrospray ionisation source in the positive ionisation mode and multiple-reaction monitoring.

The precursor ions for RAC and ISOX were the corresponding pseudo-molecular ions m/z = 302.2 and m/z 302.1, respectively. The most intensive product ion m/z 164.2 was used for RAC quantitative measurement, whereas m/z 121.2 was chosen for RAC identification. Similarly, the most intensive product ion of ISOX m/z 150.1 was used for quantification and m/z 284.1 for identification. The retention time and the ion ratio were also monitored.

4.2.5. Statistical analysis

Since the objective of the experiment was to evaluate tissue residues, the individual animal was considered as the experimental unit. RAC residue data in different tissues were subjected to an exploratory analysis to detect discrepant observations (outliers). Means ± SD were analysed using PROC UNIVARIATE and the Guided Data Analysis procedures (SAS Institute Inc 2012). Since MBM levels were equidistant, it was decided to evaluate the effects through regression analysis, using the PROC REG procedure (SAS Institute Inc 2012).

4.3. Results and discussion

4.3.1. RAC in urine

Figure 1 shows RAC residues in urine of gilts along the experimental days. When animals were fed 0% MBM, an unexpected cross-contamination might have occurred in feed mill at 28 days; at 56 days, RAC residue was below LOD (0.05 μg L^{-1}), while at 84 and 112 days, RAC values were below LOQ (0.15 μg L^{-1}).

As long as MBM addition increased, RAC residues also increased. However, comparing each treatment along the experimental days, RAC concentration decreased. The maximum concentration observed was 1.35 μg L^{-1} at 28 days of experiment, by adding 21% (w/w) MBM in the diet. At the last sampling, urine from animals receiving 21% (w/w) MBM diet showed last RAC residues compared to other treatments, probably because urine was more diluted due to higher water consumption.

The effect shown in Figure 1 may be explained by the pharmacokinetics of RAC in the gilts. Mills et al. (2003) categorised β-adrenergic receptors and observed that RAC binds mainly to the β_1 subtype in pigs. Moody et al. (2010) verified that RAC response decreases over time, due to these β_1-adrenergics receptors. Therefore, the intramuscular fat content is likely to decrease when RAC is added to the diet, probably due to rapid down-regulation of the β-receptors in the fat tissue (Dunshea et al. 2005). Almeida et al. (2012) stated that with the prolonged cell exposure to RAC, the intensity of the receptor-mediated response could be reduced despite the continued presence of the agonist.

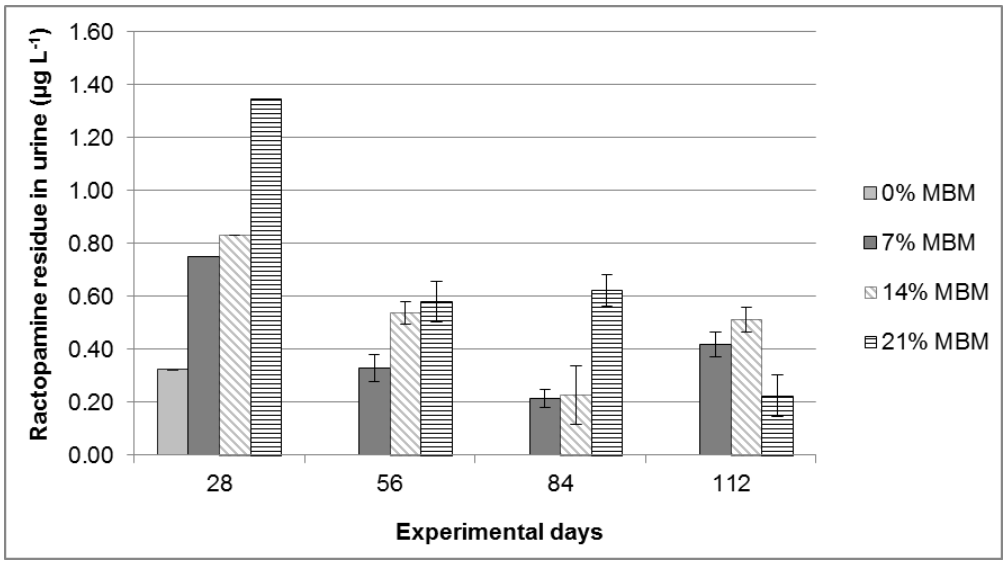

Figure 1. RAC concentration (mean ± SD) determined in the urine of gilts throughout the experiment. Concentrations are given for the four groups of animals fed 0%, 7%, 14% and 21% (w/w) MBM containing RAC (53.5 μg kg^{-1}); LOD = 0.05 μg L^{-1}; LOQ = 0.15 μg L^{-1}

RAC seems to act more efficiently within 2 weeks after its administration (Cantarelli et al. 2009), time in which animals deposit more protein (muscle hypertrophy effect). The maximum RAC concentration found may be considered low, when compared to the literature findings (Qiang et al. 2007; Thompson et al. 2008). Qiang et al. (2007) fed pig with 18 mg kg^{-1} RAC for 28 days, twice a day. They observed 650 µg L^{-1} RAC in urine (collected from the urinary bladder at slaughter). Thompson et al. (2008) reported that pig fed with the same amount of RAC for 10 days showed > 500 µg L^{-1} RAC in urine immediately after the withdrawal of this additive from feed.

4.3.2. RAC in muscle, liver, kidneys and lungs

The limits of quantification (LOQ) of the method applied herein was 0.5, 2.5, 2.5 and 2.5 µg kg^{-1} for muscle, liver, kidney and lung tissues, respectively (Feddern et al. 2018; Gressler et al. 2018).

RAC residues were below the LOQ in all tissues, including those animals sacrificed in the middle of the experiment. Therefore, MBM inclusion in the diet, even administered over a longer period in pig diet, did not lead to significant RAC residual deposition in the evaluated tissues.

Figure 2 shows the estimative of RAC consumption administrated via MBM (containing 53.5 µg kg^{-1} RAC) at the levels of 7%, 14% and 21% (w/w) compared to commercial RAC consumption, at doses (5-20 mg kg^{-1}) recommended by regulatory agencies worldwide, in the last 28 days at the finishing phase, without withdrawal period, without MBM.

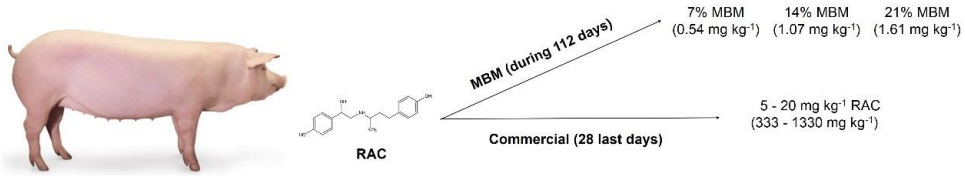

Figure 2. RAC consumption per animal weight by each gilt via MBM (53.5 µg kg^{-1}) added at 7%, 14%, and 21% (w/w), compared to estimated commercial RAC (5-20 mg kg^{-1}) given in the diet.

The cumulative feed consumption was estimated in 143.22 kg/animal for the four treatments during all experiment (112 days), based on the growth curve of the genetic line of the animals. The total RAC intake from MBM was calculated multiplying the amount of 143.22 kg/animal by 7%, 14% and 21% (w/w) totalling 10.03, 20.05 and 30.08 mg kg^{-1} of MBM ingested per animal. Then, multiplying these values by 0.0535 mg kg^{-1} (the amount of RAC originally present in MBM) resulted in 0.54, 1.07 and 1.61 mg, respectively, of RAC intake per animal during the experiment for all treatments (Figure 2).

The inclusion of 14% and 21% (w/w) MBM levels in the diet forced the presence of RAC residues and thus not represent the practical levels (3-8% w/w of MBM) in a regular pig diet in pork production, once the values adopted herein are higher than recommended (Rostagno et al. 2011; NRC

2012). This preliminary study can be useful for future studies to demonstrate the safety of MBM in pig diets.

There are reports showing that RAC metabolites correspond to RAC glucuronoconjugates in the excreta and tissues of cattle and pig which account for around 10% (EFSA 2009). Thus, we believe that the low amounts of RAC residues found herein were because the RAC administered to gilts was partially bound to glucuronoconjugates. Therefore, RAC may have been less absorbed compared to free RAC added commercially as a veterinary product in pig diets in the last 28 days of life.

RAC tends to be well absorbed and rapidly excreted what results in low RAC residual concentrations in tissues (Dalidowicz and Babbitt 1986; Ungemach 2004). The authors observed higher RAC residue concentrations in kidney compared to liver and reported that more than 88% of RAC given in pig diets is excreted via urine (Dalidowicz and Babbitt 1986).

Dong et al. (2011) administered 20 mg kg^{-1} RAC in pig diet for 30 consecutive days and detected RAC at higher concentrations in lungs (599 μg kg^{-1}) than any other tissue, following the descendent order: stomach > kidney > large intestine > small intestine > liver > heart > muscle. As there is no maximum residue limit established for lungs, its safety cannot be secured when it is consumed. Also, according to Antignac et al. (2002), lung is an organ with high concentration of β-adrenergic receptors that may contribute to increased RAC deposition due to the receptor-ligand interaction.

Since RAC is present in low concentration in MBM provided through diet during all the experiment (112 days), it is estimated that RAC residue via MBM poses a lower risk when RAC is given to gilts (Figure 2). However, commercial RAC (not via MBM) given to gilts in their last 28 days of life implied the consumption of around 1330 mg kg^{-1} RAC of animal weight each, when administering 20 mg kg^{-1} RAC in feed.

RAC deposition in tissues is controversial by the available literature. By one side, according to Dalidowicz and Babbitt (1986), 88% and 8% RAC are eliminated through pig urine and faeces, respectively. On the other side, the scientific panel on additives requested by the EU (EFSA 2009) assumes that 10% and 90% of RAC are excreted via urine and faeces, respectively. Therefore, from the total amount of 1330 mg kg^{-1} mentioned before, 4% and ~0% would be in pig tissues according to the first and second literature references, respectively. From this point of view, MBM addition up to 21% (w/w) in feed may be considered safe in the countries that allow pig production with this additive. On the other hand, MBM as feed ingredient should be avoided in the countries that forbid RAC use in any cycle of pig production, because the possibility of RAC residues exist and may be easily detected through LC-MS/MS. Even though low RAC concentration does not cause adverse health effects, according to the Codex, it does become a trade barrier.

4.4. Conclusions

All levels of MBM (7%, 14% and 21% w/w) added to pig gave no quantifiable concentrations of RAC residues in their offals (kidneys, liver and lungs) and muscle at slaughter. In this sense, these tissues can be considered safe for consumers. Besides, low residues were eliminated through urine.

When MBM presents higher RAC residue concentration than evaluated herein (53.5 µg kg^{-1}), the meat tissues should be evaluated for RAC residue deposition, once this is a preliminary study and others are necessary to investigate the amounts of RAC in various types of MBM. More research is needed regarding MBM-containing RAC and its relation to ensure safe levels of MBM added to animal diet.

However, when considering restrictive markets, such as those with zero-tolerance policy for RAC residue, MBM should not be recommended to be used as a feed ingredient.

Disclosure statement

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Supplementary material

Supplementary data associated with this article can be accessed on the publisher's website.

Supplementary material

Urine method extraction. Urine samples were homogenised and transferred into 50 mL polyethylene tubes. Aliquots of known negative urine were used for spiking and to provide blank chromatograms. A summary of the procedure used for urine sample pre-treatment and subsequent extraction and clean-up is illustrated in **Figure S1.**

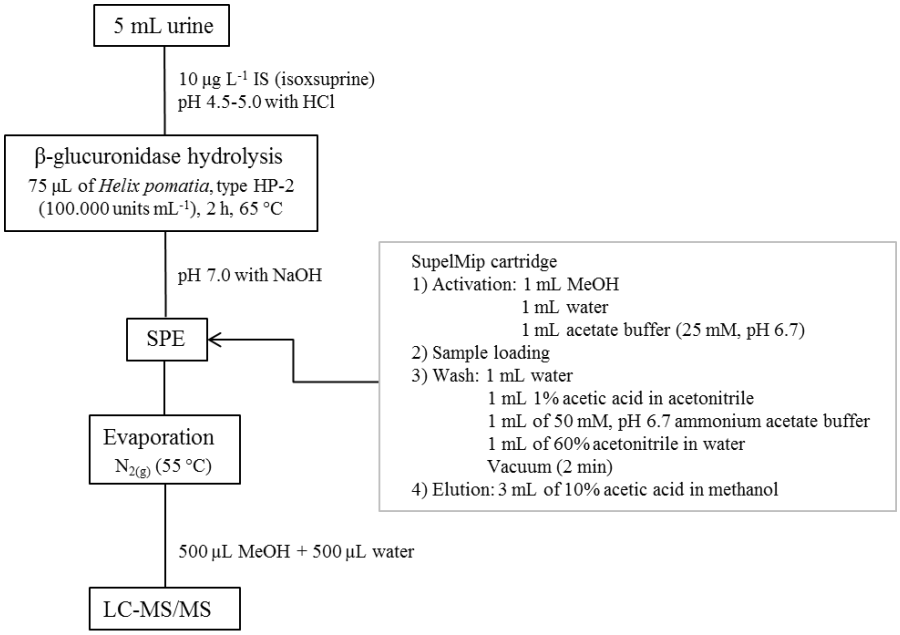

Figure S1. Overview of the complete sample-preparation and clean-up procedure for analysis of ractopamine in the urine of gilts.

LC-MS/MS analysis. The HPLC system consisted of a Surveyor Plus (Thermo Scientific). The LC column was a Kinetex C18 100A (150 x 4.6 mm, 5 µm; Phenomenex) with a C18 guard column. The mobile phase was 0.1% formic acid in methanol (A) and 0.1% formic acid in water (B). The gradient elution was: 15% A (0 to 0.5 min), 100% A (0.5 to 3.0 min), 100% A (3.0 to 7.0 min), 15% A (7.0 to 7.5 min), and 15% A (7.5 to 10.0 min). Injection volume of 10 µL, flow rate of 1.0 mL min^{-1}, and column temperature of 30 ºC were also set. The LC was coupled via an ESI probe to a MS/MS system (Quantum Access Max, Thermo Scientific). The source was maintained at 305 ºC and vaporised capillary temperature was carried out at 300 ºC. Nitrogen was used as the sheath gas and auxiliary gas at 45 and 20 psi, respectively. Spray voltage was set at 4.5 kV. Multiple reaction monitoring (MRM) was used for sample analysis. For RAC, the positive ion *m/z* 302.2 were selected as the monitor ion and three product ions *m/z* 284.2 (9 eV), *m/z* 164.2 (12 eV) and *m/z* 107.2(30 eV) for confirmation and one ion *m/z* 164.2 for quantification. Regarding IS (isoxsuprine hydrochloride), the *m/z* 302.1 ion was selected as the monitor ion and two product ions *m/z* 284.1 (9 eV) and *m/z* 150.1 (21 eV) for confirmation and one *m/z* 150.1 ion for quantification.

Urine method validation. The validation parameters evaluated were according the parameters established by the Ministry of Agriculture, Livestock and Food Supply (Portuguese acronym MAPA: Ministério da Agricultura, Pecuária e Abastecimento[1]). Specificity was investigated in 20 blank

[1] MAPA. (2011). Guia de Validação e Controle de Qualidade Analítica: fármacos em produtos para alimentação e medicamentos veterinários. (Ministério da Agricultura Pecuária e Abastecimento, Ed.), Toxicologia Analítica (1st ed.). Brasília: MAPA/ACS. Retrieved

samples by the identification and quantification of possible interferences close to RAC and IS retention times. No interference peaks were found close to RAC (5.32 min) and IS (5.70 min) retention times. The linearity of the method was evaluated in a matrix-matched calibration curve (n=3) with eight RAC concentration points (0, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 and 75.0 μg L^{-1}) and IS (10 μg L^{-1}). The equation (y = 0.885x + 0.045) obtained showed a coefficient of determination R^2 of 0.9987. Recovery was assayed by the blank sample fortification in three levels of concentration (2.5, 10.0, and 50.0 μg L^{-1}) in six replicates. The recovery rates obtained were 106.2, 102.3, and 109.7%, respectively. Repeatability (intra-day precision) were 2.67 (± 0.11), 10.72 (± 0.54) and 48.48 (± 2.76) μg L^{-1}, for 2.5, 10 and 50 μg L^{-1} respectively. Reproducibility (inter-day precision, within-laboratory) were 2.61 (± 0.32), 10.70 (± 0.50) and 48.35 (± 1.14) μg L^{-1}, for 2.5, 10.0 and 50.0 μg L^{-1} respectively. The limits of detection (LOD) were established based on a 3:1 signal to baseline noise ratio in a low-level matrix containing standard and limits of quantification (LOQ) were established based on a 10:1 signal to baseline noise ratio. LOD and LOQ levels were determined to be 0.05 and 0.15 μg L^{-1} for RAC, respectively.

A typical chromatogram of RAC residues (10 μg L^{-1}) and the internal standard isoxsuprine (10 μg L^{-1}) in urine is shown in **Figure S2.**

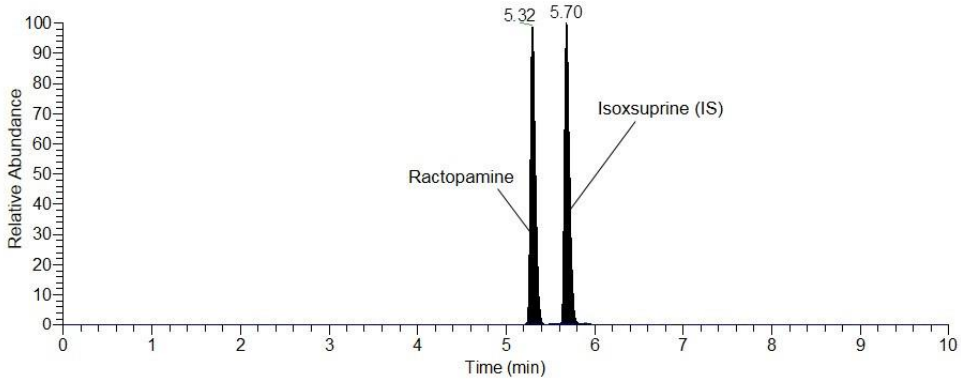

Figure S2. Chromatogram of RAC residues in spiked urine (10 μg L^{-1}), as well as the internal standard isoxsuprine (10 μg L^{-1}).

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5. GENERAL CONSIDERATIONS

At the present time, the food and agriculture sector is faced with a critical global challenge in order to ensure access to safe, healthy, and nutritious meat for a growing world population, using natural resources more sustainably (OECD/FAO 2017). In this context growth promoters can assist in meeting the increased demand for meat (Herago and Agonafir 2017). To assist this objective and increase productivity, ractopamine (RAC), a β-agonist, has been widely used in pork production systems, as a growth promotant. In a study by DeCamp et al. (2001) the use of RAC presented some environmental improvements in pork systems, as the RAC used in pig diets was responsible for a decreased overall manure output, increased nitrogen retention and decreased of nitrogen excretion per pig.

RAC is approved for use in pigs with a withdrawal time of 0 days, and it should be noted that there have been no reported cases of adverse health effects in humans exposed to livestock products containing RAC residues (Baynes et al. 2016). The approval of veterinary drugs used in food-producing animals can be made after systemic evaluation of efficacy, target animal safety, human health risk, and environmental risk assessment (Jeong et al. 2010). According to National Research Council (1999), these areas as human food safety, target-animal efficacy and safety, and environmental fate and also the worker safety are the major areas of data required in all countries for approval of a veterinary compound. However, Grommers (1996) reported that the use of β-agonists as growth promoters may result in animal health and welfare risks and raise ethical questions. Despite this some countries have banned the use of RAC in pig diets, and several laboratories have included RAC in their confirmatory methods in order to restrict and control meat products containing RAC.

The determination of residue drugs in biological matrices is often very complex, as some drugs are strongly metabolized and the metabolites might still be unknown or their standards are not available (Courtheyn 2002). A method for the detection of RAC in matrices of biological origin was satisfactorily developed in this study and shown as a useful, practical and accurate method. Once the methodology was established, the aim was to investigate RAC levels in the tissues of pigs fed meat and bone meal (MBM) at different levels.

It is known that the cereals contribute to the higher prices of pork production systems and that MBM is able to decrease cereal levels and increase the competitiveness of this sector. The cereals are the most important source of calories across the world, and these will continue to increase their share in total animal feed (OECD/FAO 2017). The MBM has been used in pig diets in order to replace soybean and increase competitiveness and also minimize environmental impacts due the recycling of co-products from slaughtered animals. However, MBM can contain RAC residue and in this study the use of MBM levels (7, 14 and 21%) above to the real levels used by the producers were designed to force the detection of RAC in pig tissue. The results showed that even at these higher levels with

MBM up to 53.5 µg kg^{-1} of RAC the values were below the maximum residues limit for the countries that permit the use of RAC in pig diets.

This is important as the challenge for pork production in Brazil will be to take advantage of the opportunity by finding ways to expand output and improve the safety of its products, taking into consideration the barriers imposed by some countries. To achieve these outcomes it is important to that productivity and efficiency keep growing, and simultaneously it is necessary to maintain the highest safety standards. These factors will be critical in the future in supporting the continued growth of the national production of pig. Thereby, this research was crucial to contribute to the surveillance programs for official control of RAC residues and also to maintain the growth of national pork production for the internal and external markets.

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6. CONCLUSIONS

A method to detect RAC in key pig tissues was improved and validated as the first phase of the study program. Results from the second phase ratify the safety of meat and bone meal (MBM) used in pig diets up to 53.5 µg kg^{-1} of ractopamine (RAC), with the purpose of complying with national legislation and other countries which have maximum residue limits for RAC. However, in countries which have restrictive markets, and those with zero tolerance, this MBM should not be recommended as a feed ingredient in pork production.

Pig diets represent more that two-thirds of the total cost of production and co-products as MBM and substances as RAC can be a good alternative to reduce them. However, the pig farming, it's known as an activity that coexists with some challenges. It should be conducted seeking the use of technologies which allow productivity gains, and consequently, lower production costs and better economic results, besides to ensure the international markets, once the global marketplace has been become very competitive.

Thereby, further researches should be elaborated on regarding MBM-containing RAC and its relation to ensure safe levels of MBM added to animal diet. The researches with focus on others strategies that may contributes for the aims above are also indicated. Strategies and programs by producers in association with national and international companies and policy makers are importants tools in order to promote the economic growth of the nation.