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Short Report

Applying Different Sample Treatment Strategies for Evaluating Phosphorus Distribution in Orange Juice

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Assessment of phosphorus in fruit juices is of great interest due to its essentiality and nutritional properties. This study applied sample treatment strategies to determine phosphorus in fractions of juices. Firstly, total phosphorus (P_{total}) was determined from digestion and sample suspension exploring direct analysis by inductively coupled plasma optical emission spectrometry, which presented no significant difference, for 95% of confidence level. Then, free phosphorus (P_{free}) was determined by spectrophotometry and represented 30-90% of P_{total} with an inverse relationship respecting P_{total} concentration. Fractioning according to particle size evidenced highest fraction of P_{free} in samples after filtration. Fractioning of phosphorus based on its charge was also performed and the high percentages of P in the anionic fraction (from 91.2 to 95.9%) are related to free inorganic phosphate in equilibrium with its protonated forms. Thus, it is more assailable after consumption, giving this food great functionality on human diet.

Keywords: phosphorus, fractioning, orange juice, spectrophotometry, ICP OES

Introduction

Citrus fruits are considered as one of the important fruit crop groups being consumed either as fresh fruit or as juice.[1] These beverages have been highly appreciated since they provide a wide array of essential nutrients for human health such as vitamins, folate, dietary fiber, and minerals, as well as many phytochemicals, including flavonoids, glucarates, terpenes, phenolic acids and carotenoids.[2,3] The consumption of citrus juice has been related to health promotion as preventing coronary diseases and chronic asthma and, not only because of that, but also due to other beneficial aspects, it has increased over the last years throughout the world.[4,5]

Among citrus juices, the orange deserves attention due to its high consumption as compared to juices of other citrus fruits. The production of orange is one of the most important sectors of Brazilian agribusiness, which has been above 16 million tons, corresponding almost to 13% of the permanent crops in Brazil in the last three years. About 80% of the Brazilian production is intended for the juice industry.[6-8]

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Because of the beneficial properties of fruit juices, their consumption has been recommended.[9] Among the elements which have nutritional significance and are essential to people's health, phosphorus is highlighted.[10] However, high levels of this mineral in the organism can cause damages.[11] Phosphorus and calcium in an appropriate ratio is important for mineral deposition into bone, and for mineral absorption.[12]

The presence of phosphorus in juices and consequently in its derivative products is related to different sources. Among soil and foliar fertilizers, the conventional farming of fruits makes use of phosphorus compounds, such as phosphoric acid, diammonium phosphate and potassium phosphate.[13] The phosphate polymers are commonly used by the food industry as meat preservatives or additives in non-alcoholic flavored drinks.[14]

The inorganic phosphorus is the most assailable form[15] and because of that, the development of analytical methods capable of estimating phosphorus distribution in juice fruits is of great interest to promote further bioavailability studies of this mineral.

Phosphorus content in fruit juices has been determined at total level (P_{total}) mainly by atomic spectrometry.[16-18] For free phosphorus (P_{free}), also known as inorganic

phosphorus or phosphate, it is common the use of ionic chromatography[19] and spectrophotometry. In this context, the spectrophotometric method of the molybdenum blue is one of the oldest, well stablished and widely employed for P_{free} determination in different samples.[20,21] However, studies about P fractions have been conducted almost totally for geological matrix.

Several authors[22,23] have used the P fractionation technique proposed by Hedley *et al*.,[24] which uses extractors from smaller to larger extraction forces, which remove P inorganic (Pi) and organic (Po) from the most available to the most stable forms. With the modifications proposed by Condron *et al*.,[25] the extractors used in the fractionation are, sequentially, anion exchange resin, $NaHCO_3$ 0.5 mol L^{-1} at pH 8.5, NaOH 0.1 mol L^{-1} and $H_2SO_4 + H_2O_2 + MgCl_2$. The Po are determined by the difference between P_{total} and the Pi in each extractor. Şahin *et al*.[26] determined four fractions of sedimentary P, including organic bound phosphorus fraction, calcium bound phosphorus fraction, Fe + Al bound phosphorus fraction and carbonate bound phosphorus. The results indicated the proportion of organic bound phosphorus fraction estimated 90.20%. These works, in general, determine organic and inorganic phosphorus in soils and derivative samples, but fractioning based on particle size and charge of phosphorus in food samples are still required.

This way, considering the absence of results in the food samples, the aim of this work was to perform different sample treatment strategies for evaluating phosphorus distribution in industrialized orange juice samples in function of its particle size and charge (neutral and anionic fractions). Besides, the relationship of P_{total} and P_{free} was evaluated in order to estimate the assimilable fraction of phosphorus in the samples.

Experimental

Instrumentation and devices

A digestion block model MA850, Marconi (Piracicaba, SP, Brazil), was used for digestion of orange juice samples. Digested and suspension of juice samples were analyzed by inductively coupled plasma optical emission spectrometry (ICP OES) model 720 Series, Agilent Technologies (Santa Clara, CA, USA). A spectrophotometer model 700 Plus, Femto (São Paulo, SP, Brazil), was used for free phosphorus determination.

For phosphorus fractioning, it was used a C_{18} Sep-pack cartridge (Waters, Barueri, SP, Brazil), a column manufactured in laboratory packed with the cationic resin Dowex 50WX8, and a peristaltic pump model Reglo digital, Ismatec® (Wertheim, BW, Germany).

Chemicals, solutions and samples

All solutions were prepared with ultrapure water (specific resistivity of 18.2 MΩ cm, electric conductivity < 0.1 μS cm^{-1}) from a Milli-Q® water purification system (Millipore, Bedford, MA, USA). All chemical reagents used in the analytical procedures are of analytical grade.

Phosphorus standard solution was prepared from a commercial standard solution of Titrisol 1000 mg L^{-1}. Nitric acid concentrated and hydrogen peroxide 30% (v v^{-1}) were used for sample digestion. A mixed solution composed by ammonium molybdate, oxalic acid and nitric acid; ascorbic acid solution and potassium antimony(III) oxide tartrate trihydrate were used for free phosphorus determination.

Industrialized orange juice samples of different brands were purchased in commercial market of Maceió City, Alagoas State, Brazil. These samples were submitted to different strategies of sample treatment according to the objective of analysis. After opened, samples were stored under refrigeration until one week.

Analytical procedures

The analytical procedures for phosphorus fractions determination in orange juice samples were developed systematically, according to the objective of analysis, as shown in Figure 1.

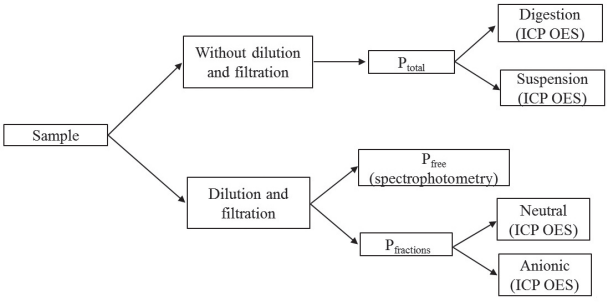

Figure 1. Scheme of analytical procedures applied to orange juice samples for fractioning of phosphorus.

Total phosphorus determination

Firstly, juice samples were digested on a digestion block based on the methodology established by Anunciação *et al*.[27] 5 mL of each sample were added to a glass vessel and then it was added 10 mL of concentrated nitric acid (14.4 mol L^{-1}) and 2 mL of 30% (v v^{-1}) hydrogen peroxide. The digestion block temperature was adjusted to 140 °C and the samples were digested for 90 min. Later, the digested samples were transferred to centrifuge tubes of 15 mL and then, the volume was adjusted to 10 mL with ultrapure water. Finally, samples were analyzed by ICP OES.

Alternatively, samples suspension were directly diluted with HNO_3 0.29 mol L^{-1} (1:1) for determination of P_{total} by ICP OES according to the methodology established by Froes *et al*.[28] The operating conditions for ICP OES were: power, 1300 W; plasma gas flow, 15 L min^{-1}; auxiliary gas flow, 1.50 L min^{-1}; nebulizer flow rate, 0.7 L min^{-1}; sample flow rate, 0.8 L min^{-1}; nebulizer system by V-groove with PTFE Sturman-masters chamber; and the selected spectral line for phosphorus was 214.618 nm. The limits of detection (LOD) and quantification (LOQ) of phosphorus were 8.70 and 28.6 µg L^{-1}, respectively.

Free phosphorus determination

For P_{free} determination, samples were diluted 125 times with ultrapure water, filtered with a cellulose acetate membrane of 0.45 µm and analyzed by molybdenum blue spectrophotometric method.[20,21] Briefly, the method consists of preparing a mixed solution composed by ammonium molybdate (5 mmol L^{-1}), oxalic acid (20 mmol L^{-1}) and nitric acid (0.2 mol L^{-1}); ascorbic acid ($C_6H_8O_6$) solution (140 mmol L^{-1}) and potassium antimony(III) oxide tartrate trihydrate (2.1 mmol L^{-1}) as catalyzer, according to the reaction described in the equations 1 and 2. In a polyethylene flask, it was added 1.5 mL of mixed solution, 1.0 mL of sample, 1.0 mL of ultrapure water, 1.0 mL of ascorbic acid and 0.5 mL of the catalyzer solution, in this sequence. After 15 min, samples were analyzed by molecular absorption spectrophotometry in a wavelength of 749 nm for determination of inorganic phosphate. The LOD and LOQ of the method were 0.028 and 0.094 mg L^{-1}, respectively.

$$PO_4^{3-} + 12MoO_4^{2-} + 27H^+ \rightarrow H_3PO_4(MoO_3)_{12} + 12H_2O \quad (1)$$

$$H_3PO_4(MoO_3)_{12} + C_6H_8O_6 \xrightarrow{Sb} \text{Molybdenum blue} + C_6H_6O_6 \quad (2)$$

Distribution of phosphorus fractions

Fractioning of phosphorus based on its particle size

For P_{free} fractioning in function of particle size, the samples were diluted 125 times with ultrapure water and an aliquot of 5 mL of each sample was filtered with a 0.45 µm cellulose acetate membrane. Then, filtered samples were analyzed by the molybdenum blue spectrophotometric method, as described previously.

Fractioning of phosphorus based on its charge

The fraction distribution of phosphorus was evaluated based on global fraction charge. For that, the neutral and cationic fractions of juice samples were separated by a solid phase extraction system. Diluted and filtered samples were submitted to a sequential extraction making use of two columns with a flow rate of 3 mL min^{-1}. The first column, a commercial one, was a C_{18} Sep-pack cartridge of 360 mg (column I), for retention of non-polar species (neutral fraction). The second, packed with ionic exchange resin Dowex 50W X8 (column II), built in the laboratory with diameter of 4 mm and length of 7.5 cm, retained the cationic fraction that contained phosphorus.

The column II was prepared based on previous procedure established by Pohl and Prusisz,[29] where 1 g of the resin was conditioned with HCl (1.0 mol L^{-1}) and NaOH (1.0 mol L^{-1}). Blanks of columns were performed before and after each analysis to verify absence of phosphorus and the efficiency of extraction system. Part of the effluent of column I was collected (effluent I) for further analysis and the rest of volume sample was pumped through column II to generate effluent II. Then, P_{total} content was determined in the effluents I and II by ICP OES.

All analyses were performed in triplicate and phosphorus concentrations were determined based on equations of the analytical calibration curves built from aqueous standard solutions of this analyte. The analytical curves for spectrophotometric analysis were composed by ten standard solutions in the range from 0 to 1.0 mg L^{-1}. Briefly, standard solutions for calibration curve for spectrophotometric analysis were prepared by addition of 1.5 mL of mixed solution (ammonium molybdate (5 mmol L^{-1}), oxalic acid (20 mmol L^{-1}) and nitric acid (0.2 mol L^{-1})), an aliquot of standard stock solution of phosphorus corresponding to the desired concentration, 1.0 mL of ascorbic acid, 0.5 mL of the catalyzer solution and completed with ultrapure water for a final volume of 5.0 mL. The analytical curves for ICP OES analysis were composed by eight standard solutions in the range from 0 to 100 mg L^{-1}. These solutions were prepared by dilution of an aqueous stock standard solution of phosphorus (1000 mg L^{-1}). In graduated tubes of 15 mL, it was added an aliquot of phosphorus stock solution according to the desired concentration of the standard and then, the volume was adjusted to 10 mL with ultrapure water.

For both atomic and molecular spectrometric determinations, the analytical parameters were calculated from the analysis of ten blank solutions on a basis of calibration curve data. LOD was defined as 3sb/a, where sb is the standard deviation of analytical signal of blank solution and a is the slope of calibration curve. For LOQ the mathematic expression is defined as 10sb/a.

Results and Discussion

Total and free phosphorus determinations

The results obtained for the total P concentration (P_{total}) in the digested samples or measured directly in the suspension are shown in Table 1. When evaluating the correlation between P_{total} concentrations in the suspension ($C_{P\text{-}susp}$) and in the digested sample ($C_{P\text{-}dig}$) through the equation $C_{P\text{-}dig} = (1.01 \pm 0.01)\ C_{P\text{-}susp} - (2.89 \pm 1.63)$, it was evidenced the resemblance between these two procedures by the obtained slope (a = 1.01) and Pearson's r coefficient (r = 0.9999) (Figure 2). The values from each sample treatment were statistically compared and revealed no significant difference at 95% confidence level, since the experimental *t*-value (t_{exp} = 2.92) was lower than the critical value ($t_{critical}$ = 3.18). This similarity between these two procedures is in accordance with previous results from other authors.[16,17] For example, Akpinar-Bayizit[17] verified that the direct analysis of pomegranate juice suspensions for several mineral species, including phosphorus, were consistent with the results provided by other studies.[30-32] In a similar way, Szymczycha-Madeja and Welna[16] compared different sample treatments prior determination of the mineral composition of fruit juices by ICP OES, and demonstrated that there were no significant differences at 95% confidence level between the results obtained by analyzing the suspension as compared to sample digestion.

Table 1. Concentrations of P_{total} (suspension and digestion) and P_{free} in juice samples

Sample	P concentration / (mg L^{-1})		
	P_{total}		P_{free}
	ICP OES		Spectrophotometry
	Digestion	Suspension	
S1	104 ± 9	104 ± 1	47.7 ± 3.4
S2	119 ± 7	112 ± 5	56.8 ± 4.9
S3	24.0 ± 8.9	21.0 ± 1.1	18.9 ± 3.3
S4	274 ± 28	269 ± 6	81.2 ± 6.9

Based on these results, the suspension procedure was considered more suitable for further analysis since it is more representative concerning original conditions of the sample and also possess several other advantages including less reagents consumption, less waste generation, lower sample manipulation, and thus, a significant increase of the sampling rate. Besides, digestion procedure presented lower reproducibility as evidenced in all samples as compared to confidence intervals of suspension data, especially in sample S3.

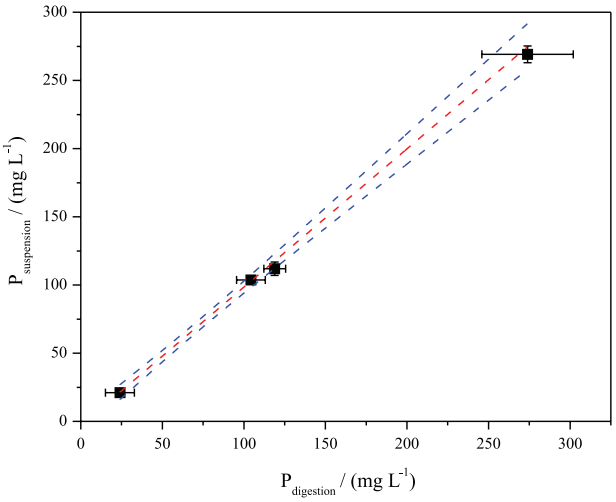

Figure 2. Correlation of digestion and suspension procedures for P_{total} determination in orange juice samples by ICP OES.

The P_{total} concentrations in the orange juice samples analyzed in the present study were comparable to the ones found by other authors in several other fruit juice samples (Table 2). According to this comparison, it is confirmed that P_{total} concentrations in the other fruit juice samples varied from 6.6 to 190 mg L^{-1} and, in the case of orange juice, this range was within 0.2 and 269 mg L^{-1}. The phosphorus levels of fruit juices depend on the nature of the fruit, the mineral composition of the soil from which it is originated, the composition of the irrigation water, the weather conditions, and the agricultural practices, such as the types and amounts of fertilizers used, among other factors.[39]

As for the chemical composition of orange juices, there were significant differences found in the labels for the different brands analyzed. The concentration of proteins, carbohydrates and vitamins containing phosphorus were different from one sample to another, which is in good agreement with the wide range of P_{total} concentration found in our study (Table 1). For example, some of the analyzed juice were enriched with vitamins whose composition contains phosphorus, like phosphate riboflavin, which is an additive used for food coloring, and pyridoxine, which also contains phosphorus in organic form in its structure.[40]

It must also be considered the addition of different amounts of polyphosphates to commercial orange juice, which is used to stabilize vitamin C; as well as the presence of ions such as Zn^{II} and Ca^{II}, which might precipitate as their respective phosphates.[5] All these features can contribute to differences in the total P_{total} concentration, thus making complicated to predict or to establish the exact concentration range of this element in industrialized orange juices.

Results of P_{free} concentrations in orange juice samples are presented in Table 1. According to these results,

Table 2. Comparison of phosphorus concentrations obtained in different juice samples after analysis by different techniques

Sample	P fraction	Concentration range / (mg L^{-1})	Analytical technique	Reference
Orange juice	P_{total}	170.0-190.0	spectrophotometry	33
Apple juice	P_{total}	46.4-80.6	ICP OES	34
Orange juice		152.8-220.0		
Lemon derivatives		6.6-69.3		
Orange juice		158.0		
Brazilian orange juice	P_{total}	119.0-190.0	ICP AES	35
Orange juice	P_{total}	197.0-209.1	ICP OES	36
Apple juice	P_{total}	75.0	ICP OES	16
Grape juice		136.0		
Orange juice		235.0		
Pineapple juice		71.0		
Orange juice	P_{total}	0.2-94.5	ICP OES	37
Orange juice with milk	P_{free}	52.1-168.1	ion chromatography	38
Pineapple, apple and grape juice with milk		51.8-90.8		
Fruit juice with soy		145.4-428.3		
Orange juice	P_{free}	19.0-81.0	spectrophotometry	this work
Orange juice	P_{total}	21.0-269.0	ICP OES	this work
	anionic fraction	15.5-67.8		
	cationic fraction	< LOD-2.1		
	neutral fraction	< LOD-1.5		

LOD: limit of detection; ICP OES: inductively coupled plasma optical emission spectrometry; ICP AES: ICP atomic emission spectrometry.

P_{free} represented a range from 30 to 90% of P_{total} content in the analyzed juices, with a directly proportional relationship in matter of concentration, as shown in Figure 3a. However, Figure 3b evidenced an inverse and exponential relationship between concentration and percentage of P_{free}, probably due to the formation of precipitates with low solubility such as calcium and zinc phosphate, since these ions are present in some commercial orange juices. Besides, aggregation of P_{free} to suspended particulate matter, proteins, carbohydrates and vitamins present in juices can occur, thus changing the relation of P_{free} and P_{bonded} content.

Distribution of phosphorus fractions

Fractioning of phosphorus based on its particle size

Phosphorus fractioning according to particle size was estimated based on the P_{free} content obtained with the molybdenum blue method. The results obtained (Table 3)

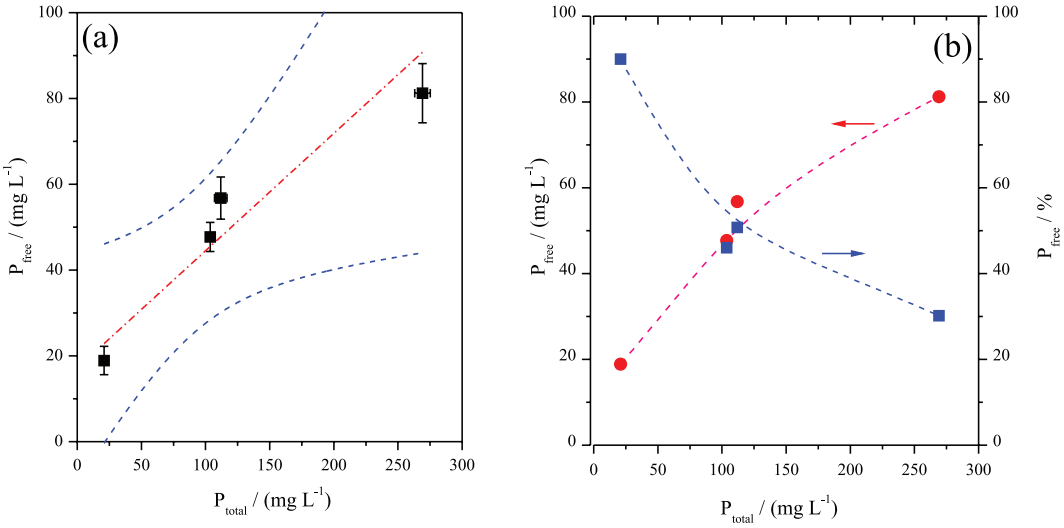

Figure 3. Correlations of phosphorus fractions in orange juice samples: (a) P_{total} and P_{free} concentration; (b) P_{total} concentrations and P_{free} percentage.

evidenced that a range from 71 to 93% of P_{free} present in orange juices had diameter below 0.45 μm. So the fraction of P_{free} with diameter above this size varied from 7 to 29% according to the brand and the composition of juice, once different brands make use of different kinds of additives and some of these compounds have phosphorus in their composition or can react with P_{free} so that some aggregates can be formed.

Table 3. P_{free} fractions in juice samples according to particle diameters

Sample	P concentration / (mg L^{-1})		Variation / %
	Without filtration	Filtered with 0.45 μm	
S1	67.8 ± 1.0	56.3 ± 2.0	83.03
S2	90.7 ± 2.1	64.4 ± 2.1	71.00
S3	29.0 ± 0.7	27.0 ± 0.7	93.10
S4	89.9 ± 6.7	66.4 ± 3.8	73.86

Fractioning of phosphorus based on its charge

For determining the fractions of phosphorus contained in the juices, as a function of their charges, firstly, an aliquot of each diluted sample was analyzed by ICP OES and the result for P_{total} was used as primary reference. Then, other aliquot of each diluted sample was filtered through 0.45 μm membranes and also analyzed by ICP OES for determination of P_{total}. This information about P_{total} was used as a reference (100%) for evaluation of the fractions distribution of this element depending on the charge and interaction with both columns employed in the solid phase extraction (SPE) procedure.

After percolating through the columns I and II, the effluents obtained were analyzed by ICP OES for phosphorous determination in the fractions neutral, cationic and anionic, respectively.

It is worth mentioning that solid phases employed in this work (C_{18} and Dowex 50WX8) have already been reported in the literature for retention of neutral and cationic fractions, respectively. The C_{18} phase has been reported for retention of neutral species such as antibodies and proteins in biologic samples;[41] and carotenoids in algae.[42] Cationic resins such as Dowex 50W were already employed for retention of Fe^{III}, Mg^{II}, Ca^{II} and Zn^{II} in milk samples;[29] and Mn^{II} in samples of wine.[43] Thus, the phosphorus fraction analyzed in this work is really is in the anionic form.

Table 4 shows the results obtained for P_{total} concentrations in diluted samples, diluted and filtered, and effluents I and II, besides the percentages of neutral and anionic fractions obtained after the interaction between the sample and the solid support of each column. According to this table, as we compared P_{total} content of filtered sample with the just diluted one, it was shown a maximum retention of 26% of P in particulate matter with size larger than 0.45 μm (sample S4). This retention is probably due to the way in which phosphorus is distributed in samples, either in organic compounds such as vitamins, carbohydrates and phosphate proteins,[40] or adsorbed in suspended particles like aggregates; thus acquiring size larger than the cut-off porosity of the membrane used for filtration. By comparing P concentrations in both effluents (I and II), it was possible to calculate the percentage of cationic fraction of each sample obtained from the difference of P_{total} concentration. The distribution of these fractions is shown in Figure 4.

According to the obtained data, we can infer that there was no interaction between phosphorus species of sample S4 and column I (C_{18}, neutral). For the other samples, the interaction was confirmed since the range obtained was up to 8.9%. On the other hand, the cationic fraction varied up to 8.8%, indicating that part of phosphorus contained in juice is bonded to species or macro aggregates of positive charge.

The high percentages of P in the anionic fraction (from 91.2 to 95.9%) are related to free inorganic phosphate in equilibrium with its protonated forms, due to the acid characteristic of orange juice, whose pH varied between 2 and 3. These data are in agreement with previously published results, in which phosphorus was mostly found in its inorganic form in food samples, either by natural occurrence or by inclusion as additive in the food industry.[44]

Table 4. Phosphorus distribution in juice samples according to the charge of fractions

Sample	P concentration / (mg L^{-1})				P percentage / %	
	Diluted	Diluted and filtered	Effluent I (C_{18} column)	Effluent II (Dowex column)	Neutral fraction	Anionic fraction
S1	38.7 ± 1.3	32.6 ± 4.1	32.0 ± 2.0	30.8 ± 6.6	1.84	94.48
S2	88.8 ± 4.9	71.1 ± 3.6	69.9 ± 3.2	67.8 ± 2.8	1.69	95.47
S3	16.9 ± 4.4	16.9 ± 2.3	15.4 ± 2.2	16.2 ± 0.4	8.88	95.86
S4	23.0 ± 4.0	17.0 ± 0.8	17.0 ± 3.2	15.5 ± 1.3	< LOD	91.18

LOD: limit of detection.

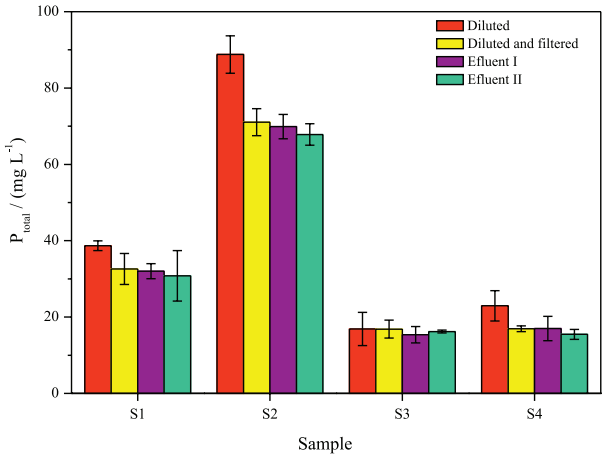

Figure 4. Phosphorus distribution present in fractions of orange juice.

Thus, it can be concluded that the P contained in industrialized orange juice samples is mostly present in its inorganic form, and because of that, it is more assailable after consumption, giving this food great functionality on the human diet. On a basis of the daily-recommended intake of phosphorus for an adult (700 mg *per* day), the average content of P_{free} present in the analyzed orange juice samples represents 1.6% considering the intake of 200 mL of juice.

Conclusions

This study applied different strategies of sample treatment to evaluate phosphorus distribution in industrialized orange juice samples. At first, procedures of digestion and suspension analysis were compared for P_{total} determination and the obtained data revealed no significant difference at 95% confidence level. So, suspension was selected for further analysis.

The concentration of P_{free} was directly proportional to the concentration of P_{total}, varying from 30 to 90% in relation to the total content. Meanwhile, percentage of Pfree was inversely proportional. Meanwhile, concentration and percentage of P_{free} were inversely proportional.

In the fractioning step, it was verified that most of the phosphorus present in juices were associated to particulate matter with size under 0.45 µm. Therefore, this study allowed the unprecedented comparison among P_{total} concentration and their fractions in industrialized orange juices with the highest phosphorus percentages in the anionic fraction, which is related to the inorganic form of this element.

It is worth mention the use of molybdenum blue method which, although is restricted to P_{free} determination, presents the advantage to be applied to previous evaluation of phosphorus availability, since this is the assailable form of this element by the organism.

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