Characterization of Phosphorus in Poultry Litter and Determination of Phosphorus Extraction Efficacy of Extractants

by

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Abstract

Poultry litter (PL) is used as a nutrient source for row crops, horticultural crops, and pasture production. Typically, PL is a mixture of poultry feces, bedding materials, spilled grain and feather than can potentially enrich soils with primary nutrients [nitrogen (N), phosphorus (P), potassium (K)), and secondary nutrients [sulphur (S), aluminum (Al), iron (Fe), calcium (Ca), magnesium (Mg)]. Poultry birds lack phytase enzyme, hence they are unable to digest complex organic molecules such as phytic acid present in the feeds. This leaves greater proportions of undigested P in the bird's feces. Additionally, PL is bulky and has low density which causes its long distance transportation uneconomical. This practice results in repeated land application of PL and increases the soil background P levels. Runoff losses from these P hotspots can cause eutrophication and aggravate water quality issues in the surface waterbodies. Therefore, there is a need to understand the P forms present in PL and develop methods towards extracting and recycling P safely for agricultural purposes. The overall goal of this study was to investigate different P forms present in litter and determine the P extraction efficiency of various chemical reagents to extract P from PL. Specific objectives of this study was to i) quantify various P fractions in poultry litter of four PL clean out frequencies (CF) (0-6 months, 6-12 months, 12-24 months, and 5-year-old) using sequential P fractionation ii) understand the difference in P forms among 4 CF of PL iii) to quantify the phosphorus dissolution efficacy of selected acidic (HCl, citric acid), neutral (water), and basic extractants $NaHCO_3$ iv) quantify the co-release of metals during the extraction cycle. Sixteen PL samples were collected from eight counties in Alabama. Dried and ground PL were used for general chemical characterization and sequential P fractionation. 0.3g of PL was extracted using 30ml of de-ionized water (DH_2O), sodium bicarbonate (0.5 M $NaHCO_3$), sodium hydroxide (0. 1

M NaOH), and 1 M HCl in a sequential order. Total P in PL was characterized as DH_2O P, $NaHCO_3$- P, NaOH-P and HCl-P and residual P fractions, where each fraction represent P of increasing stability. Results showed that greater residence time of PL in the poultry house caused recalcitrant organic fraction to decline with time possibly due to the breakdown of organic P. Whereas, in 6 months old PL, the recalcitrant P was significantly greater than 5 year old PL, there was no significant difference in inorganic P fraction (P_i) between PL of different clean out frequency. Repeated independent extractions were performed with 4 selected acid and basic extractants: de-ionized water (DH_2O), $NaHCO_3$ HCl and citric acid. 0.3g PL was repeatedly extracted with 30ml of individual extractant. Filtrates were analyzed for dissolved total P (DTP) and molybdate reactive P (MRP). Co-release of nutrients and heavy metals were recorded with P release (Al, Fe, Ca, Mg, As, Cs, Cd, K, S). Acids like HCl and citric acids took 4 extraction cycles to extract upto 90% of the P, whereas $NaHCO_3$ took 5 extraction cycle and extracted only 56 %. Similarly, deionized water took 7 extraction cycles and extracted 40% P. Comparing the DTP and MRP recovery rates of the acidic and basic extractants, HCl recovered significantly greater percentage (95%) of DTP, whereas citric acid recovered significantly greater percentage (66%) of MRP .The phosphorus extraction efficiency can be ranked as 1 M HCl > 1 M citric acid > 0.5 M $NaHCO_3$> deionized water. The residual P left after water and sodium bicarbonate extraction was 50% of the total phosphorus (TP) whereas up to 7% of the TP was left after acid extractions. Negligible concentrations of heavy metals like Arsenic (As), cadmium (Cd), chromium (Cr) and lead (Pb) were found.

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List of Abbreviations

Al	Aluminum
As	Arsenic
BDL	Below detection limit
Cd	Cadmium
Ca	Calcium
Cr	Chromium
Conc.	Concentration
Cu	Copper
DOP	Dissolved Organic P
DTP	Dissolved Total P
HCl	Hydrochloric acid
Fe	Iron
Mg	Magnesium
MRP	Molybdate reactive P
$NaHCO_3$	Sodium bicarbonate
NMR	Nuclear Magnetic Resonance
P_i	Inorganic P
P_o	Organic P
K	Potassium

Res P	Residual P
S	Sulphur
TP	Total P
WEP	Water Extractable P
WSP	Water Soluble P
XANES	X-ray absorption near edge structure
Zn	Zinc

Literature Review:

Introduction

The element phosphorus (P) and importance:

Phosphorus (P) is the 11th most abundant element accounting for about 0.12 % of the earth's crust (Greenwood and Earnshaw, 1984). The most abundant naturally occurring source of phosphate (PO_4^{3-}), an anion of P, is the mineral apatite (Lamprecht et al., 2011). Phosphorus is essential for the survival of life because it is a crucial constituent of several biomolecules such as DNAs, RNAs and ATPs. It is present in bones and living tissues (Lamprecht et al., 2011) and a nutrient source for all living organisms including plants, animals, and human beings (Lamprecht et al., 2011). Phosphorus is rarely found in it's free inorganic state but is found in greater abundance in organically bound forms. In fact, phosphorus has the slowest matter cycle on earth, spanning all geological frames of time span (Lenntech, 2015). Unlike carbon and nitrogen (N) cycles, P does not exist in gaseous phase in its cycle (Reddy, 2008). Due to its close associations with living systems, the decomposition of living organism releases P into the environment. Hence, all organic wastes such as, sludge and sewage, municipal wastewater, human and animal excreta. Manures of non-ruminant animals, such as swine and poultry, are particularly rich in P due to lack the phytase enzyme in their gut. Hence, they accumulate larger portions of P in their excreta (Bedford, 2000).

Broiler Industry in USA and Alabama

Intake of poultry products is an intergral part of the human diet and has led to an exponential growth in broiler industry in the recent decades. In fact, the growth in the broiler sector by 2050 is projected to be more than that of beef and other meat industries (FAO, 2005). Arkansas, North Carolina, Georgia, Alabama and California contribute to more than half of the poultry production in the United States (Moore,1998). Alabama ranks second after Georgia (USDA, Agriculture statistics, 2018). Being the second largest agricultural commodity in the state, it contributes employment to the more than 86,000 residents in Alabama (ALFA, 2017). Yearly 6 million pounds of meat is produced by the broiler industry, generating an income of 3 billion dollars to the poultry farmers of Alabama (NASS- USDA, 2016). In the process, 1.5 million tons of poultry waste is generated which contains 19,560 tons of P (typically 1 ton of PL contains 60 lbs P_2O_5). Proper management and disposal of poultry litter is important to avoid environmental issues such as water quality degradation of natural water bodies.

Role of phosphorus in the eutrophication

Runoff of nutrients from the manure has the potential to cause eutrophication in lakes and water bodies. A classic incidence was the study conducted in the experimental lakes in Canada that observed the effects of eutrophication on introduction of nutrients such as N, P and C. Results indicated that P played a key role in regulating algal bloom and eutrophication (Schindler,1974). Thereafter the U.S. federal government legal regulations were imposed on the transport of detergent phosphate into the surface waterbodies. Imposing regulations on point sources (detergent phosphate) reduced algal bloom and eutrophication to some extent. The reason being most of the water bodies and systems are P limiting (Baldwin, 2013), whereas the non-point P sources also significantly contributes to eutrophication and algal bloom. Agricultural runoff from fertilizers and manures are the examples of non-point sources of nutrients that can be one of the leading causes of eutrophication. The federal government of United States stressed upon reducing the non-point sources in “the Great Lakes” basin (Rohlic and O’Connor, 1980) after 1970’s. A study in Netherlands found that eutrophication was caused by animal wastes, so they limited the P entry from animal waste into water system to control eutrophication (Breeuwsma and Silva, 1992).

Chicken litter being rich in P aggravates the risk, as runoff P leads to pollution in surface waters (Sharpley and Menzel,1978). Eutrophication is a global environmental pervasive problem in waterbodies and ought to be checked as once a lake turns into toxic by harmful algal blooms (HABs) and cyanobacterial abundance, it is very difficult to restore it. The difficulty in restoration supported by "the theory of alternative states" has two factors i) feedback mechanisms of the alternative stable states ii) hysteresis is involved in the shift (Scheffer et al., 2001). Hence, prevention of eutrophication is imperative as cure is almost impossible.

Numerous studies have mentioned the usage of poultry litter (PL) for the agronomic practices (Bosch and Napit,1992). Application of PL as manure to improve organic matter and nutrient nourishment to crops is an age-old practice. Poultry litter has always been extensively studied for its agronomic values (Miller et al.,1991; Salter and Schollenberger 1939; Smith and Wheeler,1979; Stephenson et.al 1990), but several studies has shown occurrences of problems with the direct application of the poultry litter to the soil. These problems can be categorized as production problems and environmental problems. Runoff and leaching of the nitrates and phosphates are enlisted under the environmental problems (Kingery et al.,1994). Recent studies have shown that repeated application of chicken litter in the same piece of land has increased the background P levels in the soils (Sharpley et al., 2007). Over half of Alabama's broiler litter production is concentrated within the northeastern portion of the state known as Sand Mountain which has resulted in large amounts of litter applied in relatively small agricultural areas. According to an investigation carried by Kingery et al., (1994) in Cullman, Blount, Dekalb and Marshall county of Sand Mountain region in Alabama, P has accumulated and moved downward to approximately 60 cm in littered pastures. The study showed that long term litter application of broiler litter has increased the soil test P levels by an average of 30% in the 0-60 cm depth as compared to pastures that never received litter. The study also revealed that P concentrations measured in the 0-15 cm depth in litter sites have a rating of “extremely high” according to the Auburn University Soil Testing Laboratory. Elaborate discussions on the management and problems associated with PL application and handling are accounted in literature (Cabrera and Sims, 2000; Sims and Wolf 1994; and Williams et al; 1999).

“Phosphorus availability is a function of chemical equilibrium – controlled by solubility and rate limited processes” (Tiessen and Moir, 1993). The availability of phosphorus is heavily related to its chemistry, predominantly regulated by binding strength, oxygenation and pH. Under anoxic environment, P is available, hence lake sediment and ocean floor are the vast reserves of phosphorus (Reddy, 2008).

Geochemical and biological processes in soils regulate the availability of P. The stability and availability of different P forms is variable. In natural ecosystems the long term and short term distributions of P are controlled by geochemical and biological processes respectively. Therefore, the movement of P through a natural ecosystem is termed as its ‘biogeochemical cycle’ (Richey 1983, Sclesinger 1991). The biogeochemistry of P determines its stability and distribution in soils and other natural ecosystems (Ramirez and Rose,1992).

Phosphorus fractionation studies

Phosphorus binds with elements like calcium (Ca), iron (Fe), aluminum (Al), and hydrocarbons. Phosphorus dynamics are governed by binding, where P binds with different binding strength in organic (P_o) and inorganic (Ca bound P, Fe bound P, Al bound P) molecules. In fact, P exists in different pools of mobility (water soluble P, bioavailable P, potentially bioavailable P) based on binding strength. The various pools of P can be estimated by fractionation studies. Extraction techniques can be employed using chemical reagents to release P as the reactive phosphate or orthophosphate. Hence, phosphate pools can be differentially extracted and characterized according to the relative ease of extraction.

Phosphorus fractionation is a classical approach for phosphorus characterization. The approach was first suggested and tried in soil (Dean, 1938). This approach has been heavily used in soil P and remains in use till date. A fractionation procedure was developed having a series of extractions beginning with 1 M NH_4Cl following 0.5 M neutral NH_4F, 0.25 M H_2SO_4, CBD and 0.5 M NH_4F (Chang and Jackson, 1957). The underlying concept was these reagents preferentially extracted P from soil samples. The procedure was based on the hypothesis that 1 M NH_4Cl extracts the water-soluble P and the loosely bound P, 0.5 M neutral NH_4F extracts majorly the Al - bound P and 0.25M H_2SO_4 successively the Fe- bound P; while the CBD extracts the occluded P mainly. The hypothesis had some limitations particularly pertaining to calcareous soils and sediments and whether the distinction between Al-P and Fe-P is substantial. Studies were conducted thereafter which involved several modifications of the Chang and Jackson (C and J) method. One of the modified schemes included addition of NaCl along with NaOH to correct reabsorption (Williams et al.,1967). The modifications included alteration of the name of the fractions associated with one or more chemical reagent(s) and shaking times. Modifications to C and J procedure were proposed by other studies. (Syers and Smillie ,1972; Peterson and Corey,1966; and Fife 1962).

Another procedure was proposed employing a different set of chemical reagents (Hedley et al.,1982). This scheme of fractionation quantified the extractable P as organic and inorganic pool. It uses the following sequence of reagents in order: i) Anion Exchange Resin; ii) 0.5 M $NaHCO_3$; iii) 0.1 M NaOH; iv) 0.1 M NaOH after sonification; v) 1M HCl; v) Residues digested with H_2SO_4+ H_2O_2. The first two extractants namely the resin and $NaHCO_3$ supposedly extracts the most bioavailable P or the most soluble and labile P fraction, followed by labile organic and inorganic P. The NaOH and sonification extracted Fe-bound P and Al-bound P occluded in the interiors of amorphous Al/Fe hydrous oxides. The more stable inorganic P (P_i) associated with Ca and primary minerals and some organic P(P_o). The oxidizing agents H_2SO_4 and peroxide solubilizes the most chemically stable organic P (P_o) and occluded inorganic P in the residue. Originally, this procedure was used to study the mineralization and immobilization of organic and inorganic P as a result of changing cropping practices. Thereafter, several studies have been conducted time and again with modifications of the Hedley scheme, with approximately 1300 'peer reviewed' studies reference to it.

Although the fractionation techniques provide significant information about the bioavailability of P and its transformation in soil, interpretation of which reagent extracts what fraction can be ambiguous in some cases (Toor et al., 2006). Lack of clarity remains with the fact that what discrete forms of P can be expected in the labile and occluded fractions. A knowledge gap still exists about exact forms of P associated with particular extractant.

The organic pool of P is comprised of the phospholipids from animal and plant debris, nucleic acid, sugar phosphates, inositol phosphates etc. Some of the species may be abundant than other species depending on the type of soil or substrate. For example, the predominant organic species in black soil might be different from some organic P species found in chicken litter. Research conducted on the grassland soils, which is highly organic, to study the transformation and nature of organic P pools (Anderson,1967) classified the pools into i) labile pool extracted by 0.5 M $NaHCO_3$, ii) acid soluble and alkali soluble organic P, iii) moderately resistant organic fraction (fulvic acid fraction), and iv) highly resistant (humic acid fraction). Other studies have also attempted to quantify fractions of the organic P (P_o) (Halstead and Anderson, 1970; Stott and Tabatabai,1985). According to these studies, 0.5M $NaHCO_3$, and various concentration of NaOH and HCl were ideally suitable to extract the organic pool of P. These fractionation methods underwent several modifications over time.

P Fractionation studies in animal manures and organic wastes

Fractionation hypothesis used originally in soils was extensively used for the organic wastes and chicken litter applied on soils. Studies have also been conducted which employed the fractionation methods in animal manures and organic wastes (Sharpley and Moyer, 2000; Self Davis and Moor, 2000). The modification of the C and J method was used with swine, cattle and dairy manure (Tiecher et al., 2014). The results of this study interestingly showed that most of the P present in the manures are highly bioavailable. In addition, studying the different pools of P individually provides a more comprehensive understanding of the total P in manures.

The earliest reference of the phosphorus fractionation scheme used in manures is found in experiment conducted decades ago (McAuliffe and Peech, 1949). This pioneer experiment segregated the P present in manures as i) lipid P fraction extracted with 3:1 alcohol ether mixture, ii) inorganic and acid soluble P fraction removed by 5% TCA, iii) residual P by ashing the manure. Other significant modifications were proposed to this method while conducting a research on various range of animal manures (Barnett, 1994). The study conducted by Barnett (1994) evaluated the P status of animal waste and corresponding effects on animal rearing was a steppingstone. He analyzed the total P present in the beef, swine and poultry manures as total P (Pt), inorganic P(P_i), acid soluble organic (P_{aso}) and lipid soluble organic P (P_l) by chemical extraction. One of the most popular procedure that was proposed as a modification of the Headley's scheme of fractionation was Dou et al. (2000).

Phosphorus Characterization

Phosphorus is most commonly studied from an environmental viewpoint as thr total P and molybdate reactive P. Conventionally, total P is measured in the organic wastes. Since the inception of clean water act, researchers have shown more inclination towards measurement of the molybdate reactive P. Water soluble P/water extractable P (WEP) is the amount of P that is readily soluble in water and can be obtained with shaking the manure sample with deionized water. Attempts to measure and quantify water soluble P in organic wastes (Kleinman et al., 2002; Wolf et al.,2005) and biosolids (Brandt et al., 2004) can be useful to estimate the P susceptible to runoff and leaching loss. Research has also been conducted comparing different dilutions, shaking times and filter paper types (Kleinman et al., 2002) for WEP determination. Results indicate that the strongest correlation for WEP determination is observed at 1hour of shaking.

The Total P

Total P analysis involves digestion of the manure samples via acids and peroxide followed by analysis. Inductively coupled Plasma - Optical Emission Spectroscopy (ICP-OES) is more suitable to be used for the determination of total P in liquid samples. Colorimetric methods like Murphy and Riley are also used to read the TP values in digested samples. Upon acidification the acid labile organic compounds get hydrolyzed into mono phosphate molecules which can then be measured to estimate TP (McKelvie et al., 1995). The digestion process involves a strong oxidizing agent, generally an acid or combination of acids. (i) HNO_3 and $HClO_4$, (ii) H_2SO_4 and HNO_3, and (iii) H_2SO_4 and $(NH_4)_2S_2O_8$ or $K_2S_2O_8$ (APHA, 1989). Commonly used acid mixture for digestion are H_2SO_4–H_2O_2–HF (Bowman,1988). Some researchers have used sodium compounds as Na_2CO_3 fusion (Olsen and Sommers, 1982), NaOBr–NaOH oxidation (Dick and Tabatabai, 1977), and ignition (Saunders and Williams, 1955). According to the classic hypothesis, digestion helps to break down all the organic P bonds which are not easily extracted by any extractant, hence the determination of Total P is the estimate of both the organic and the inorganic pools (Po + Pi). The limitation of these methods is the requirement of specific and safe equipment to carry out the process of digestion. Methods can call in question the efficiency in cleaving of the organic P bonds. More elaborately how it can be ensured that the method used properly estimates the organic P fraction. U.S Environmental Protection Agency uses the most robust method which involves the digestion by HNO_3 followed by H_2O_2, referred to as EPA3051 and EPA3052. It is more generally used for the estimation of TP in animal waste and biosolids in the US.

Water extractable P (WEP)

Water soluble P (WSP)/ Water Extractable P (WEP) are the acronyms used to indicate the fraction of TP that can be extracted by water or that is soluble in water. WEP serves as a quick test of the relative availability of P in waste and thus became more popularly used. High values of P in biosolids has been reported in several studies (Brandt. et al.,2004). In dairy, poultry and swine manures the WEP values can be as high as up to 75% of the TP (Angel et al., 2005; Dou. et al.,2002; Toor 2005a). The concentration of WEP can vary with sample type and their composition. Water extractable phosphorus measurement provides a significant insight into the potential risk of P loss into waterbodies due to inappropriate waste disposal practices.

Sequential and repetitive extractions in different types of manure:

With popularization of the sequential fractionation, a modified Hedley scheme was developed for the manures and waste with research over the years. In Hedley sequential fractionation, WEP is dissolved inorganic P, $NaHCO_3$ represents labile P and NaOH quantifies the fractions composed of the amorphous and crystalline Fe-P and Al-P with some organic P. The acid labile fraction (HCl or H_2SO_4) indicated the relatively stable Ca-P (Toor et al., 2006b). However, an important consideration for both soil and organic waste is the difference in chemical nature or composition of the two. Soils are more dominated by mineral phases and contain more Al and Fe than manures. Manures on the other hand, contain more Ca and Mg than soils. According to study by Duo et al (2000), the majority of P extracted from manures was WEP and labile P, extracted by H_2O and $NaHCO_3$ respectively. In contrast, a greater amount of P is extracted by the NaOH and HCl in soil fractionation studies (Sharpley 1996; Tiessen et al.,1984).

The possibility of the variation in P forms with manure types has also been studied where the relationships of different types of manure P was compared (Leinweber et al., 1997). Leinweber's study reported higher percentage of WEP in poultry litter 27%, whereas in case of swine slurry it was 10%.

Sharpley and Moyer studied the forms of P in swine, dairy and poultry manures and composts by sequential fractionation procedure. The study also involved the investigation of the release of P under rainfall simulated conditions over time. The graph plotted for P concentration in runoff water in the simulated rainfall events with repeated independent extractions with water showed a high degree of correlation ($r^2 = 0.98$). Thus, results indicated that WEP concentration is a good estimate of the P released as leachate during rainfall.

With the usage of chemical reagents as extractants to extract P in the solution form, H_2O, $NaHO_3$, NaOH and TCA were used as individually to carry out independent repeated extraction cycles to observe P release patterns from swine, dairy and poultry manure (Dou. et al.,2000).

P Characterization through NMR Spectroscopy:

Nuclear Magnetic Resonance (NMR) spectroscopy is one of the advanced approaches to study the P species present in organic wastes like PL. It is based on the principle of detection of the spin quantum number of atoms. Thus, it helps to draw the outline of the molecular structure of the complex functional groups embedded in the long chain of hydrocarbons.

Undisturbed manure samples (without extraction) can be analyzed in a solid-state NMR method. In this case air drying and grinding is necessary to ensure the homogeneity in the sample for the efficient spinning of the rotor in the magic angle spinning probe. Whereas, in liquid state NMR it requires the extraction with an alkaline extractant. Solution phase NMR has an advantage of facilitating the extraction of paramagnetic metal cations (Fe and Mn). This technique is most effective for the characterization of organic P. Disadvantage of solution phase NMR is the relative overestimation of the orthophosphate fraction (Leinweber at al.,1997) due to the possible hydrolysis of organic phosphate esters (Cade-Menun et al.,2002; Turner et al., 2003).

Considerable amount of P exists as organically bound forms in PL. Hence, the use of advanced methods to study the organic P chemistry of manures can be useful.

Approaches for phosphorus removal and recovery from poultry litter

Hydrothermal Reactions

Phosphorus bonds can be cleaved by the help of physical, chemical, or biological agents. The recovery of phosphorus from organic waste and biosolid by employing physical agents like heat in hydrothermal carbonization reactions. More specifically, it can be termed as a physicochemical process that involves the hydrothermal reaction followed by acid extraction of the hydro charcoal (hydrochar). An experiment conducted in fresh manure (without bedding materials and straw) with hydrothermal carbonization followed by the acid extraction of hydrochar found that the phosphorus extracted was 8- 10% for poultry manure (Heilman et al., 2014). The percentage yield of hydrochar was dependent on temperature while the hydrochar mass loss caused by acid extraction was 26%. Although this was a novel approach for P reclamation from manures and biowastes it calls in question the economic viability of this method for future large scale P reclamation.

Microbial degradation of Poultry litter

Many microorganisms present in the soil like bacteria, fungi, actinobacteria have the capabilities of mineralizing P. Several bacterial and fungal strains have been isolated which helps to solubilize the P present in the soil (Glick, 1995). They are known as PSM (phosphate solubilizing microorganisms). *Pseudomonas* and *Bacillus* are the two most abundant genera in soil, which also act as PSM's (Illmer and Schinner,1992). The mechanism underlying the release of P, are i) the production of an extracellular enzyme phosphatases which can cleave the phosphate bonds, releasing P into the environment and ii) (McGill and Cole, 1981). The microbes use this mechanism to break down organic material and derive nutrition. Various strains of bacteria and fungi have been explored and studied extensively for their P mineralization capabilities (Sharma et al, 2013).

Apart from external microbial hydrolysis of P, the PL is also exposed to microbial load in poultry houses. The microbial concentration in PL can exceed 10^{10} cells/g (Acosta Martinez and Harmel, 2006; Cook et al., 2008; Rothrock et al., 2008a). Studies recorded the abundance of gram-negative bacteria like actinomycetes, clostridia or eubacteria (Lu et al., 2003; Enticknap et al., 2006; Lovanh et al., 2007). Bacillus sp. (Ritz et al., 2004) and Arthrobacter spp. (Schefferle, 1965; Kim and Patterson, 2003) were identified as the most prominent uric acid mineralizing microbes in PL.

Struvite

Struvite is a naturally occurring mineral of P. Struvite precipitation technology has been employed in recent years to recover phosphate from waste sludge and sewage. Solid state anaerobic digestion (SSAD) of poultry manure was experimented with anaerobic leaching bed (ALBR)/ Plug-flow digester (Farrow et al.,2016). In case of poultry litter, it also helps in reduction of ammonia emission (Zhang and Lau, 2007). Chemically struvite is magnesium ammonium phosphate hexahydrate, therefore, formation of struvite takes up available P and can be converted to a more usable fertilizer product for field application. Both N and P are recovered as nutrients in the process, which has been experimented with a plug flow anaerobic digestion (Field et al.,1985). Anaerobic digestion poultry manure and biosolids has been conducted in various studies for ammonia and methane management (Bujoczek, 2000).

Acid extraction

Organic and inorganic acids were used in a batch of experiments to study the P recovery efficiency of acids to recover P from PL (Szogi et al., 2008). “Quick wash” was the nickname of the set of experiments, that employed PL and extractant in the ratio (1:25 w/v). The acids used in the experiments were citric acid, acetic acid and HCl in varying concentrations. Results indicated that HCl and Citric acid had comparable efficiencies of recovering P from PL.

Significance

According to reports, the projected use of phosphate mineral reserves, phosphate rocks, could be exhausted by 2050 (Steen 1998; Smil 2000b; Gunther 2005). The projected global demand of phosphorus is 3-4% annual increase (Maene, 2007; FAO, 2007a). The growth in the demand of phosphate fertilizer could result in the excessive rate of use of the P reserves. Practically useful P mineral deposits for extraction are geographically concentrated in few countries. Only a small fraction of 4 $x10^{15}$ tons of P present in the earth’s crust of total P reserves are present in Morocco (85%), China (6%) and USA (3%) (Cordell et al 2009). The mineral reserves in the earth’s crust are being mined for the extraction of the phosphate; the rate of extraction is increasing by 2.3% yearly. The availability of phosphate through extraction is variable and dynamic depending upon the current technology and prices. In the long run, these non-renewable sources of P have the risk of getting exhausted. Phosphate is included as the three primary nutrients required by the plants for metabolism. There is no substitute for phosphorus in crop growth and production. If global P security is at risk, it would have further implication on the global food security in future (Cordell and White, 2009). According to the definition put forward by UN's Food and Agricultural Organization (FAO) food security ''exists when all people, at all times, have access to sufficient, safe and nutritious food to meet their dietary needs for an active and healthy life'' (FAO, 2005b, p1). Securing food is considered as a global priority, hence there is a crucial need to identify and substantiate secondary and sustainable sources of phosphate for plant fertilizers.

Research Objective

Depleting inorganic phosphorus reserves continues to be an emerging crisis, that needs integrated solutions through interdisciplinary approaches. As P is a non-renewable resource like oil and fossil fuels, renewable or recyclable phosphorus from manures will function as a restorative approach. While the management of phosphorus in organic, biowaste, and manure remains critical towards the water quality issues, exploring methods of recycling the phosphorus from waste and manures for usage as a source of nutrients for crops will be useful. Various approaches to reclaim phosphorus from manure and waste have not been able to reach a recovery rate of more than 60%. Therefore, there is a need to develop an economically feasible and viable method to recover P from poultry litter. Characterization and understanding the phosphorus forms in poultry manure will be a crucial step towards development of a method to recycle phosphorus from PL. Exploring the chemical reagents that can extract phosphorus can be a step ahead towards an economically viable method to extract and concentrate the P from PL at the source. The goal of this project is to understand the P forms in chicken litter and explore chemical reagent that can extract 90% or more P from PL.

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I. Phosphorus characterization in poultry litter of various ages by sequential fractionation procedure

Abstract

Poultry litter (PL) is the waste generated from the broiler houses typically consisting of poultry manure, bedding materials, spilled feed and feathers. Poultry litter is primarily used as a nutrient source for row crops, horticultural crops, and in pasture lands. Poultry litter is rich in phosphorus (P) since chickens are unable to break down complex organic P in their gut due to the lack of enzyme phytase. By the elemental nature of P, it exists in different pools/fractions based on its binding strength with metals like calcium (Ca), magnesium (Mg), iron (Fe) and aluminum (Al). Phosphorus fractionation procedures are used to quantify association of P with different metals by employing chemical reagents. The different P fractions directly correlate to the bioavailability of the P in PL. It is known that nutrients in PL can vary greatly between flock clean out frequency (number of flocks between total cleanout), type of bedding material, and composting method, but very less is known about the fractions of P present in PL of different cleanout frequencies (CF). Understanding the P fractions in PL of various CF will be useful towards a better understanding of P availability from agronomic and environmental viewpoint. The objectives of this research were i) to quantify the P fractions in PL of 4 CF (0-6 months, 6-12 months, 12-24 months, and 5 year old) ii) quantify the difference between the P forms (dissolved total P (DTP), inorganic P (Pi : molybdate reactive P (MRP)), dissolved organic P (P_o) and Residual P (Res P)) and their concentrations among the four PL CF. Sixteen PL samples were collected from 8 counties across Alabama, dried, and processed. Samples were categorized into 4 CF. Dried PL was extracted by de-ionized water (DH_2O), 0.5 M $NaHCO_3$, 0.1 M NaOH and 1.0 M HCl in a sequential order following Dou et al. (2000) which was a modified Hedley procedure (Hedley et al., 1982). Results

showed that P fractions in PL changed with CF. The DH_2O-P and HCl-P fractions were greatest among all P fractions, although they did not differ significantly between CF. The fractions $NaHCO_3$-P, NaOH-P and Res-P differed significantly between CF. Percent Res-P decreased whereas $NaHCO_3$-P increased with decreasing CF. This indicated that with increase in residence time of PL in houses (5 years) recalcitrant P undergoes mineralization causing subsequent decrease in Res-P and increase in labile P ($NaHCO_3$-P) fractions. Percent DTP increased with reduced CF, however there was no significant difference between Pi among the 4 CF. The driving factor for greater DTP was due to increase in Po. The percent Po increased significantly with reduced CF. The percent Res-P was significantly greater in the 6-month-old litter ($p< 0.001$) than the other CF group and decreased with reduced CF. In summary, higher residence time of PL in poultry house leads to mineralization causing greater proportion of dissolved Po and lesser concentrations of recalcitrant P.

Introduction

Poultry is the second-largest agricultural commodity in the state of Alabama. It contributes $15.1 billion in revenue and employs more than 86,000 citizens in Alabama (ALFA, 2017). Each year, Alabama's broiler industry produces an estimated 6 billion pounds of meat, generating approximately $3 billion in income for Alabama's poultry farmers (NASS-USDA, 2016). However, the industry also generates an estimated 1.5 million tons of poultry litter containing approximately19,350 tons of phosphorus (P). Poultry litter (PL) is used as a nutrient source for row crops, horticultural crops, and pasture lands. Typically, PL is a mixture of chicken feces, spilled feed along with the bedding materials. Previous literature elucidated the importance of nutrient nourishment of soil through PL (Bosch and Napit,1992). Poultry litter is considered rich in phosphorus (P) compared to other nutrients since chickens (*Gallus gallus domesticus*) are unable to break down a larger fraction of the complex organic P present in their diet due to lack of the phytase enzyme in their guts (Bedford, 2000). Therefore, repeated land application of poultry litter enriches the soil with primary soil nutrients, particularly P, as well the secondary and micronutrients. The nutrient composition of PL can vary widely depending on the age of litter/length of storage, number of flocks between litter cleanouts, amount and type of bedding material, moisture content of litter and pH (Prasad and Stanford, 2019). In the last 15 years, poultry production practices have changed such as house clean-out schedules, windrowing of litter between flocks, and phytase use in feed to aid in P availability (Tom Tabler et al., 2018).These production practices have significant influence on nutrient composition of the litter (Tom Tabler et al., 2018). Litter nutrient content can also vary due to postproduction handling and treatment (Ndegwa et al., 1991) operation. For example, the nutrient content in PL has been found to vary with compost treatment conditions (Day and Funk 1998; Osei et al. 2000). Composition of bedding material can be another reason for variation in the nutrient content in litter (Robinson and Sharpley, 1995). Robinson and Sharpley (1995) found that the nutrient content varied between the two types of PL studied; total P content in the PL with bedding material composed of pine shavings were greater than PL composed of wheat straw bedding material. Variation in nutrient content of PL can have both agronomic and environmental implications. For example, PL with greater fraction of mineralized P will be quickly available for plant uptake. However, from an environmental viewpoint, excess soluble P can trigger or accelerate the eutrophication process in surface water bodies (Schindler, 1974). Eutrophication and harmful algal blooms (HAB's) lead to the impairment of the water quality in surface water bodies (Sharpley, 2000; Boesch et al., 2001) and have far stretched consequences like anoxia, fish kills, and dead zones in rivers.

The bioavailability of P in soils or manure is "controlled by solubility and rate limited processes" and depends upon the lability of P (Tissen and Moir, 1993). Phosphorus fractionation has been a classical approach to separate P into various pools of lability. In biosolids, available P rather than total P is more relevant (Maguire et al., 2001). Since the P pools are directly correlated to the availability of P (bioavailability) there is a need to understand and identify the various P fractions/ pools in litter of different cleanout frequencies.

The phosphorus fractionation concept was first tried and tested in soils by Dean in 1938. The principle is stationed on the assumption that various chemical reagents preferentially extract discreet forms of P. The underlying hypothesis of the concept is that phosphate pools can be differentially extracted and characterized according to their relative ease of extraction. A primitive and widely used scheme classified the total P pool as water soluble P or loosely bound P, aluminum bound P (AL-P), iron bound P (Fe-P) and Ca bound P (Ca-P) and occluded Fe-P and Al-P (Chang and Jackson, 1957). Chang and Jackson scheme (1957) has undergone several modifications over time (Smillie and Seyers ,1972; William et al., 1967). The most widely used P fractionation procedure for soils was developed by Hedley in 1982. Modified version of Chang and Jackson procedure were used for P fractionation in manures and biowastes thereafter (Tiecher et al., 2014). The study conducted by Barnett (1994) evaluated the P status of animal waste and corresponding effects on animal rearing was a steppingstone. He analyzed the total P present in the beef, swine and poultry manures as total P (Pt), inorganic P(P_i), acid soluble organic (P_{aso}) and lipid soluble organic P (P_l) and residual P (P_r) by chemical extraction. Results indicated that inorganic P (P_i) and residual P (P_r) fraction in the non-ruminant animals like poultry were more variable than that of the ruminant animals. One of the most popularly used scheme was developed by Dou et al (2000) as adaptation of the Hedley procedure to characterize phosphorus in animal manures by sequential P fractionation. This scheme used a modified Hedley fractionation procedure to preferentially extract various fractions of P. According to the hypothesis, the first extractant **de-ionized water (DH$_2$O)** extracts water soluble and loosely bound P, followed by **0.5 M NaHCO$_3$** which extracts the labile P (P fraction also available for crop uptake) in the second step , **0.1 M NaOH** extracts amorphous and crystalline Al-P and Fe-P along with some amount of chemically and physically protected P forms in the third step, and lastly **1 M HCl** extracts the relatively stable Ca-P. The residual P left after the completion of the fractionation represents the recalcitrant organic P in manures.

While several studies have been carried in the past to understand the chemical composition of manures, most of these studies compared different types of manures (broiler, swine, and dairy) and their differences in P fractions by employing sequential P fractionation procedure (Moyer and Sharpley 1998; Li et al.,2014). Research on how the P fractions vary with PL cleanout frequency is lacking and unexplored. The information about the P fractions of various types of PL will be useful towards gaining a better understanding of P availability both from an agronomic and environmental viewpoint. Hence the objectives of this study

were i) to quantify the P fractions in PL of 4 CF (0-6 months, 6-12 months, 12-24 months, and 5 year old) ii) quantify the difference between the P forms (dissolved total P (DTP), inorganic P (Pi : molybdate reactive P (MRP)), dissolved organic P (P_o) and Residual P (Res P)) and their concentrations among the four PL CF.

Materials and Methods

Sixteen broiler litter samples (mixture of bird excreta and bedding material) were collected from commercial poultry farms across 8 counties in Alabama (Figure 2.1). Poultry litter samples collected varied by flock clearance frequencies. Detailed information regarding bedding material and clean out schedule of samples collected in this study are presented in Table 2.1.

Poultry litter was collected in buckets by using sharp shovel/auger. Three to five samples were collected at each sampling point and composited, mixed in a bucket to obtain representative samples from each location. The samples were collected in plastic bags and stored at 4° C until processed.

Characterization of poultry litter

The PL samples were processed by drying in bulk at 70°C in a forced air oven for 24 hours (Peters et al., 2003) followed by grinding and sieving through a 2 mm sieve. General chemical characterization included determination of pH, total N, P, K, Fe, Al, S, Ca, Mg, Mn, Zn, Cu, and moisture content. Concentration of total elements (P, K, Fe, Al, S, Ca, Mg, Mn, Zn, Cu) was determined using Andersen's method (Andersen 1976). Briefly, 0.5 g of ground and sieved PL samples were taken in 50ml Pyrex glass beakers and burned to ash in a muffle furnace at 550 °C for 4 hours (Peters et al., 2003). The ash was digested with 6 N HCl in a hotplate at 110 °C (Andersen 1976). After cooling, the digested sample was filtered through a Whatman No. 42 filter paper and the final volume was made to a 100 ml. Elemental concentration (Fe, Al, K, S, Ca, Mg, Mn, Zn,) in the digested solution (10% 6 N HCl) was determined using the inductively coupled plasma-atomic emission spectrometry (ICP-AES; Spectro Ciros, Spectro Analytical Instruments Inc. Mahwah, NH). Total N was determined by dry combustion LECO C/N analyzer (LECO Corp., St. Joseph, MI). Determination of pH was done by taking 5-gram PL and 50 ml water in 1:10 ratio (Li et al, 2014).

Sequential Phosphorus Fractionation

The modified procedure of Hedley et al. (1982) as outlined by Duo et al. (2000) was followed to sequentially extract P. Briefly, 0.3 g of dried and sieved PL sample was sequentially extracted with 30ml of de-ionized water, 0.5 M $NaHCO_3$, 0.1 M NaOH and 1.0 M HCl (Dou et al., 2000). Shaking time of 1 hour and PL to solution ratio of 1:100 was maintained in all steps of fractionation. One hour shaking time was followed by centrifugation at 4000 rpm and filtration through Whatman number 42 Filter paper. One-hour timing was selected based on Duo et al. (2000) procedure who found no difference in 16 hr and 1 hr extraction time for dairy and poultry manure samples. While P characterization is typically performed with filtration by 0.45µ membrane filter, the comparison of Whatman No 42 and 0.45µ membrane filter was done following centrifugation at 4000 rpm. No significant difference in P concentrations between the two filtrates was found (Table S.1.). An aliquot from each of the filtrate was diluted and analyzed for dissolved total P (DTP) using the ICP- AES, which indicated the total soluble P. Diluted samples were also used to determine the dissolved molybdate reactive P (MRP) which indicates the inorganic P (P_i) (referred hereafter as P_i). The chemical determination of Pi was performed using the ammonium-molybdate ascorbic acid method (Murphy and Riley, 1962) via a microplate reader (Gen 5 µQuant; BioTek, BioTek Instruments Inc. Winooski, VT). The difference between

DTP and MRP (Pi) represents dissolved organic P (P_O). Finally, 0.1 g of air-dried residues were transferred to a 50 ml Pyrex glass beaker and burned to ash in a muffle furnace at 550 ºC for 4 hours. Residual ash was digested with 6 N HCl in a hotplate at 110 °C (Andersen 1976). After cooling, the digested sample was filtered through a Whatman No. 42 filter paper, the final volume was made to a 25 ml final volume. The residual P concentration was measured using the ICP-AES, which estimated the concentration of recalcitrant P (RP).

Dilution and pH adjustment

The ascorbic acid method is a pH sensitive chemical reaction, which is mediated by several critical factors, one of which is Riley ratio (Pai and Yang, 1989). Riley ratio is defined as the ratio of [H+] ions to that of the [Mo] ions for stable and rapid reaction for the blue colored complex formation. Hence, the samples were diluted or acidified adequately as needed to obtain the desired pH range of 2-3. For analysis of molybdate reactive P (Pi), the samples with alkaline pH ($NaHCO_3$ and NaOH samples) were diluted with acidified water 0.00225 M HCl and 0.12 M HCl, respectively, for ease of the ascorbic acid reaction of the blue colored molybdate complex formation. Dilution of the HCl extracted samples with a factor of 10 were also done to slightly neutralize the acidity.

Phosphorus analysis and calculation

Phosphorus analysis values read in instruments were measured in mg L^{-1} or ppm and were converted to mg kg^{-1} using a multiplication factor. The total P (TP) was estimated by adding the dissolved total P (DTP) and the residual P.

Total P (TP) = Dissolved Total Phosphorus (DTP) + Residual P (Eq.1)

The final values were expressed as percentage of the total P. Other parameters determined were:

i. Dissolved Organic P (Po) = Dissolved Total P (DTP) – Molybdate reactive P (MRP or Pi)

ii. Dissolved Total P (DTP) % = (DTP /TP) X 100, where DTP and TP were expressed in mg kg^{-1}

iii. Inorganic P (MRP(P_i)) % = (Pi/TP) X 100, where Pi and TP were expressed in mg kg^{-1}

iv. Dissolved Organic P (P_o)% = (P_o/TP) X 100, where Po and TP were expressed in mg kg^{-1}

v. Residual P (Res P) % = (Res P /TP) X 100, where Res P and TP were expressed in mg kg^{-1}

Statistical Analysis

Analysis of Variance (ANOVA) was performed on a mixed model using PROC GLMMIX in SAS 9.4 (SAS institute, 2013) to test if there was a significant difference in the P fractions (DTP, Pi and Po) extracted sequentially between the four CF of PL. The location of the sample was treated as as a random factor. The interaction of CF with location (CF*location) indicated that observation with a combination of CF group and location were the unique identifiers of the experimental units from which samples were collected. Post hoc test TUKEY was used for means separation at α = 0.05. DDFM = SATTERTH option was used in the model statement along with ADJDFE = ROW to allow for unequal variances due to unequal sample sizes. Those data sets where the assumptions of ANOVA were not satisfied, arc sin transformation was applied to transform data into a normal distribution data.

Results and Discussions

Poultry litter general characteristics

The nutrient concentrations varied among the litter CF (Table 2.2). The average pH of PL was near neutral except for litter that had a CF of five years (pH 8.04). The moisture content in litter of 0-6-month CF was lowest (6%) among all CF and increased with decreasing CF (29 % for 12-24 months and 25 % for 5 year). The mean Fe, Al, Ca, and Mg concentration was greatest in litter of 0-6 month CF and least in 5 years, however due to wide variability in composition and the fact that litter is highly heterogeneous, these nutrient concentrations were not significantly different among CF groups ($P>0.05$). The mean N, P, and K concentrations showed no clear pattern of increase or decrease and were not significantly different among the four CF groups studied. Mean zinc (Zn) concentration increased with decrease in cleanout frequency however the change in concentrations among CF were not significant ($P>0.05$). Large variability in the nutrient concentrations was observed in PL of all CF.

Phosphorus fractions in poultry litter

The relative percentages of P fractions showed unique differences among CF (Figure 2.3). The DH_2O-P and HCl-P fractions were greatest among all P fractions, although they did not differ significantly between CF. The fractions of Res-P differed significantly between CF. Percent Res-P decreased whereas $NaHCO_3$-P and NaOH-P remained unchanged with change in CF. This indicated that with increase in residence time of PL in houses (5 years) recalcitrant P might have undergone mineralization causing subsequent decrease in Res-P.

The total P recovered from the sequential P fractionation was (93.31±3.81 %). Turner and Leytem (2004) also reported the P recovery from the sequential P fractionation process to be 94% in case of PL. The relative percentage of P extracted in all the PL samples studied showed HCl extracted P had the greatest percentage (42±12.94%) amongst the 4 sequential extractants. The greatest P fraction being associated with the HCl fraction was reported in the previous literatures as well (Dou et al., 2003; He et al., 2006b, 2008). As HCl extracted P represents the Ca bound P, greater percentage of HCl-P is a possible indication of the predominance of relatively stable Ca-P. There was no significant difference in HCl extracted P fraction among the four PL cleanout frequency groups indicating that the Ca and Mg associated P fraction is least affected by the cleanout frequency of PL. The DTP fraction extracted by water was not significantly different in any of the

CF (Fig 2.3). Pagliary and Laboski (2012) reported the range of DTP extracted as water soluble P was in the range from 8-19 % in chicken manure. The $NaHCO_3$ extractable P was not significantly different in any of CF. $NaHCO_3$ associated P fraction is also known as the labile P available for crop uptake. This indicated that as the PL stays in poultry house for a longer period there is no significant effect on the labile P fraction. Similarly, the NaOH-P fraction did not show any significant difference with CF difference. NaOH-P is assumed to extract the Fe and Al bound phosphorus along with chemically protected P. NaOH extracted P can also be termed as potentially bioavailable P (Su et al., 2007).

Order of DTP concentration in various sequential extracts was HCl (42±6) %> H_2O (30±7) %> $NaHCO_3$ (15±4) %> NaOH (6±2). Trends reported in this study are supported by previous literature. For example, He et al. (2010) reported the order of distribution of P fractions in frozen PL samples as HCL> H_2O> NaOH> $NaHCO_3$ (He et al., 2010). Similarly, Codling (2006) reported the average Pi fraction extracted by water was 55% whereas HCl extracted 34% Pi. The percentage of P_i extracted via sequential extraction was reported to be least in NaOH (3%) followed by HCl (6%), $NaHCO_3$ (12%) and most through water (29%) (Li et al., 2014). Excreta from poultry litter had the greatest fraction of DTP recovery (60±3) associated with the HCl in a study which compared 8 types of animal manure (Pagliary and Laboski, 2012). Lower amount of the NaOH associated P suggested relatively lesser abundance of Al and Fe- associated P. Pagliary and Laboski (2012) also reported least values for the NaOH associated P (5±1). Although the trends were similar, a range of percentages has been reported in literature. The P fractionation in soils differ from that of the manure in two major aspects: first, in manures relatively higher fraction of P is present in Ca-P and less in recalcitrant form whereas in case of soils the fraction of recalcitrant P is relatively greater. Second, in soils the NaOH associated P is larger as soils are dominated by

Al and Fe oxides, NaOH-P is very less in manures. Previous literature has also reported these stark differences in the P fractionations between soils and manures (Sharpley, 1996; Tiessen et al., 1984; Duo et al.,2000; He et al., 2003).

Concentrations of Phosphorus forms among four Clean Out Frequencies of poultry litter

Concentrations of DTP, Pi, Po and Res-P are presented in table 2.4. Percent DTP in 5 -year and 12-24 months CF were significantly greater ($p<0.001$) than the 6-month and 6-12-month CF indicating DTP increased with reduced CF. Comparison of percent Pi showed no significant difference ($P=0.412$) between the CF, which indicated the total reactive Pi (MRP) present in PL was not affected by the CF of PL. Similarly, the percent Po did not show significant deviation in with the change in CF indicating the percent Po was also not affected by variation in CF. Lastly, the percent Res-P was significantly greater in the 6-month-old litter ($p< 0.001$) than the other CF group and decreased with reduced CF. Less frequent clean out and longer residence time of PL in poultry house cause mineralization of P. This could also be driven due to microbial turnover period which causes the Po to decrease over time. A limitation of this study is the small sample size of five year CF litter. Also, samples collected from different locations may had variations since this experiment was not a controlled experiment but an observation study. Variation of other factors includes the possible differences in the bedding materials, poultry house management aspect, number and weight of birds etc. Additional information on litter collected from few locations are separately enlisted (Table. S2).Future studies should focus on controlled experiments with explicit information on bedding material, feed, management and harvesting the bird at same size.

Conclusions:

As the poultry industry litter management practice shifts towards a less frequent litter cleanout schedule and greater residence time in poultry house, it becomes imperative to evaluate how the P fractions vary with PL cleanout frequency. This information will be useful towards gaining a better understanding of P availability both from an agronomic and environmental viewpoint. The sequential P fractionation scheme employed on 4 PL CF indicated Res-P fraction differed significantly between CF. The DH_2O-P and HCl-P were found to be dominant fractions among all P fractions and their concentrations did not change significantly between CF. Concentrations of Res-P decreased with increase in residence time. This implied that longer the litter stayed in the poultry house; the recalcitrant organic fraction possibly underwent mineralization. Dissolved total P concentrations in PL increased with longer residence time suggesting microbial turnover and decomposition of recalcitrant P fractions. This study clearly demonstrates the implication of changing clean out frequency on P fractions in PL and their possible agronomic and environmental consequences.

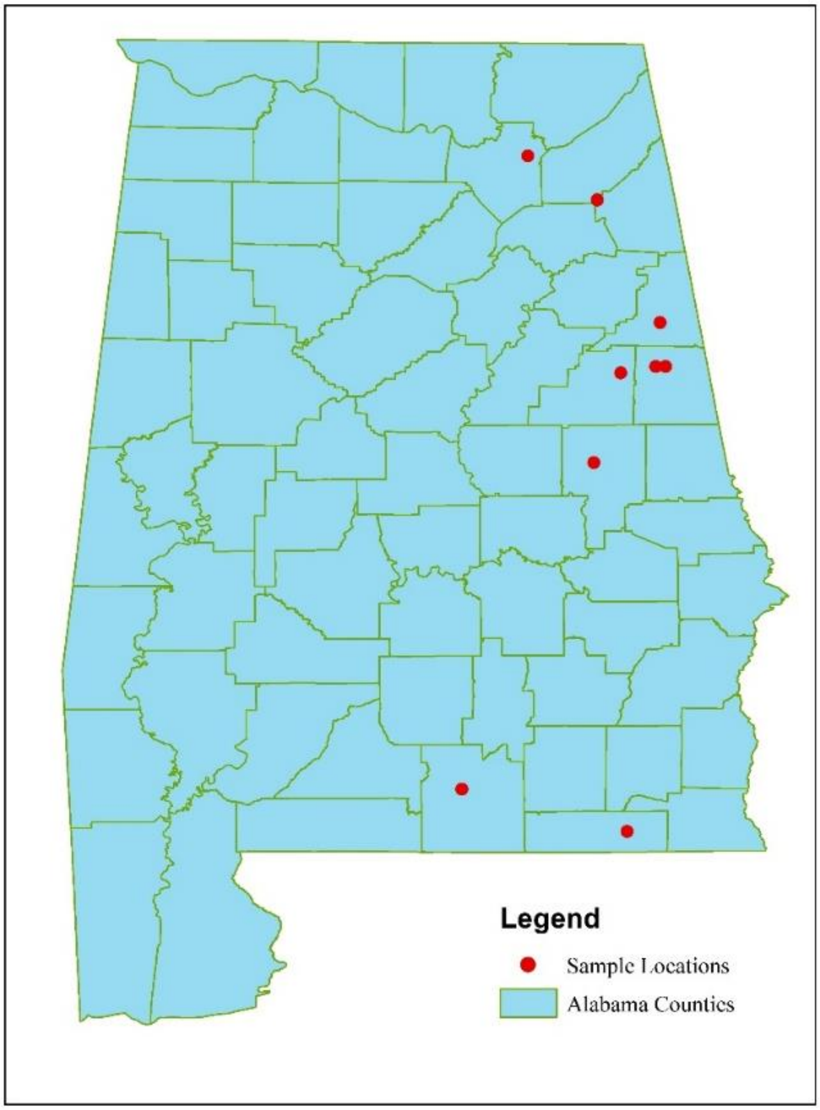

Figure 2. 1. Map showing location of sample collection in Alabama.

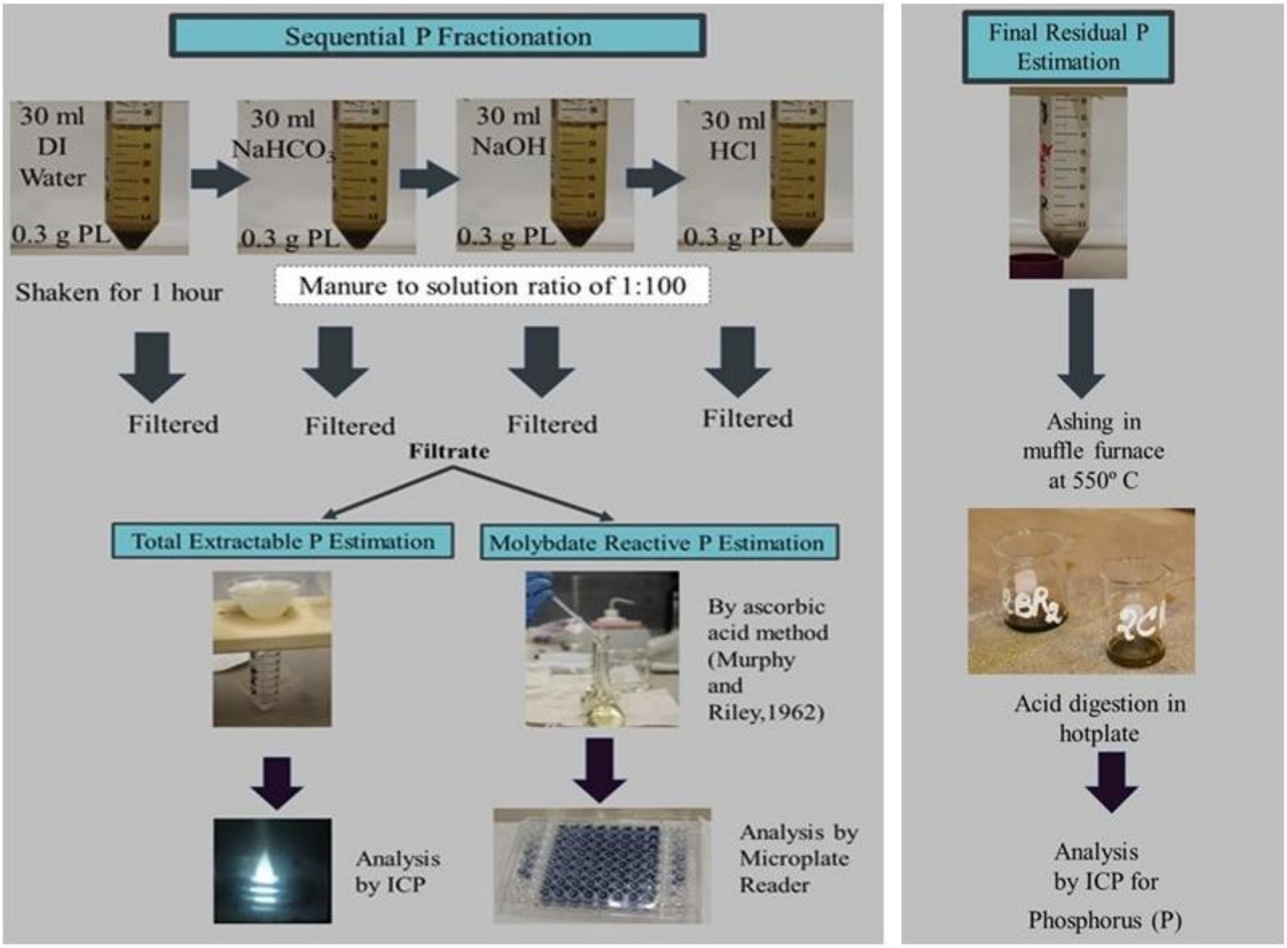

Figure 2. 2. Flowchart of sequential P fractionation

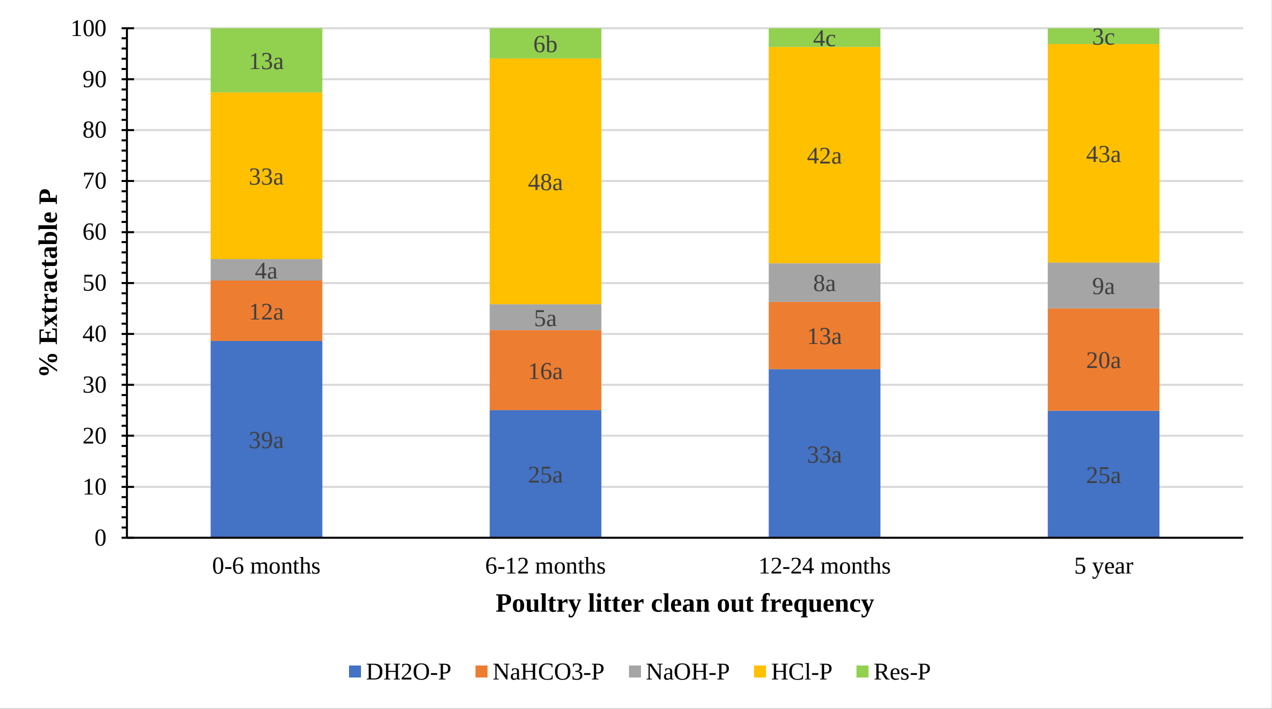

Figure 2. 3. Percentages of four sequentially extracted P forms and residual P in poultry litter of clean out frequencies (CF) at 6 months, 6-12 months, 12-24 months, and five year. The four sequentially extracted P fractions include P forms extracted with deionized water (DH_2O–P), sodium bicarbonate ($NaHCO_3$–P), sodium hydroxide (NaOH–P), and hydrochloric acid (HCl–P). The residual P (Res-P) is the P left after sequential extraction. The different letters indicate significant differences ($P < 0.05$) among four CF for a given P fraction.

Farm Number	Location	Clean Out Frequency	General Practice
1	Collinsvile, AL	(0-6 months)	3 flocks old
2	Collinsvile, AL		4 flocks old
3	Collinsvile, AL		4 flocks old
1	Widowee, AL	(6-12 months)	Cleaned annually
2	Gunterville, AL		Cleaned annually
3	Weedowee, AL		Cleaned annually
4	Jackson, AL		5 flocks old
5	Jackson, AL		Decaked annually
6	Talapoosa, AL		5 flocks old
7	Lineville, AL		Cleaned annually
1	Talapoosa, AL	(12-24 months)	10 flocks old
2	Covington, AL		12 flocks old
3	Geneva, AL		14 months old
4	Geneva, AL		12 months old
1	Covington, AL	(5-year)	Cleaned in 5 year
2	Covington, AL		Cleaned in 5 year

Table 2. 1. General information on poultry houses location and poultry litter clean out frequency

Poultry litter clean out frequency	Statistics	Moisture (%)	pH	N	P	K	Fe	Al	Ca	Mg	Mn	Zn	S
				mg kg^{-1}									
0-6 month	Mean	6.01b	7.71a	33000a	13498.5a	36321.4a	3577.5a	5321.7a	41670.7a	12002.2a	426.5a	292.1a	8679.9a
0-6 month	SD	0.7	0.1	6464.5	1995.3	6595.6	4650.8	3467.0	7034.6	2035.2	120.2	74.6	3277.8
0-6 month	SE	0.4	0.1	3732.3	997.6	3297.8	2685.2	1733.5	3517.3	1017.6	60.1	37.3	1638.9
	(N=3)												
6-12 month	Mean	11.2b	7.7a	36264.29a	17238.5a	37673.1a	2854.3a	5523.8a	46818.6a	12512.5a	663.6a	433.7a	10860.5a
6-12 month	SD	6.4	0.4	4651.0	6461.8	18635.1	2162.0	4221.5	29836.7	4211.4	469.7	262.7	5530.1
6-12 month	SE	2.4	0.2	1757.9	2442.3	7043.4	817.2	1595.6	11277.2	1591.7	177.5	99.3	2090.2
	(N=7)												
12-24 month	Mean	28.68a	7.33a	34275a	30037.2a	30037.23	1521a	3281a	30289.7a	8768a	621.2a	506.6a	10973a
12-24 month	SD	6.1	0.9	5249.2	8907.5	7409.2	1109.7	1581.8	13332.3	2728.1	293.4	253.4	3244.3
12-24 month	SE	3.0	0.4	2624.6	4453.7	3704.6	554.9	790.9	6666.2	1364.1	146.7	126.7	1622.1
	(N=4)												
5 years	Mean	25.3a	8.04a	32800a	14044.7a	36258.4a	1878.1a	3149.8a	36611.3a	9290.2a	694.1a	666.1a	10256.4a
5 years	SD	0.0	0.1	1131.4	532.8	576.7	47.5	145.8	1689.1	267.8	22.2	112.6	133.6
5 years	SE	0.0	0.0	800.0	376.8	407.8	33.6	103.1	1194.3	189.3	15.7	79.6	94.4
	(N=2)												

Table 2. 2. General characterization poultry litter used in this study. SD represents standard deviation; SE is standard error of mean; N represent total number of samples. Values followed by the same letter are not significantly different within a given column according to Tukey's HSD at α= 0.05

PL clean out history	DH_2O-P	0.5 M $NaHCO_3$- P	0.1 M NaOH -P	1.0 M HCl- P	Res-P	TP
	--mg kg^{-1}	---				
0-6 months	3448(±443)	1136(±348)	386(±115)	3351(±2026)	1201(±359)	9524(±2994)
6-12 months	3603(±747)	2181(±888)	791(±490)	8111(±4641)	904(±306)	15563(±4984)
12-24 months	4102(±912)	1996(±1283)	1018(±361)	6748(±5241)	534(±309)	14622(±7192)
5 year	2952(±111)	2404(±1051)	1066(±41)	5072(±286)	365(±17)	11861(±595)

Table 2. 3. Distribution of P fractions in poultry litter of various clean out frequencies (0-6 months (n= 3), 6-12 months (n = 7), 12-24 months (n = 4) and Five year (n=2). Values in the bracket is standard deviation of mean. D_2O-P: water extractable P; $NaHCO_3$-P: labile P; NaOH-P : Fe/Al bound P; HCl-P: Ca/Mg associated P; Res-P recalcitrant P; TP: total P (sum of DH_2O-P, $NaHCO_3$- P, NaOH –P, HCl- P, Res-P).

Table 2. 4. Phosphorus forms and their percentages (expressed as percentage of total P) extracted in sequential P fractionation.

PL clean out history	DTP	Pi	OP (Po)	Res P	TP	DTP/TP	Pi/TP	OP/TP	Res P/TP
		-----------------------mg kg^{-1}	----------------------			---------------------------%	----------------------		
0-6 months	8323	5066	3257	1202	9525	87c	52.a	35a	12a
6-12 months	14771	9043	5728	904	15675	94b	55a	39a	5b
12-24 months	13867	8296	5571	534	14401	96a	49a	46a	3c
5 year	11496	4758	6738	365	11861	96.a	40a	56a	3c

DTP: Dissolved total phosphorus; Pi: dissolved inorganic phosphorus (molybdate reactive P); OP dissolved organic phosphorus; Res P: residual phosphorus; TP: total phosphorus (sum of Pi, Po and Res P). Values followed by the same letter are not significantly different within a given column at α = 0.05

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II. Understanding Phosphorus and Nutrient Release Characteristics of Poultry Litter using Neutral, Acidic, and Basic Extractants

Abstract

Poultry Litter (PL) is used as soil amendment and contains plant macro and micronutrients. However, long-term usage of PL can increase background phosphorus (P) concentrations in soil. Extraction of P from PL can overcome the limitation of repetitive PL application. Studying the P release characteristics of PL along with co release of other nutrients will help better the nutrient management approach in crop and forage production. The objectives of this experiment was 1) to quantify the P extraction efficiency of selected acidic, basic, and neutral extractants and quantify the acid hydrolysable and acid non-hydrolysable P fractions. 2) study the co-release pattern of macronutrients (Al, Fe, Mg, Ca, K, S) along with P. Sixteen PL samples were collected from 8 counties across Alabama. Poultry litter was extracted using 4 selected extractant (1 M HCl, 1 M citric acid, De-ionized water (DH2O), and 0.5 M NaHCO3). Poultry litter to extractant ratio of 1:100 and 1 hour shaking time was used repeatedly till the molybdate reactive P fall below the method detection limit. The extracted solution was analyzed for total dissolved P, molybdate reactive P (MRP) and total dissolved nutrients (Al, Fe, Mg, Ca, K, S) after each wash cycle. Finally, the residues were ashed and analyzed for residual P and Al, Fe, Mg, Ca, K, and S concentration. Results showed that P extraction efficiency can be ranked as follows: 1 M HCl (95.37± 1.98) % > 1 M citric acid (93.0 ± 4.5) % > NaHCO3 (56.4 ± 12.73) % > DI Water (43.8 ± 10.42) %. The acid non-hydrolysable P fraction was 4.53 ± 2.04 %. Concentration. of heavy metals like As, Cd, Cr was found to be below detection limit in all water and acid extractions. Greater concentrations (10 times) of Al was released by HCl compared to citric acid and water. The cumulative mean concentration of Al and Fe in water and acid extracted solution were lesser in magnitude than that of Ca and Mg in PL. Greater abundance of Ca and Mg indicates the possible

predominance of Ca and Mg associated P. The cumulative potassium (K) and sulphur (S) released during extraction cycle was least affected by the extractant types.

Introduction

Globally, poultry (*Gallus domesticus*) production has witnessed steep growth due to rising demand of poultry products. Since the 2000's the share of broiler meat in world meat production has increased from 15 % to 30% and the growth rate in the poultry sector has increased by more than 5% per annum (FAO, 2006a) This growth rate is greater than the contemporary beef (*Bos taurus*) and pork (*Sus scrofa domesticus*) meats of 1.5% and 3% respectively. In fact, the United Nations projected the greatest growth rate for the poultry industry towards 2050 (UN working paper no. 12-03, p131). Not only the global poultry industry, but also in the United States, consumption of poultry products has seen promising growth. Alabama ranks second in broiler production in the United States behind Georgia. The poultry production generates an enormous amount of poultry waste also known as poultry litter (PL) which is a mixture of bird feces, bedding material, spilled feeds, and feather. Alabama poultry industry alone generates 1.5 million tons of poultry litter annually (ALFA, 2016). Management strategies for poultry waste are becoming imperative towards a sustainable food production system.

One of the biggest challenges in handling PL comes from the fact that PL is bulky and hence long-distance transportation is uneconomical. This limitation in transportation causes repeated land application of PL in pasture and agricultural lands – a practice that increases the background soil phosphorus levels due to positive P balance (Sharpley et al., 1994; Sharpley et al., 2007). In areas of intensive agricultural and localized animal feeding operations, soil samples had exceeding high levels of STP (soil test phosphorus) than required for optimum agricultural yields (Alley, 1991; Sims, 1992). As the P accumulation in soil increases, the potential for P enrichment in agricultural runoff water also tends to increase.

Phosphorus runoff from non-point sources is one of the key water pollutants that leads to accelerated eutrophication in surface water bodies and causes impairment of the water quality (Schindler, 1977). According to U.S. Environmental Protection Agency (USEPA), nutrient runoff from the agricultural fields and other non-point sources contributed to the water impairment of 55% surveyed river length, and the lake area suffered from the water quality issue (USEPA, 1990). The pervasive problem of eutrophication has many more consequences of further environmental degradation by triggering anoxia, fish kills and dead zones.

Poultry litter is rich in P, in fact, the 1.5 million tons of PL generated from the Alabama poultry industry contains19,665 tons of phosphorus. Runoff water from poultry litter applied fields can be the cause of potential water quality degradation of surface waters. Development of methods to extract phosphorus from litter could be an important step towards reducing the P transport into the environment and extracted P can be safely reused for agricultural purposes.

Phosphorus is present in organic and inorganic forms in animal manures including PL. Sequential fractionation studies have been conducted in animal manure to quantify various Inorganic and organic P forms (Ajiboye at al., 2004; Codling 2006; Haygarth and Sharpley, 2000; Dou et al., 2000). Another attempt of characterizing the organic and inorganic forms of manures includes coupling of the P fractionation procedure with enzymatic hydrolysis to distinguish between labile organic P and recalcitrant organic P (He and Honeycutt 2001; He et al.,2004a; He et al., 2004b) . He et al (2006) performed sequential P fractionation along with enzymatic hydrolysis to identify inorganic and organic P in poultry manures. As poultry birds lacks the enzyme phytase in the intestine, PL is rich in complex organic molecule such as inositol hexaphosphate (Church, 1979; Cunha, 1977; Simons and Jongbloed 1981). With the aid of solution ^{31}P NMR studies Toor et al.

(2005a) reported occurrence of other organic P forms in PL such as pyrophosphate and the phospholipids monoester, diesters, and DNA-RNA-like P (McDowell and Stewart, 2005).

With use of more advanced techniques like XANES (X-ray absorption near edge structure) the minerals associated with manures (Shober et al., 2006) were found to be calcium minerals like dicalcium phosphate, octacalcium phosphate and hydroxyapatite. In some cases, magnesium (Mg) phosphate like struvite and aluminum (Al) phosphate like variscite can be found in manures (Peak et al., 2002; Toor et al., 2005b)

Different chemical, physical and biological agent mediated methods were documented to extract phosphorus from animal manures and biowastes like struvite precipitation (Zhang and Lau, 2007), anerobic digestion (Farrow et al., 2016) and hydrothermal carbonization (Heilman et al., 2014). While generally these methods either have an expensive instrumentational setup or the recovery percentage is not satisfactory for large scale transformation. A two-stage system was developed to separate the swine slurry which employed the use of nitrification bioreactor followed by P precipitation induced by slaked lime addition (Vanotti et al., 2005, 2010). The recovery percentage in this case was 95% P in swine slurry. Davis (2011) developed a two-step system to recover nitrogen (N) and P from dairy parlor wastewater. The two-step process utilized addition of slaked lime to recover calcium bound P in the first step followed by biological conversion of ammonia to nitrite in the second step was. Church et al. (2016) developed a system call MAPEX for manure P extraction that was tested in dairy manure with a future prospect of adapting in a field scale. It consisted of several steps of auger press, fiber filters and centrifuge to liquid and solid particles in the dairy manure.

Chemical extraction of P from PL needs the first step of exploring chemical reagents that can effectively extract P from PL to obtain a target recovery yield of upto 90%.

The overall goal of this project was to identify and utilize the chemical reagents that can effectively extract P from PL with a vison to recycle P from poultry waste. As different organic and inorganic forms of P in PL can have varying degree of dissolution capacity, the estimation of P extracted in solution phase can potentially help us to understand the feasibility of developing a method to remove P from PL. The specific objectives of the study were i) to quantify the P dissolution efficacy of selected acidic (HCl, citric acid), neutral (water), and basic extractants ($NaHCO_3$). and ii) quantify the co-release of metals during the extraction cycle.

Materials and Methods

Collection of broiler litter

Sixteen PL samples were collected from broiler farms located in 8 counties in Alabama (Figure 3.1). The collected samples varied in age, composition, and flock clearance frequencies. General characteristic of the litter is presented in chapter 1 (Table 2.2)

Method of collection and storage

Three to five PL samples were collected from the six-inch-deep bedding floor, mixed in a bucket to obtain a representative sample from each location. Collected samples were stored at 4° C until processed. The PL samples were dried in bulk at 70°C in a forced air oven for 24 hours (Peters et al., 2003) grinded to homogenize and sieved through a 2 mm sieve.

Repeated independent extractions

The neutral, basic, and acidic, extractants selected were de-ionized water (DH_2O), 0.5 M sodium bicarbonate ($NaHCO_3$), 1 M HCl (Dou et al., 2000) and 1 M citric acid (Szogi,et al., 2008). 0.3 g of PL samples were shaken with 30ml (PL: solution ratio = 1:100) of each extractant for 1 hour followed by centrifugation at 4000 rpm for 20 minutes and filtration through a Whatman No. 42 filter paper. While P characterization is typically performed with filtration by 0.45µ membrane filter paper, the comparison of Whatman No 42 and 0.45µ membrane filter was performed following centrifugation at 4000 rpm. No significant difference in P concentrations between the two filtrates was found (Table S.1.). The extracted filtrate was analyzed for DTP (dissolved total P) in ICP- AES, and molybdate reactive P(MRP) using the ascorbic acid method in a microplate reader (Murphy and Riley; 1962). The extraction cycles were repeated and continued until the concentration of P was below the method detection limit. Number of extraction cycle varied between different extractants. The residue left at the end of extraction cycle was used for determination of residual P (RP) by transferring 0.1g litter residues to a beaker, air dried followed by ashing in a muffle furnace at 550° C and acid digestion with 6 N HCl in a hotplate (Andersen,1957) . Elemental P concentration in the solution was analyzed using ICP-AES.

pH adjustment for Molybdate reactive P analysis

Riley ratio refers to the ratio of acidic ions in the solution to the molybdate ions in the solution. Since the molybdate reactive P reaction is a pH sensitive reaction, pH adjustment step was incorporated for the acidic and basic extractants (Pai and Yang, 1989). The dilution factor also varied with the sequence of extraction cycles as dilution factor decreased with falling P concentration in solution phase. Prior to the analysis with ICP-AES and microplate reader, the $NaHCO_3$ extracted samples were diluted with acidified water (0.00225 M HCl) to adjust the pH of the samples within the range of 2.5-3. Whereas the acid samples of HCl and citric acid were diluted with regular de-ionized water, to ameliorate the high acidity. Also, 1:1 diluted coloring reagent (2.5 M H_2SO_4) was used for the colorimetry in the molybdate reactive P analysis. Citrate/citric acid as a media had negative interactions with the molybdate complex formation in colorimetric method (Nagul et al., 2015; Zou et al.,2005). Hence, it took more time to develop blue colored phosphomolybdate complex. The dilution factors were determined according to the range and order of magnitude of P concentration. Dilution factor of 20 and 10 was used for the first and second extraction cycle with all four extractants, followed by 10 in the 3rd and 4th extraction cycle for the acids. For water, a dilution factor of six was used for the 3rd extraction cycle followed by no dilution from 4th to 7th extraction cycles.

Phosphorus analysis and subsequent calculation

Phosphorus concentrations in the extracts were in mg/l or ppm, which was converted to mg/kg using the multiplication factor of 100 (as the soil to solution ratio was 1:100). The difference between the Dissolved Total P (DTP) and Dissolved Molybdate Reactive P (MRP) represented the soluble organic P in each extraction cycle. The Total P (TP) in each sample was estimated by adding the DTP obtained in individual extraction cycle using individual extractant and the residual P (Res P) (Dou et al, 2000).

1. Dissolved organic P (DOP) = dissolved total P(DTP) - dissolved molybdate reactive P (MRP)
2. $\text{Total P (TP)}_{\text{HCl}} = \Sigma\{(\text{DTP})_{\text{each extraction cycle}}\}_{\text{HCl}} + \text{Res P}_{\text{HCl}}$
3. $\text{Total P (TP)}_{\text{Citric}} = \Sigma\{(\text{DTP})_{\text{each extraction cycle}}\}_{\text{Citric}} + \text{Res P}_{\text{Citric}}$
4. $\text{Total P (TP)}_{\text{NaHCO3}} = \Sigma\{(\text{DTP})_{\text{each extraction cycle}}\}_{\text{NaHCO3}} + \text{Res P}_{\text{NaHCO3}}$
5. $\text{Total P (TP)}_{\text{Water}} = \Sigma\{(\text{DTP})_{\text{each extraction cycle}}\}_{\text{Water}} + \text{Residual P}_{\text{water}}$

All values were expressed as percentage of the total actual P.

1. Total P recovery (TPR) % = (DTP/TP) X 100
2. Dissolved molybdate reactive P (MRP) % = (MRP/TP) X 100
3. Dissolved organic P (P_o)% = (P_o/TP) X 100
4. Residual P (Res P) % = (Res P /TP) X 100

Nutrient analysis and subsequent calculation:

The concentration of nutrients co-released at each extraction step (Al, Fe, Ca, Mg, Zn, S and K) was measured in ICP-AES. The concentrations in mg/kg were further converted into moles. The molar ratio of P to metals associated with P (such as Al, Fe, Ca, Mg) were also calculated.

Statistical Analysis:

Analysis of Variance (ANOVA) was performed using PROC GLM for the P concentrations for 4 parameters **Σ**DTP, **Σ**MRP (Pi) and **Σ**DOP P (P_o) and Residual P between the four individual extractants. Separation of means were done using TUKEY-KRAMER post hoc test at $\alpha = 0.05$. Tukey Kramer analysis was used to account for the experiment wise error II as 16 heterogenous PL samples were used in the experiment.

Repeated extraction cycles of all extractants were analyzed using repeated measures. PROC GLM was used to distinguish between the P and other nutrients (Al, Fe, Ca, Mg, S, K, Zn and Fe) concentration of all the extraction cycles. Separation of means was done using TUKEY. WELCH test was used as robust for unequal variances in the parameters where assumptions of ANOVA was not satisfied. Where necessary, Arc sin transformation was used for non-normal data set.

Results and Discussions

a) Total P recovered using four extractants

The P release and recovery in this study was done using four extractants. The mean values for TP_{HCl} was 16210±6038 mg kg^{-1}, TP_{Citric} was 14354 ±8583 mg kg^{-1}, TP_{NaB} was 13046± 6038 mg kg^{-1}, which was significantly different from TP_{WEP} (20957± 7948 mg kg^{-1}) (Table 3.1). The total P estimated via the additive method of all the 7 extraction cycles for water the was significantly greater than the other three extractants. The dilution factor used in the seven set of samples might have caused overestimation of the DTP values. The total P reported in previous studies were in range with our study (Sharpley and Moyer 2000). Barnett (1994b) reported mean concentration of P in poultry manure to be 8.63 kg ton^{-1} with a coefficient of variance of 41%.

b) P release characteristics of PL in acidic and basic extractants

The P release characteristics of all the four extractants followed an exponential decay trend where the concentrations of P decreased gradually with each successive extraction cycle (Figure 3.3). The P release pattern was similar as reported by Dou et al (2000). It took four extraction cycle for HCl and citric acid before the concentration of MRP fell below the method detection limit. Duo et al. (2000) reported three extraction cycles for 1 M HCl and 5% TCA, for hen litter. The DTP released in the 4 extraction cycles of the HCl and citric was 15801 (± 7305) and 13,653 (± 8493) mg/kg respectively whereas Dou et al (2000) reported 20300 mg/kg of P released. Szogi (2008) found organic citric acid was more efficient in solubilizing P than mineral acid HCl from PL and attributed it to fast conversion of insoluble P to soluble P by rapid hydrolysis reactions. Citric acid extracted P fraction that represented the citrate soluble P. In 5 extraction cycles 6989 (2711) mg kg^{-1} of DTP was extracted from PL by the alkaline reagent $NaHCO_3$. Water took 7 extraction cycles to extract 8493.8(±2082) mg kg^{-1} of DTP from PL. The MRP released by water over the 7 extraction cycles was 6935 (±1729) mg kg^{-1}. Similar inorganic P (Pi: 7400 mg kg^{-1}) was reported by Sharpley and Moyer (2000) in their manure leachate experiment where they discussed how the concentration of MRP(Pi) especially in poultry manure decreased less slowly in the successive extraction cycles, whereas the P concentrations decreased more rapidly in contemporary beef and swine manures. The magnitude of P released in case of acids was approximately two-fold greater than that released by the water or sodium bicarbonate.

The total dissolved MRP represents the inorganic P fraction Pi. Dissolved MRP released was approximately 10217.57(± 7601.97) and 9,741.8(± 7103.52) mg kg^{-1} and for HCl and citric acid, respectively (Table 3.1). Over the course of 4 extraction cycles, both acids were able to extract more than 90% of P present in PL (Table 3.2). While the slope of DTP release curve of HCl is greater (Figure 3.3), the slope of MRP release curves of HCl and citric acids overlap (Figure 3.4). MRP extracted by NaHCO3 over the 5 cycles was 5422 (2637) mg kg^{-1}. The trend of P concentration released in the extraction cycles for sodium bicarbonate and water were similar as reported by previous literature in fractionation studies (Dou et al.,2000; Sharpley and Moyer 2000).

As P availability from animal manures largely depends on its labile organic and inorganic forms (Leytem et al., 2002), the MRP or inorganic P(P_i) present in the PL reflects the bioavailability of P. Bromfield (1960) found that plant yield uptake responses are directly related to manure P_i content. He also found that with increase in the TP content in PL, the proportion of inorganic P perpetually increased. The MRP or reactive P is the orthophosphate P form which can potentially cause eutrophication. Edward and Daniel (1993) found 80-90% of the P in runoff from pastures receiving PL was MRP.

c) Percentage phosphorus recovery per extraction cycle

Greater percentage of DTP extracted in the first extraction cycle for HCl suggests relatively stronger extraction potential of HCl. Although HCl extracted the highest % of DTP (95%) summed up for 4 extraction cycles, DTP for the first extraction cycle was greater for citric acid (85%) (Table 3.3). This indicates that even though total P recovery was greater for HCl, citric acid is quicker to extract a greater percentage of P in the first wash cycle (Table. 3.3).

Concentrations of MRP extracted in the third and fourth extraction cycle of HCl were very negligible compared to the first and second cycle (Table 3.3). The inorganic fraction (MRP) of the HCl extractable P in the first cycle was 10% lesser than of citric acid, which was 92 %. (Table 3.3) Hence, in case of acids there was a constant declining trend, whereas in case of water and sodium bicarbonate extraction, a considerable amount of P in the fifth, sixth, and seventh extraction cycles was observed. Previous studies have indicated that approximately 65-70% of the DTP was extracted in the first extraction cycle of the water and $NaHCO_3$ (Dou et al., 2000). In this study the first extraction cycle of the $NaHCO_3$ extracted 64.8 % of DTP, whereas for water the first extraction cycle extracted 43.9 % DTP which is comparatively less than the previous reported values literature. Also, the trend of constant decrease in the P concentration for successive extraction cycles was contradicted with the fifth extraction cycle when there was an abrupt rise in the P concentration. This might be due to release of some the soluble Ca-, Mg- bound P

The DTP extracted in the first extraction cycle of the water extraction was significantly greater than the second extraction cycle, which in turn was significantly greater than the third and fourth cycles. The last three extraction cycles did not show any significant deviation suggesting smaller amounts of P can be released in the last three cycles.

Comparison of the various P forms in PL showed the DTP % was highest in HCl followed by citric acid, water, and 0.5 M $NaHCO_3$. According to previous literature most of the P in manures were reported to be in inorganic forms (Barnett 1994a, Leinweber 1996). The soluble organic fraction of the P extracted by water and 0.5 M sodium bicarbonate was lesser that that extracted by acids. Water and sodium bicarbonate extractable P are considered bioavailable P pools. The MRP(Pi) extracted by citric acid was significantly greater than other extractants.

d) Phosphorus dissolution efficacy and comparison of P recovery from all extractants

Mineral acid like HCl and organic acid like citric acid was most efficient in extracting more than 90% of P from PL. The DTP extracted by HCL was 95% which was significantly different from the DTP recovered by citric acid (92%) (Fig 3.5). Thus, it can be inferred that most of the P present in litter was acid hydrolysable. The total DTP recovered from the basic $NaHCO_3$ was 56% which was significantly greater than total DTP extracted by water (40 %). The total MRP extracted by citric acid (66 %) was significantly greater than that extracted by HCl (55 %) (Fig 3.8). The total DOP (Po) extracted by the acids differed in their magnitudes. Significantly greater amount of total DOP was extracted by HCl (40%) than by citric acid (26%). The percent total DOP extracted by the alkaline and neutral extractant were not significantly different from each other. The residual P left at the end of extraction cycle for HCl represented the acid non-hydrolysable P (4.3 %). The residual P of the citric acid represented the citrate insoluble P (7%) in PL. The residual P left after repeated extractions was significantly least in acids amongst the four extractants. The Barnett (1994) found the residual P associated with monogastric animals like hog and poultry were most abundant. when the comparative analysis of different types of animal manures was studied. The relative P extracting power of the four extractants can be ranked as HCl > Citric acid > 0.5 M $NaHCO_3$> deionized water. Results are supported by the previous literature. Dou et al (2000) reported the power of extraction of the extractants as HCl> TCA > $NaHCO_3$ > H_2O > NaOH.

e) Co-release of nutrients

The co-release of macronutrients and micronutrients was also determined at each P extraction cycle. Previous research involving phosphorous reclamation in biowastes and manures have no documentation or records on the magnitude of nutrients recovered from chemical/physicochemical extraction process. Although the concentration of Ca, Mg, Al, and other nutrients were documented as co-released in phosphorus fractionation studies (Turner and Lytem, 2004; Li et al., 2014).

Cumulative recovery of nutrients:

The concentration of micronutrients boron (B), copper (Cu) and heavy metals arsenic (As), chromium (Cr), cadmium (Cd), and lead (Pb) were found to be below detection limit (BDL).

Typically metals such as arsenic, iron, copper, and zinc are added in the feed for poultry used as growth promoting agent for poultry birds and to control intestinal parasites. (Bolan et al., 2004; Jackson et al., 2003). Heavy metals are environmental concern because they can remain bound to the soil (Ferket et al., 2002), and is dependent on the absorption desorption ability of the soils. United States geological survey reported the mid-west region of intensive poultry production units in the Delaware, Maryland and, Virgina introduce 20-50 tons of arsenic in the environment annually (Christen, 2001). Heavy metals elements can become toxic to plants and have further health impacts on the organisms consuming the plants. In our study recovery of heavy metals was reported to be nil/negligible.

Cumulative mean concentration of Zn and Fe were BDL over the 7 extraction cycles with water whereas, Zn and Fe extracted by the acids were significant. Mean cumulative Zn concentration extracted by HCl 331(±211) mg kg^{-1} and the citric acid 186(±255) mg kg^{-1} were similar in magnitude (Figure 3.7). Although relatively greater mean iron concentration of 703(848) mg kg^{-1} was extracted by citric acid than that extracted by HCl 266(±685) mg kg^{-1} there were no significant differences between the cumulative Fe and Zn concentration recovered from the two acids. The mean cumulative moles of Al extracted by the HCl 1242(±1397) mg kg^{-1} was significantly greater (10 times) in magnitude than extracted by the citric acid 91(±48) and water 37 (±8) (Figure 3.9). The ratio of mean number of Al : P moles released by the HCl is 2.12, higher compared to the 0.18 and 0.12 released by citric acid and water respectively. The mean moles of Al and Mean cumulative moles of Ca released by HCl 633 (±319) and citric acid 514(±297) did not vary significantly, whereas water extracted significantly lesser 112 (±79) moles. Mean cumulative number of Mg moles released by HCl was significantly greater 843 (±589) mg kg^{-1} but that released by citric acid 165(±77) was not significantly different from that extracted by water 324

(±87) . In fact, the mean cumulative Mg released by the water in 7 extraction cycles was relatively greater than citric acid (Figures 3.12 and 3.16).

The magnitude of recovery of calcium can be substantiated by the fact that diet of poultry birds contains at least 1 % dicalcium phosphate for bone development (National Research Council,1984). Furthermore, the dicalcium phosphate can pass through the digestive system of the birds undissolved (Isermann, 1990). Generally, two-thirds of the P in taken by the animals as feed is excreted in the manures (Hristov et al, 2006).

Acid extractable nutrients co-release pattern:

The release characteristics of various nutrients during the repeated extraction cycles varied between HCl and citric acids. The release pattern of iron (Fe) was similar uptill the 4th cycle of extraction. The mean concentration of aluminum (Al) released by the HCl in the first extraction cycle was 3 times greater than the mean concentration of the Al extracted in the first extraction cycle by citric acid. The release pattern of Al with HCl showed an increasing trend over the four extraction cycles (Figure 3.9), whereas in case of HCl it showed a declining trend (Fig. 3.9). Greater mean concentration of zinc (Zn) was extracted in the first extraction cycle by HCl compared to the mean Zn concentration extracted by citric acid. The mean concentration of Ca released in the first extraction cycle was comparatively higher in case of HCl but after the second extraction cycle the release rates coincided and declined (Fig 3.11). While the mean Mg concentration extracted in the 1st extraction cycle of the citric acid was 1.5 times lower than the mean Mg concentration of the HCl extraction (Fig 3.12) the Mg release curves of HCl and citric acid did not coincide or overlap (Fig 3.12). 21099 (±6664.9) mg/kg potassium (K) was released in the first extraction cycle compared to the 22953 (±6664.9) mg/kg extracted by citric acid (Fig

3.13).Higher mean concentration of S was extracted by the HCl as compared to citric acid consistently over the 4 extraction cycles. (Fig 3.14).

Water extractable nutrients co-release pattern:

Micronutrient like Zn and Fe concentration were found to be BDL over all the seven extraction cycles. Mean Zn recovery from the water-soluble P fraction in a sequential P fractionation study was reported as 35 mg/kg (He et al.,2000), this corresponds to the first extraction cycle of water extraction in this study. The mean Al concentration showed a constant steady decrease along the 7 extraction cycles (Fig 3.15). Whereas the Ca and Mg concentration in water extraction cycles decreases, and peaks and coincides on the fourth extraction cycle, thereafter, decreases and increases slightly in the last extraction cycle (Fig 3.16). The pattern of the release, which slightly increased toward the later extraction cycle suggested that as the more labile phosphorus was extracted by shaking for 4-5 extraction cycles, the release of the moderately labile or non-labile fraction of P can be associated with the release of Ca bound P and Mg bound P. Similar extraction curve trends were observed for mean K and S concentrations (Fig 3.17; Fig. 3.18). Most of the K was released in the first water extraction cycle (28539.94 (±8675.309) mg/kg), tand negligible amount were extracted from the 4th cycle onwards. The mean S concentration extraction curve showed no or negligible release after the 2nd extraction cycle.

Conclusion:

The P extraction efficiency can be ranked as 1 M HCl > 1 M citric acid > 0.5 M $NaHCO_3$> deionized water. Acidic extractants are efficient in dissolving organic P present in poultry manure. Approximately 7% of the total phosphorus (TP) was acid non-hydrolysable P, which means the complex organic molecules make up 7% of the bulk of litter. The cumulative recovery of Al, Fe, Mg, Ca, K, S substantiate the previous studies which suggested PL is a rich source macro and micronutrients. Concentration of heavy metals like As, Cd, Cr was found to be below detection limit in all water and acid extractions. Greater concentrations (10 times) of Al was released by HCl compared to citric acid and water. The cumulative mean concentration of Al and Fe in both water and acid extracted solution were lesser than Ca and Mg in PL. Greater abundance of Ca and Mg indicates the possible predominance of Ca and Mg associated P. The cumulative potassium (K) and sulphur (S) released during extraction cycle was least affected by the extractant types.

Table 3. 1. Comparison of total P recovery using four extractants in mg kg $^{-1}$

Extractant	Dissolved Total P (DTP)	Dissolved molybdate Reactive P (MRP)	Dissolved Organic P	Residual P	Total P
--mg/kg --					
Sodium bicarbonate	6989 (2711[†])	5422 (2637)	1567 (1217)	6062 (4352)	13045 (6037)
HCl	15801(7305)	10217 (7601)	5583 (902)	589 (110)	16390 (7296)
Citric acid	13653 (8548)	9741 (7103)	3911 (3297)	693 (128)	14354 (8583)
Water	8493 (2081)	6935 (1729)	1558 (657)	12463 (6686)	20957 (7948)

† Standard deviation of the mean of the mean given in parenthesis.

Table 3. 2. Concentration of DTP and MRP released in the repeated extraction cycles in 1 M HCl, 1 M citric acid, 0.5 M $NaHCO_3$, and de-ionized water. Values in parenthesis represents standard error of means. Percentage recovery is expressed as percentage of TP. Percentage P values of each parameter followed by the same letter are not statistically significant to each other at $P \leq 0.05$.

Extractant	Extraction cycle number	Dissolved total P (DTP)	Molybdate reactive P (MRP)
		(mg/kg)	
1 M HCl	1	12860(1701[†])	9208(1902)
	2	1880(105)	934(75)
	3	536(61)	32(7)
	4	525(72)	43(9)
	Σ	15801(1826)	10218(1900)
Residual P		590(27)	
TP		16391(182)	
Percent recovery (%)		95a	55b
1 M citric acid	1	12171(2068)	9076(1692)
	2	1178(99)	500(71)
	3	176(10)	133(31)
	4	128(10)	35(10)
	Σ	13653(2137)	9745(1775)
Residual P		693(128)	
TP		14354(2145)	
Percent recovery (%)		93a	66a
0.5 M NaHCO3	1	4300(340)	3586(347)
	2	1190(200)	504(88)
	3	628(132)	637(147)
	4	347(79)	257(59)
	5	524(115)	585(169)
	Σ	6990(1509)	5569(650)
Residual P		6062(1088)	
TP		13046(1509)	
Percent recovery (%)		56c	43c
water	1	4418(313)	3338(251)
	2	1805(240)	1541(199)
	3	895(112)	813(123)
	4	543(70)	461(70)
	5	398(144)	347(44)
	6	246(30)	218(28)
	7	192(23)	217(60)

Σ	8498(627)	6935(519)
Residual P	12463(1730)	
TP	20957(2152)	
Percent recovery (%)	40d	35.8d

† Standard error of the mean given in parenthesis.

Table 3. 3. Percentage dissolved total phosphorus (DTP) and molybdate reactive P (MRP) released per extraction cycle (DTP/Σ DTP and MRP/Σ MRP) in the repeated extractions. Percentage P values of each parameter followed by the same letter are not statistically significant to each other at $P \leq 0.05$. ANNOVA was performed using PROC GLM in SAS 9.4 version (SAS institute, 2013).

Extraction cycle	DTP/Σ DTP $_{\text{HCl}}$	DTP/Σ DTP $_{\text{Citric acid}}$	DTP/Σ DTP $_{\text{NaHCO3}}$	DTP//Σ DTP $_{\text{Water}}$
	%			
1	79a	85a	58a	47a
2	13b	11b	17b	26b
3	3c	1c	9c	10c
4	3c	0.9c	5cd	5cd
5			10de	4de
6				2de
7				2e
Extraction cycle	MRP/Σ MRP $_{\text{HCl}}$	MRP/Σ MRP $_{\text{Citric acid}}$	MRP/Σ MRP $_{\text{NaHCO3}}$	MRP//Σ MRP $_{\text{Water}}$
	%			
1	82a	92a	64a	48a
2	16.b	5b	9b	27b
3	0.6c	1c	8b	10c
4	1.0c	0.59c	3.6c	6c
5				4c
6				2d
7				3d

Table 3. 4. Cumulative recovery of number of moles of nutrients (Al, Ca, Mg, K and S) from acidic and alkaline extractants in the repeated extractions. Ratio of nutrients to the total P released.

	Al	Ca	Mg	K	S
			number of moles		
HCl	1242.5	633.8	843.6	655.9	340.9
Citric	91.3	514.6	165.1	636.5	162.1
Water	37.7	112.02	324.2	881.41	338.7
	Al /P	Ca /P	Mg /P	K /P	S /P
HCl	2.12	1.61	1.30	1.62	0.69
Citric	0.18	1.51	0.29	1.82	0.38
Water	0.12	0.53	0.93	4.06	1.28

Figure 3. 1. Location of sample collection in Alabama.

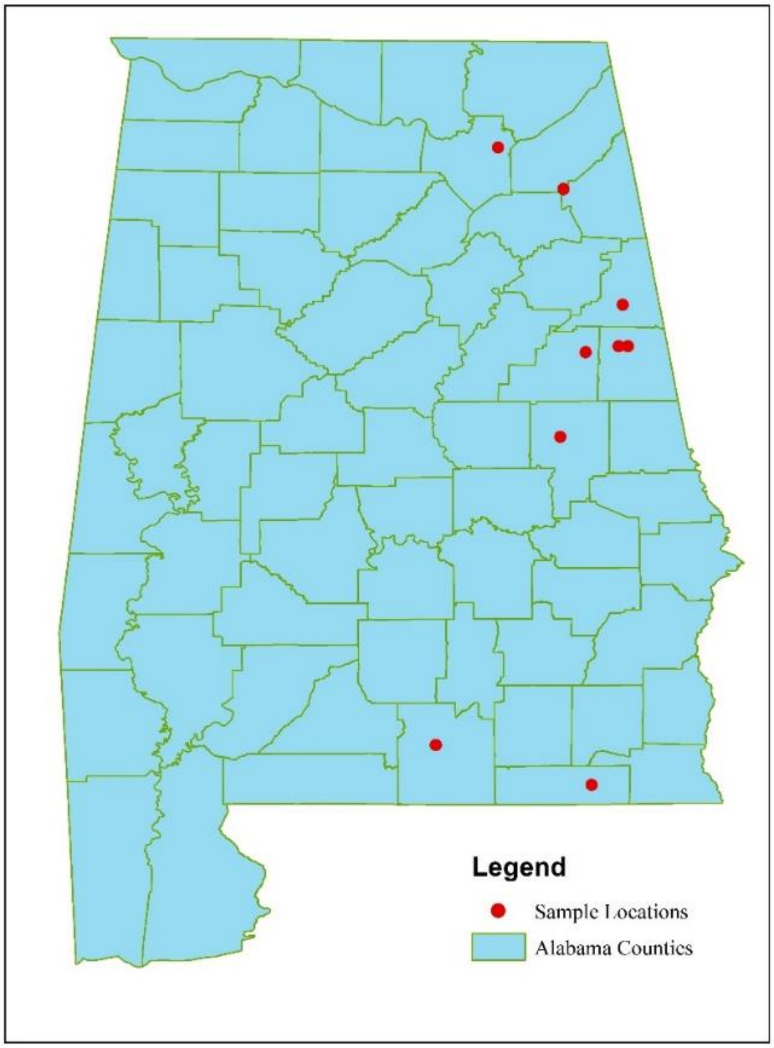


Figure 3. 2.Flowchart for repeated P extraction.

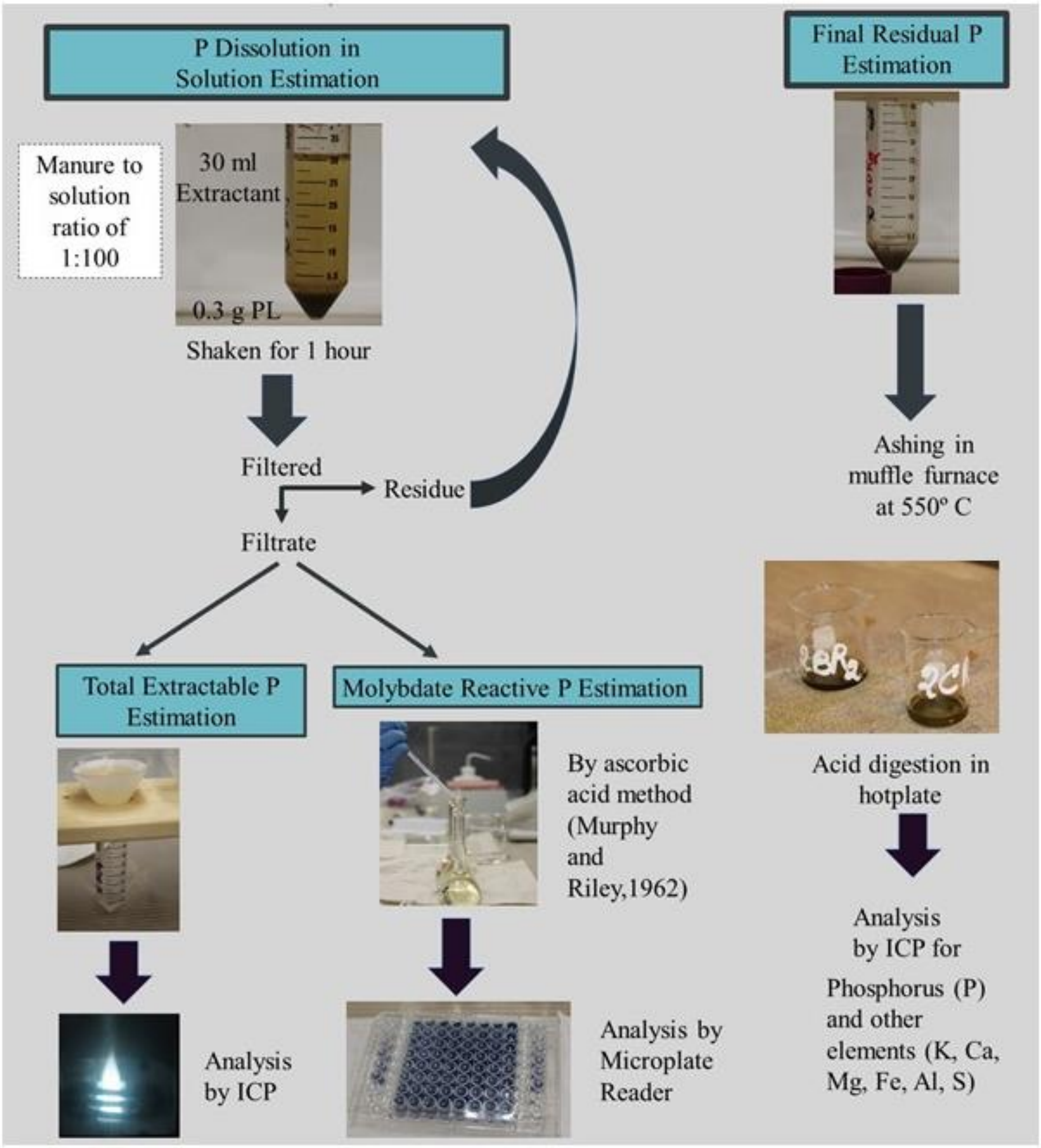


Figure 3. 3.Comparison of DTP release characteristics from acidic (1 M HCl and 1 M citric acid), neutral (de-ionized water) and basic ($NaHCO_3$) extractants. Error bar is the standard error of the means

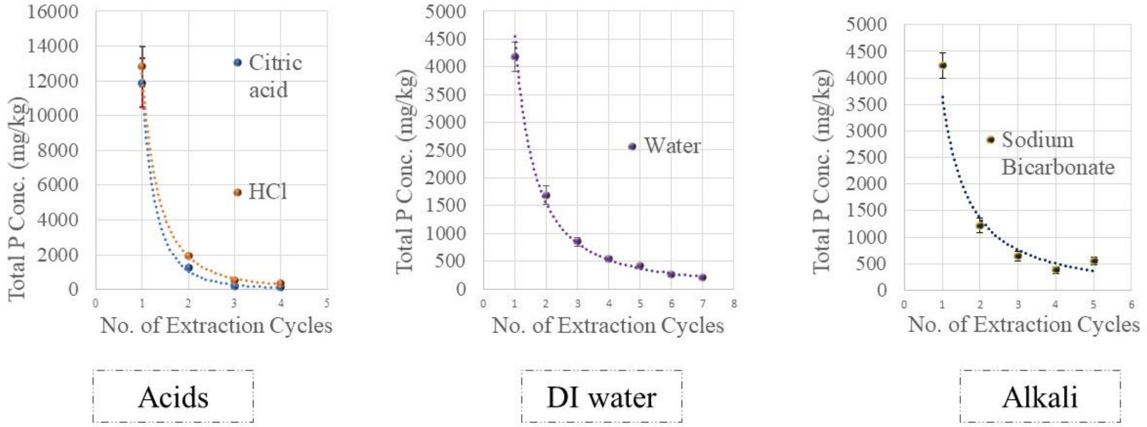


Figure 3. 4.Comparison of MRP release characteristics from acidic (1 M HCl and 1 M citric acid), neutral (de-ionized water) and basic (NaHCO$_3$) extractants. Error bar is the standard error of the means.

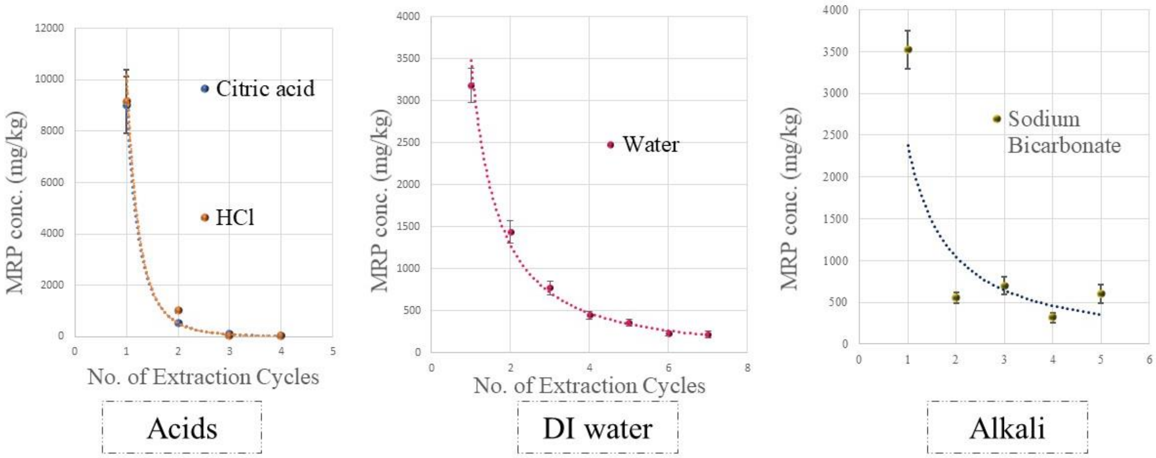
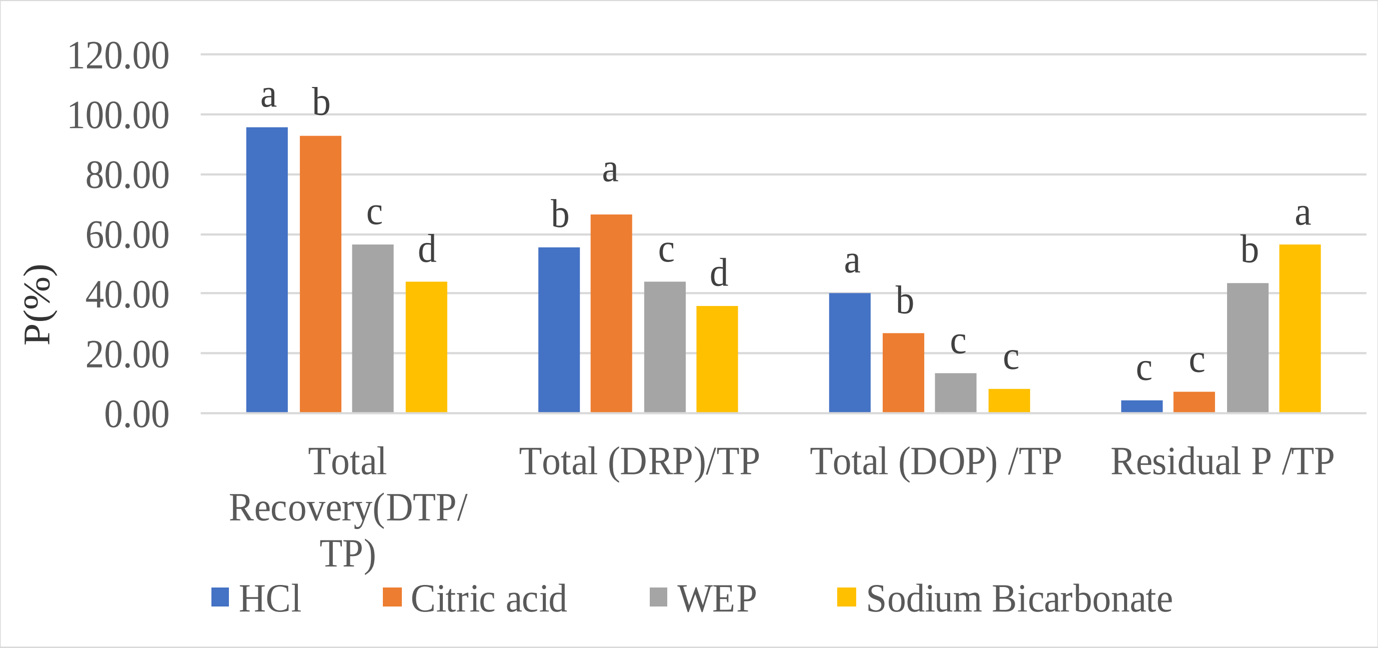


Figure 3. 5.Comparison of total DTP, MRP and DOP recovered from 1 M HCl, citric acid, water, and sodium bicarbonate from repeated P extractions. ANOVA was performed using PROC GLM in SAS 9.4 version (SAS institute, 2013). Means separation was done using TUKEY-KRAMER.



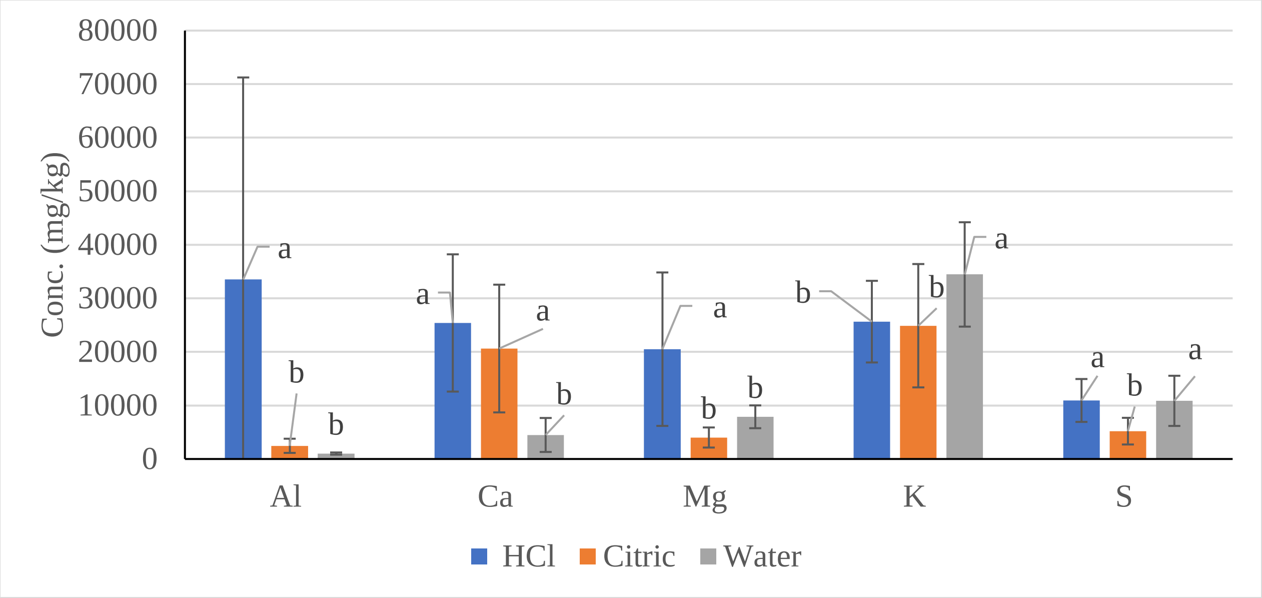


Figure 3. 6.Cumulative recovery of nutrients (Al, Ca, Mg, K and S) from acidic and alkaline extractants. Values of each parameter followed by the same letter are not statistically significant to each other at P ≤0.05. ANNOVA was performed using PROC GLM in SAS 9.4 version (SAS institute, 2013).

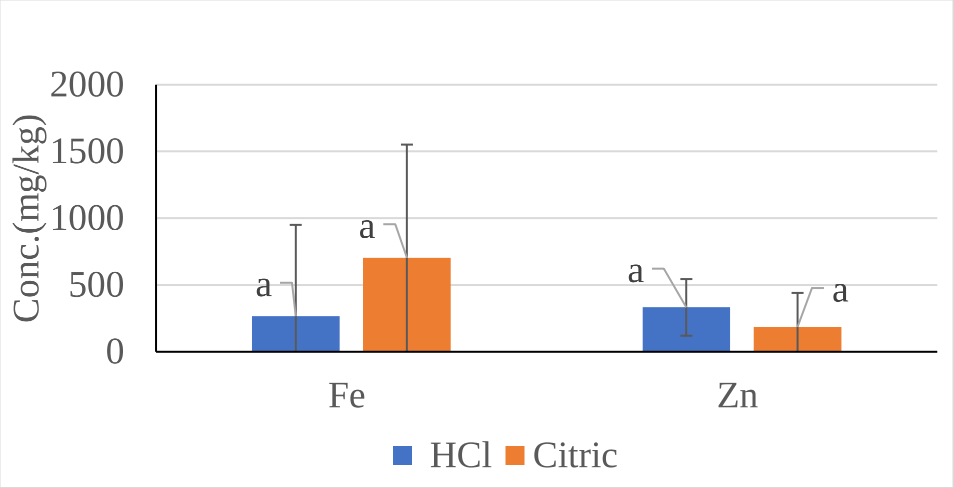


Figure 3. 7.Cumulative recovery of micronutrients (Fe and Zn) from the acids HCl and citric acid

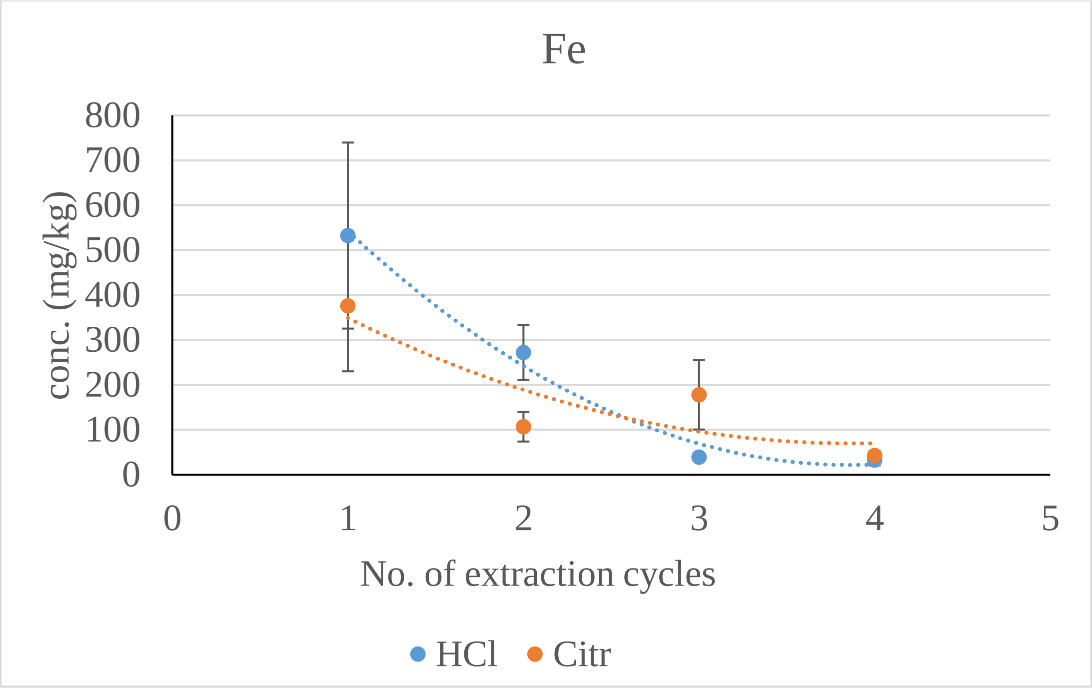


Figure 3. 8.Release characteristics of Fe with the acids HCl and citric acid

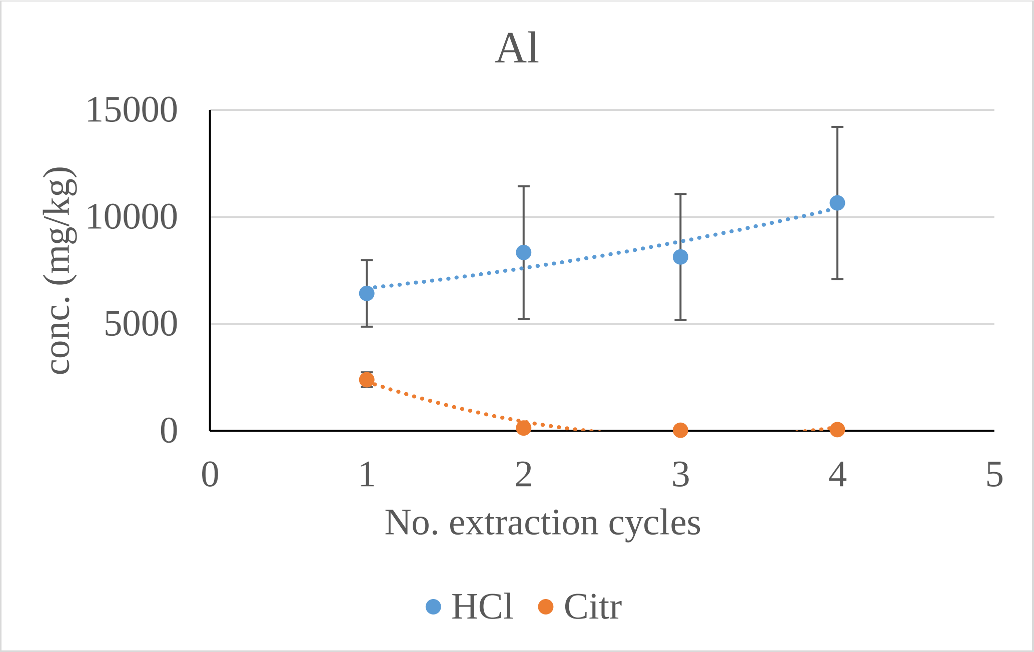


Figure 3. 9.Release characteristics of Al with the acids 1 M HCl and 1 M citric acid

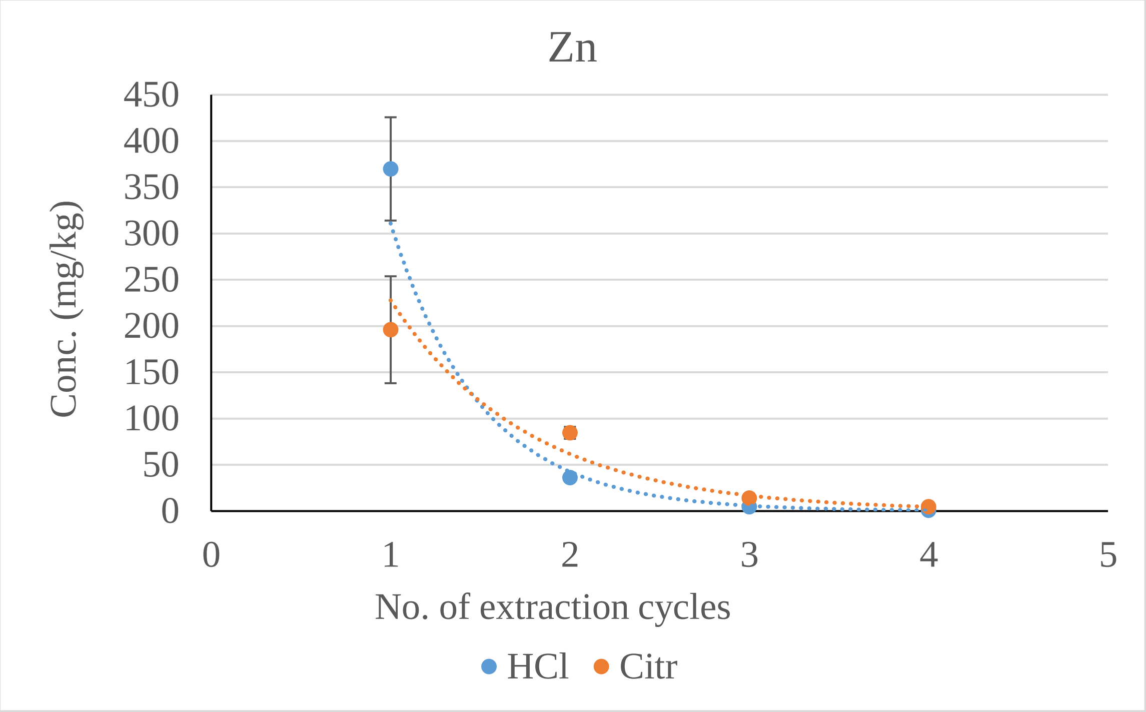


Figure 3. 10.Release characteristics of Zn with the acids 1 M HCl and 1 M citric acid

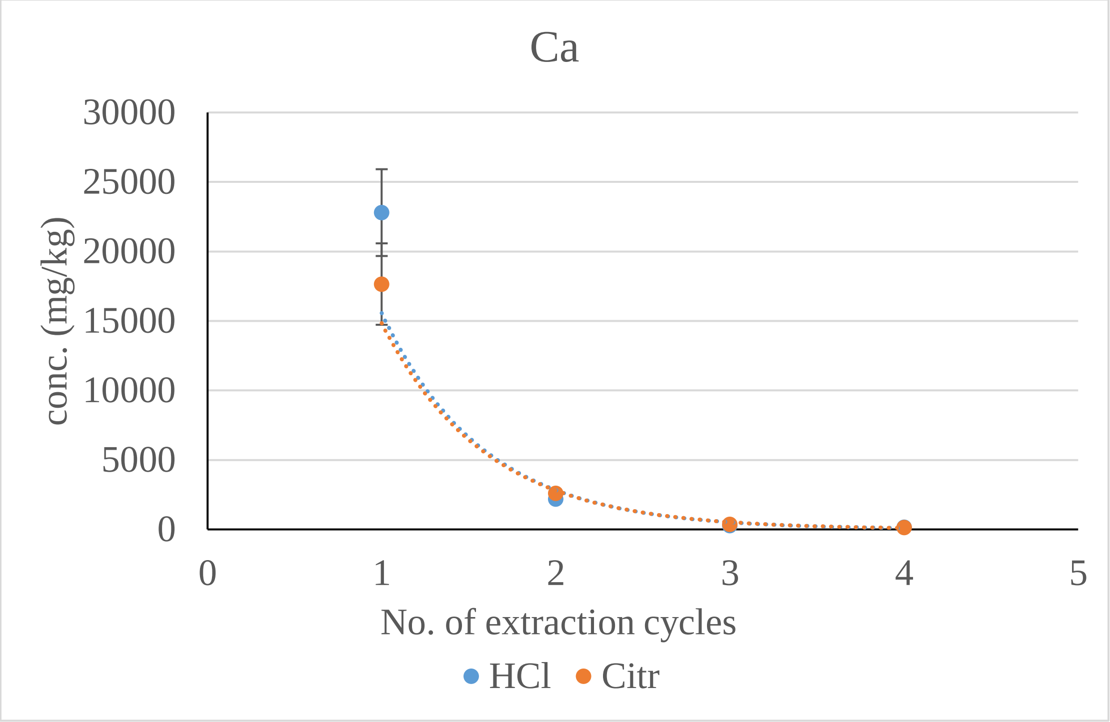


Figure 3. 11.Release characteristics of Ca with the acids 1 M HCl and 1 M citric acid

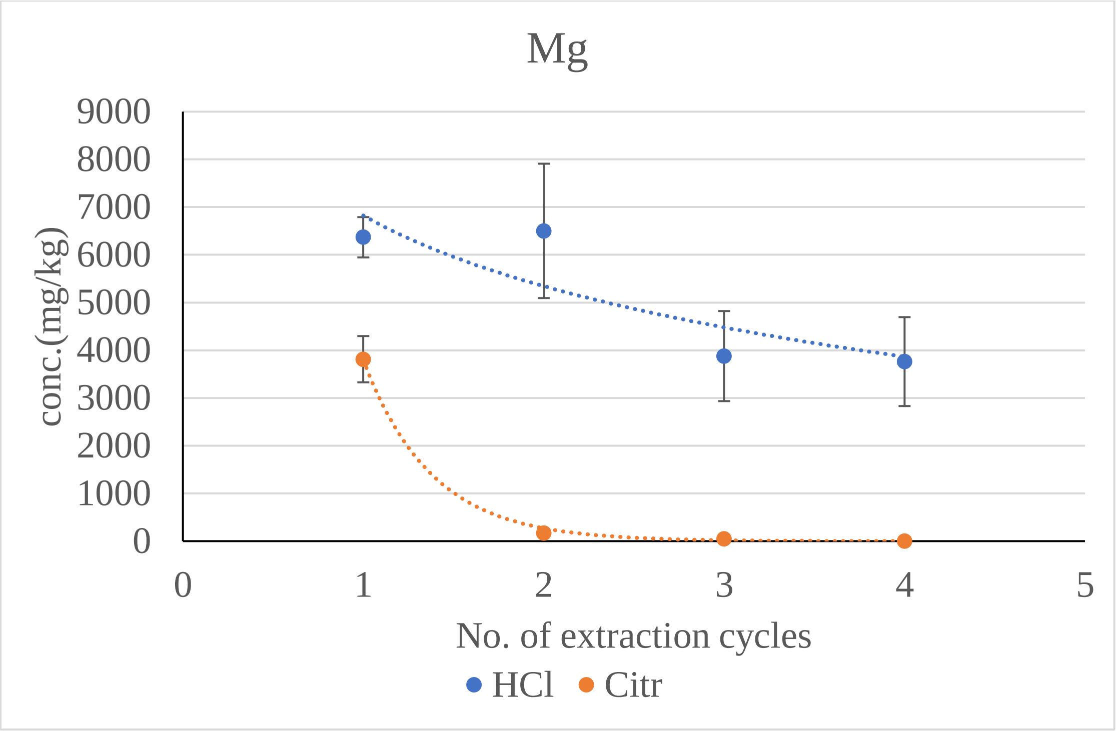


Figure 3. 12.Release characteristics of Mg with the acids 1 M HCl and 1 M citric acid

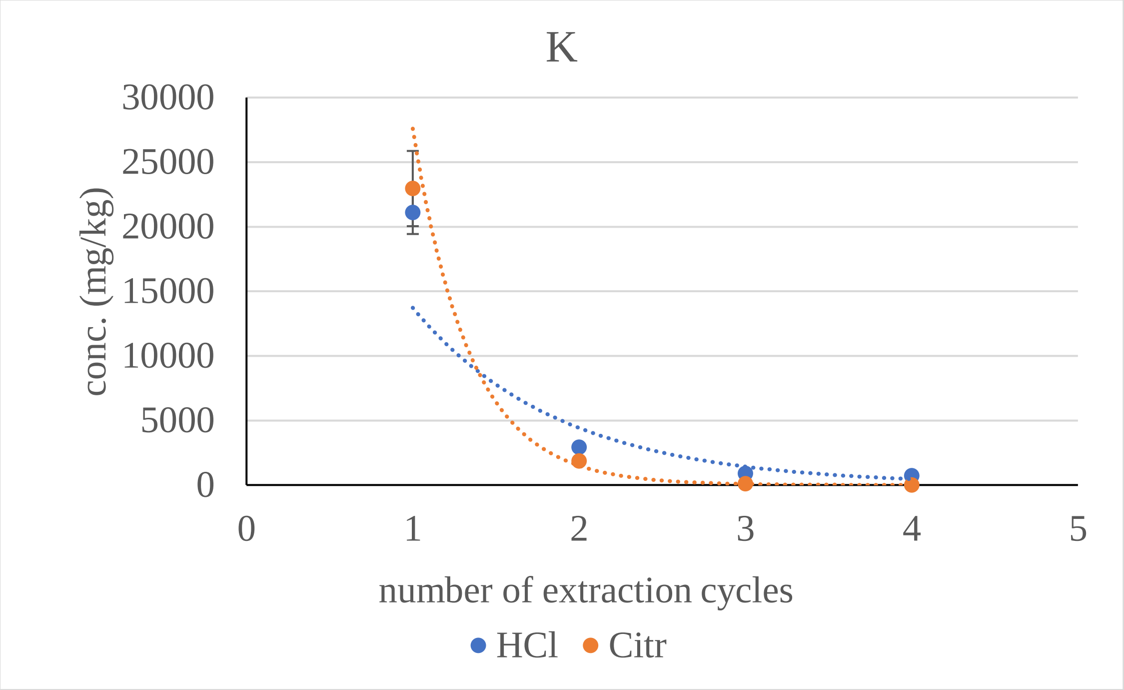


Figure 3. 13.Release characteristics of K with the acids 1 M HCl and 1 M citric acid

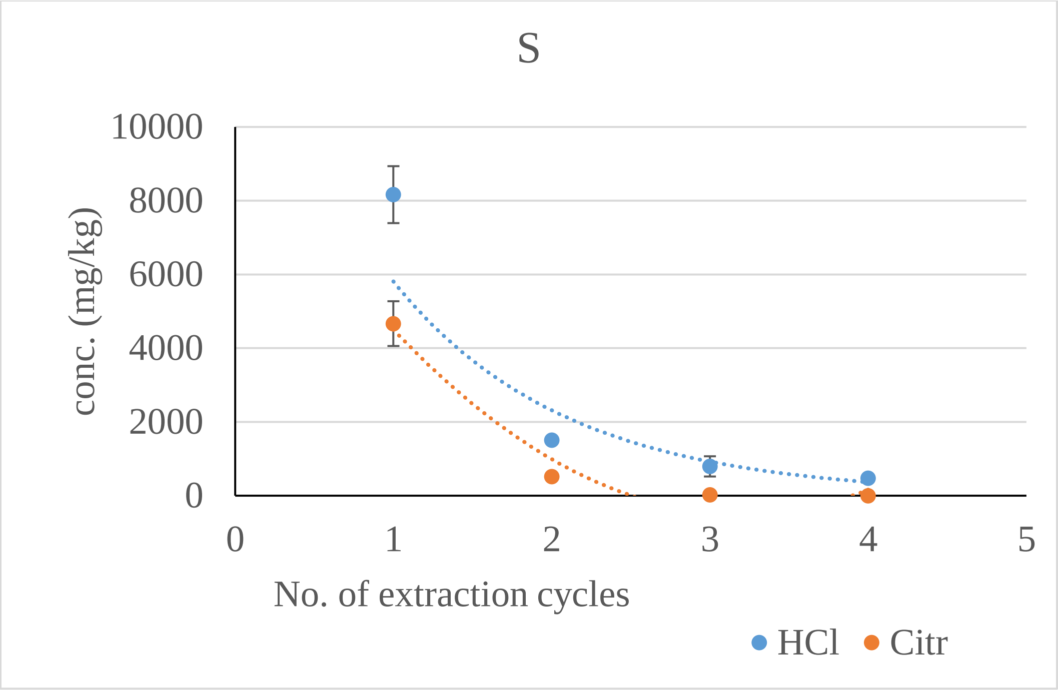


Figure 3. 14.Release characteristics of S with the acids 1 M HCl and 1 M citric acid

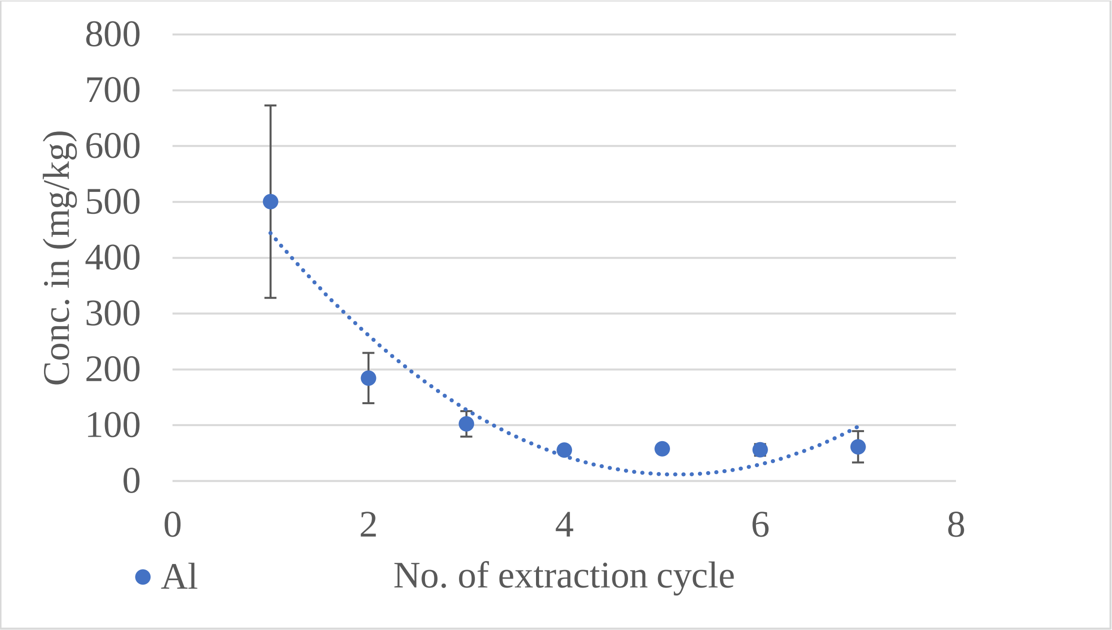


Figure 3. 15.Release characteristics of Al with repeated extraction with de-ionized water

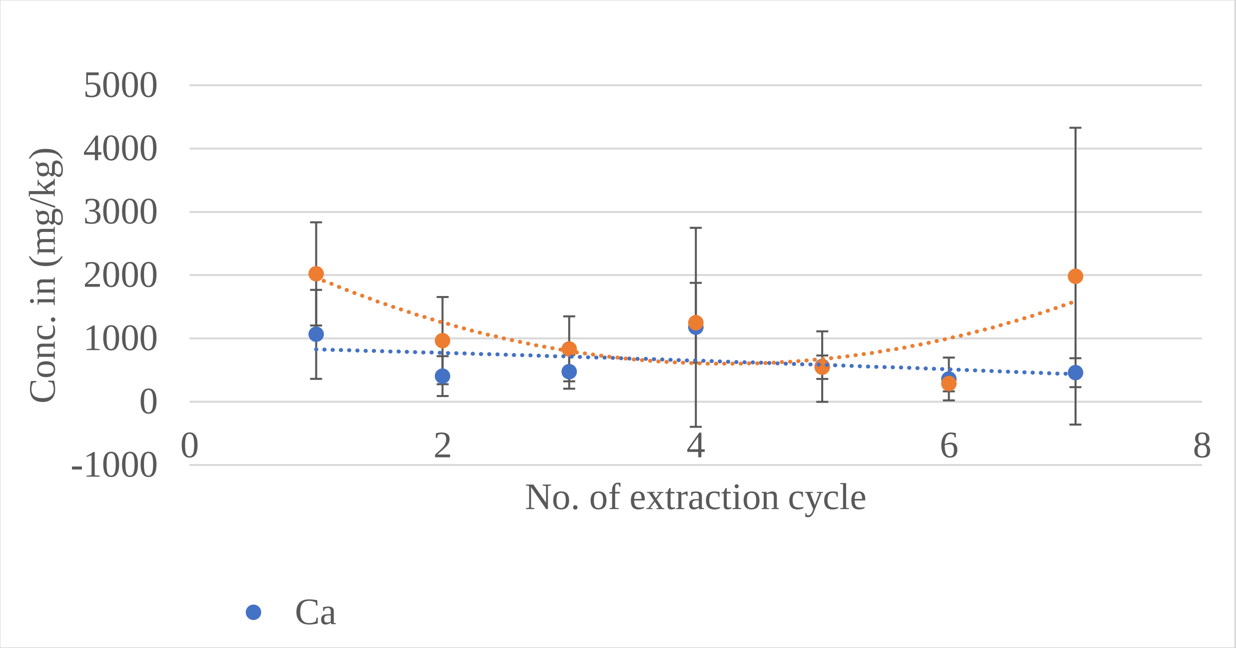


Figure 3. 16.Release characteristics of Ca and Mg with repeated extraction with de-ionized water

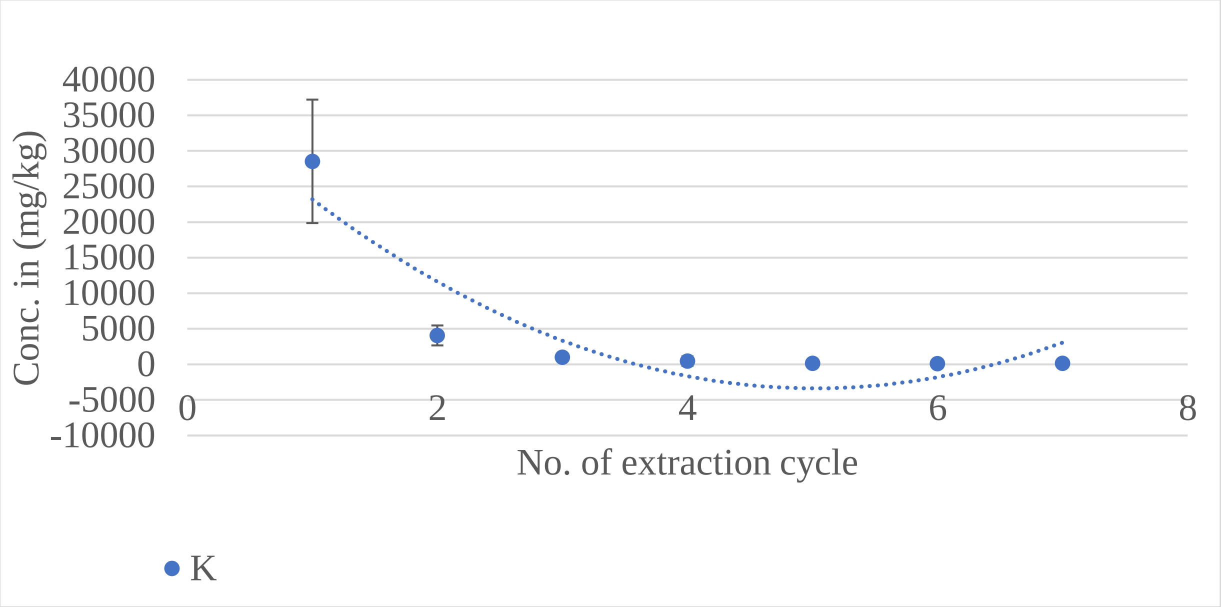


Figure 3. 17.Release characteristics of K with repeated extraction with water

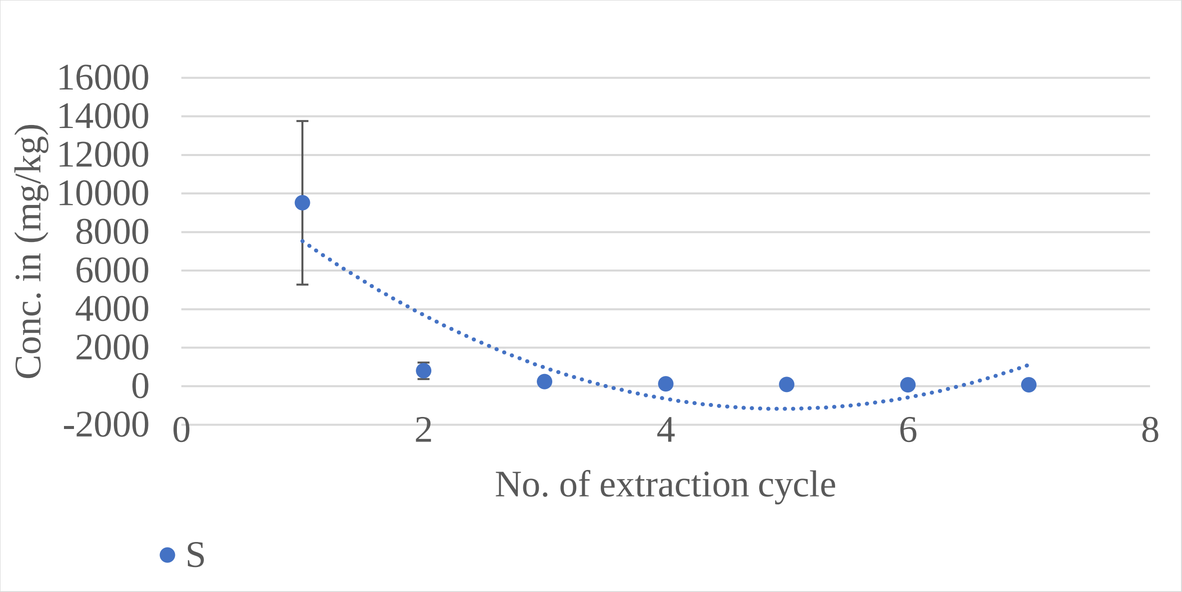


Figure 3. 18.Release characteristics of S with repeated extraction with water

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Table S. 1. Comparison of two methods of filtration 1) Whatman number 42 filter paper 2) 0.45µ filter membrane

	Filtration with Whatman no. 42 filterpaper	*Filtration through 0.45µ filterpaper*
Mean	4.2	4.1
Variance	4.9	4.9
Observations	8	8
Hypothesized Mean Difference	0	
df	14	
t Stat	0.081	
P(T<=t) one-tail	0.468	
t Critical one-tail	1.76	
P(T<=t) two-tail	0.936	
t Critical two-tail	2.14	

Table S. 2. Additional information on Poultry Litter Cleanout Frequency.

Clean out frequency (0-6 months)			
Farm number	Location	Bedding material	Description
1	Collinsvile, AL	Pine shaving and Sawdust	
2	Collinsvile, AL		
3	Collinsvile, AL		4 flocks old
Clean out frequency (6-12 months)			
Farm number	Location	Bedding material	Description
1	Widowee, AL	Pine shavings	
2	Gunterville, AL	composted litter	
3	Weedowee, AL	Pine shavings	
4	Jackson, AL	composted shed PL	120,000 chicken, 3.75 lb
5	Jackson, AL	Decaked PL (dry stacked)	
6	Talapoosa, AL	Decaked PL (dry stacked)	140,00 Birds, 9 males
7	Lineville, AL	Pine shavings	
Clean out frequency (12-24 months)			
Farm number	Location	Bedding material	Description
1	Talapoosa, AL		140,00 Birds, 5.5lb
2	Covington, AL		
3	Geneva, AL	Peanut Hull	Pulverized litter
4	Geneva, AL	Peanut Hull	Pulverized litter
Clean out frequency (5-year)			
Farm number	Location	Bedding material	Description
1	Covington, AL		Weight of bird 3.75lb Flock size 30,000
2	Covington, AL		