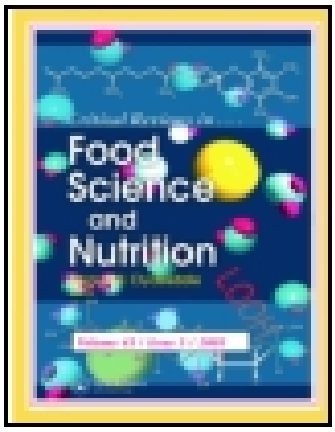

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Statistical approaches to assess the association between phenolic compounds and the in vitro antioxidant activity of Camellia sinensis and Ilex paraguariensis teas

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Statistical approaches to assess the association between phenolic compounds and the *in vitro* antioxidant activity of *Camellia sinensis* and *Ilex paraguariensis* teas

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Abstract

Tea presents a diverse phenolic composition which is responsible for its alleged biological activities, including the *in vivo* and *in vitro* antioxidant capacity. It is very usual to find researches applying univariate statistical methods, such as analysis of variances (ANOVA) and linear Pearson correlation coefficients to analyze the strength of correlation between phenolic composition and the *in vitro* antioxidant activity of teas from *Camellia sinensis* (green, black, white, oolong, red, and yellow teas) and *Ilex paraguariensis* (Yerba mate), which are the most produced and consumed types of teas. However, evidence has shown that these approach are not as suitable as multivariate statistical approaches once it does not depict nor show association among all results and variables simultaneously, making it difficult to understand clearly the data structure and patterns. Then, the objective of this work is to review and explain some univariate and multivariate statistical techniques used to assess the association between phenolic compounds and the *in vitro* antioxidant activity of green, white, black, red, yellow, and oolong and Yerba-mate teas. Moreover, this paper provides an overview on some assays used to estimate the *in vitro* antioxidant capacity of teas.

Keywords: Correlation analysis, chemometrics, flavonoids, cluster analysis, PCA, Yerba mate.

1. Introduction

Recent epidemiological studies have shown that some age-related diseases occur because of the oxidation of cells because of the reaction between cell components and free radicals/reactive species. Antioxidant compounds may be an effective protection of body cells by scavenging these reactive species or chelating pro-oxidant metals, such as iron (Halliwell & Gutteridge, 2006). Once there is a constant formation of nitrogen, carbon, and oxygen reactive species on account of aerobic metabolism, the cells tend to respond to the oxidation promoted by such reactive species by increasing the activity of catalase, glutathione peroxidase, and superoxide dismutase (Xu et al., 2012). In many cases, this endogenous protection may not be sufficient to buffer the free radicals and other reactive species produced in the body. Hence, the consumption of foods rich in antioxidant compounds, such as red wines, fruit juices and teas, should be included in the regular diet.

Tea, the most widely consumed beverage aside from water, is an infusion prepared from dried leaves of many species such as *Camellia sinensis* O. Kuntze (Theaceae) and *Ilex paraguariensis* (Aquifoliaceae), and has been considered a source of health-promoting phenolic compounds (Moderno et al., 2009). Tea has a worldwide *per capita* consumption of approximately 40 liters per year and three types of tea are predominantly manufactured: black, green and oolong tea (Steele et al., 2000). However, Yerba-mate tea, an infusion made from the leaves of *Ilex paraguariensis* widely consumed in South America (especially in Uruguay, Brazil, Argentina, and Paraguay) is gaining a rapid introduction into the world market, including the

United States of America and many countries in Europe, not only due to its health-promoting benefits but also because of its sensory properties (Heck & de Mejia, 2007).

Several *in vitro* and clinical protocols have suggested that different types of teas may exert cancer protective effects on humans. For example, Steele et al. (2000) concluded that extracts from black and green teas successfully inhibited neoplastic transformation in mouse mammary organ cultures, rat tracheal epithelial cells and human lung tumor epithelial cells, showing thus strong evidences of anti-mutagenic, anti-proliferative and anti-neoplastic. Carvalho et al. (2010) found that green tea extract strongly inhibited the growth of renal carcinoma cell lines, corroborating the fact that green tea is an effective anticancer beverage. Ramirez-Mares et al. (2004) verified that Yerba-mate tea inhibited 65% of human antitopoisomerase II activity whereas this inhibition was about 15% for green tea. De Mejia et al. (2005) verified that the consumption of Yerba-mate extracts at concentrations higher than 375 μg extract/mL inhibited the growth of oral cell carcinoma. Therefore, teas from *C. sinensis* and *I. paraguariensis* are suitable alternatives for consumers interested in phenolics health benefits.

It is widely accepted that phenolic compounds present a considerable antioxidant capacity both in *in vivo* and *in vitro* protocols, and the correlation analysis between variables is usually carried out by univariate statistical techniques, such as the Pearson linear correlations (Berté et al., 2011). However, multivariate unsupervised statistical techniques, such as Principal Component Analysis (PCA) and Hierarchical Cluster Analysis (HCA), also seem to be suitable techniques to assess the association among individual and total phenolic compounds and the antioxidant capacity of beverages with high antioxidant power such as teas and wine, once by using these approaches it is possible to compare all results simultaneously, highlighting the

similarity and association among samples and experimental techniques (Pękal et al., 2012; Deetae et al., 2012; Macedo et al., 2012).

Based on these considerations and taking into account that there is an increasing number of publications regarding the antioxidant capacity of teas, juices, beers and other beverages, the objective of this work is to review and explain some statistical techniques used to assess the association between phenolic compounds and the antioxidant activity of green, white, black, red, yellow, and oolong (*Camellia sinensis*) and Yerba-mate (*Ilex paraguariensis*) teas. Moreover, this paper provides an overview on four assays widely used to estimate the *in vitro* antioxidant capacity of teas.

1.1. Some insights regarding C. sinensis and Yerba-mate teas

More than half of the total world's tea production is consumed in the countries where it is produced, which is the case of India, the world's largest producer of tea and the world's biggest consumer. Whereas tea is the most common beverage for 2/3 of the world's growing population, there is a great potential for an increasing demand. In accordance with Wu and Wei (2002), around 78% of the total amount of tea produced and consumed in the world is black tea, while around 20% is composed by green tea and less than 2% is oolong tea. According to Mejía et al. (2010), the size of the tea market reached up to $6.8 billion in 2005, while in 2010, about 4.1 million tons of different types of tea were produced worldwide. The demand for green tea has increased and its market is estimated to grow with a compound annual growth rate of more than 10% in the next five years. In Germany, for example, the average consumption of tea is around 26 liters per capita per year, and the most consumed teas are black and green teas (Deutscher

Teeverband, 2011). In Brazil, the most consumed types of tea are Yerba-mate, followed by black and green teas. Overall, the five countries that consume a greater quantity (kg/person/year) of tea are United Kindom, Ireland, New Zealand, Japan and Australia (NationMaster, 2012).

One possible reason to the growth of the tea market is the increased awareness of the health-promoting effects from bioactive phenolic compounds, such as anticholesterolemic (Chen et al., 2001), anticancer (Kuroda & Hara, 1999), anti-inflammatory (Bacquer et al., 2006), hepatoprotective (Xu et al., 2012), anti-mutagenic (Yen & Chen, 1994), anti-atherosclerotic activities (Kawai et al., 2008), and also by reducing the blood pressure and improving parameters related to insulin resistance (Bogdanski et al., 2012), reducing the risk of developing Parkinson's disease (Prakash, & Tan, 2011), and also by improving the plasma antioxidant activity in healthy fasted subjects (Silva et al., 2008). Other clinical, *in vitro*, *in vivo* and epidemiological evidences of functionality of teas against malignancies have been extensively revised by Tijburg et al. (1997), Higdon & Frei (2003), Butt & Sultan (2009), and Huang et al. (2012). These beneficial effects are believed to be mainly due to the antioxidant activity attributable to phenolic compounds, especially flavonoids and phenolic acids.

During the commercial production, leaves of *Camellia sinensis* undergo different degrees of processing, giving rise to various types of teas (Carter et al., 2007). In accordance with Kim et al. (2011), the infusions came from the genus *Camellia* can be divided into three categories based on the fermentation process: green tea (unfermented), oolong tea (partially fermented), and black tea (fully fermented). Herein, the aerial parts of Yerba-mate (*I. paraguariensis*) are used to prepare a tea-like beverage, the so called *mate*. In accordance with Fraga et al. (2000), mate-based beverages may be prepared by two different ways: i) by the simple addition of

boiling water to the dry plant material; ii) by repeated additions of close to boiling water to the dry plant material. Both preparations extract almost all water-soluble bioactive compounds.

Green and Yerba-mate teas are blanched, in which green tea leaves are steamed or pan-fried and Yerba-mate leaves are flash-heated over open flame. This process deactivates polyphenol oxidase and avoids prolonged fermentation, what is in contrast to the production of black teas, where the leaves are allowed to wither and ferment, oxidizing the polyphenols to form dimerized compounds, namely catechins to theaflavins (Heck & de Mejia, 2007; Hara, 2001). The reddish-black color of black tea, its reduced bitterness and astringency, and the low perception of leafy and grassy flavor are derived from the oxidation process, giving black tea a marked distinction as compared to green tea (Cheng, 2006). White tea is composed only by the bud or first leaves that are plucked, being the least processed tea, as it is directly dried after harvesting and suffers no fermentation. In this way, the delicate white leaf hairs are left intact, leading to a pale yellow-colored infusion (Hilal & Engelhardt, 2007). The production of yellow tea is similar to green tea's production, but instead of immediate drying after fixation, the leaves are stacked, covered, and heated in a humid environment. This condition propitiates the chlorophyll oxidation by means of non-enzymatic and non-microbial reactions, which results in a yellowish or greenish-yellow color.

The major difference between green tea and Yerba-mate is the drying method, in which green tea is dried by a fast and high temperature drying air, retaining the fresh leaves characteristics and developing their characteristic flavor. Yerba-mate is dried very slowly, usually using wood smoke, developing new flavor characteristics and contributing to changes in the chemical makeup and physical appearance (Heck & de Mejia, 2007). Semi-fermented oolong

tea is generally fermented from 20% to 60% to avoid green tea's characteristic (leafy and grassy notes) while obtaining black tea's sweet and bold flavor. Black tea is more popular in North America and Europe, whereas oolong tea is more produced and consumed in China (Leung et al., 2001).

Teas, such as green/black/red/yellow and Yerba-mate, contain a considerable amount of catechins, which are flavonoids that present considerable antioxidant-related effects. There are several catechin present in *Camellia sinensis* and Yerba-mate leaves, but the major tea catechins are (-)-catechin, (+)-epicatechin, (-)-epigallocatechin, (-)-epigallocatechin gallate, (-)-epicatechin, and (-)-epicatechin gallate (Song et al., 2012). Whereas catechins are the most abundant polyphenols in green tea, the typical pigments in black tea are theaflavins, thearubigins and theabrownins, which are derived from the oxidation of catechins during the fermentation process (Leung et al., 2001). Out of these tea varieties, green tea has been proven to present the highest amount of flavonoids and total polyphenols as compared to oolong, black, and Yerba-mate teas (Vinson, 2000; Berté et al., 2011).

In order to avoid losing ground to soft drinks, the tea industry is actively trying to promote consumption by emphasizing tea's health benefits and also demonstrating that ready-to-drink teas are healthier than soft drinks (OXFAM, 2002). Another key-point to increase the consumption of teas is the development of 'exotic teas', which are a mixture of a tea with fruit juices and herbal extracts. This technological innovation has been applied to many types of teas in order to dissipate negative off-flavors and to enhance aroma and fresh taste.

1.2 Overview on some in vitro *assays to evaluate tea's antioxidant activity*

The antioxidant activity of teas has been demonstrated in *in vitro* and *in vivo* biological systems, and researchers usually find a direct and positive correlation between antioxidant activity and the content of phenolic compounds, especially flavonoids (Granato et al., 2011; Macedo et al., 2012). Antioxidants can buffer free radicals mainly by three mechanisms: hydrogen atom transfer, electron transfer and combination of both mechanisms (Prior et al., 2005). Following this, the hydrogen atom transfer mechanism measures the ability of an antioxidant compound, gallic acid for example, to quench free radicals by hydrogen donation. The electron transfer measures the ability of antioxidant compounds to transfer one electron to reduce free radicals, pro-oxidant metals and carbonyls (Huang et al., 2005). In accordance with Prior et al. (2005) and Huang et al. (2005), Ferric Reducing Antioxidant Power (FRAP) is an electron transfer assay, while oxygen radical absorbance capacity (ORAC) is a common hydrogen atom transfer assay and the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay combines both hydrogen atom transfer and electron transfer mechanisms.

There are many *in vitro* antioxidant assays available to evaluate the bioactivity of teas, such as ferrous ion chelating activity (Wang et al., 2012), lipid peroxidation inhibition assay (Naithani et al., 2006), deoxyribose assay (Chen et al., 2005), photoreduction of nitro blue tetrazolium assay (Chen et al., 2005), superoxide dismutase mimetic activity (Naithani et al., 2006), assay of hydroxyl and superoxide radicals scavenging activity (Xu et al., 2012), among others. A detailed review on these methods is accurately described by Alves et al. (2010). However, the most used methodologies are: DPPH, ORAC, FRAP and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid). These methods differ among each other in relation to the

principles of the chemical reaction, the target molecule, the way to express the results, the pH of the medium, reaction time, among others. Moreover, once many chemical mechanisms and multiple reactions are involved, no single method will accurately reflect the antioxidant capacity of complex matrices, such as teas. It is necessary, therefore, to assess the *in vitro* antioxidant capacity of teas by using different assays that use distinct mechanisms, principles, and chemical reagents. It is also important to take into consideration the polarity of the antioxidant compounds present in the food matrix, that is, the methods used to assess the antioxidant capacity should be able to measure the antiradical capacity of both water-soluble and hydrophobic compounds.

DPPH assay: DPPH is one of the most used assays to determine the electron transfer between the DPPH radical and the antioxidant compound(s) present in the medium because of its low-cost and also because it does not require sophisticated equipments. This assay is based on the ability of antioxidant to scavenge the DPPH cation radical, determining the hydrogen donating capacity of the antioxidant, and does not produce oxidative chain reactions nor does not react with free radical intermediates. This method is highly dependent on the experimental conditions employed, such as the final concentration of the extracts, the concentration of the DPPH solution, the incubation time, and the solvent used for the DPPH solution (Dudonne et al., 2009).

In this assay, the 2,2-diphenyl-1-picrylhydrazyl radical (purple), which absorbs in a wavelength of 517 nm, is reduced and a yellow compound (2,2-diphenylpicrylhydrazine) is formed, decreasing thus the absorbance (Brand-Williams et al., 1995) (Figure 1A). Then, it is possible to determine the percentage of antioxidant activity and/or the remaining DPPH radical present in the medium (Sousa et al., 2007). In recent studies regarding the DPPH method,

Sharma and Bhat (2009) and Hartwig et al. (2012) stated that one drawback of the comparison of data obtained by this assay is due to different experimental conditions employed by different laboratories, such as the DPPH concentration (4 – 500 μm), presence and intensity of light, reaction time (5 to up 1440 min), solvent (ethanol, methanol, toluene, methanol buffered solution), and pH of the final mixture. These conditions hinder the comparison the data obtained in different laboratories and point out the need for standardization of this assay.

ORAC assay: The ORAC assay system uses an area-under-curve technique and thus combines both inhibition time and inhibition degree of free radical action by an antioxidant into a single quantity. The ORAC assay measures the peroxyl-scavenging activity of compounds/extracts (Huang et al., 2002). This method involves thermal degradation at 37^{o}C, and carbon-centered radicals are generated by the azo-initiator (usually 2,2'-azobis-2-amidinopropane-dihydrochloride - AAPH), where peroxyl radicals (ROO$^{\bullet}$) are formed from the breakdown of AAPH. Peroxyl radicals can oxidize fluorescein probe (3',6'-dihydroxy-spiro[isobenzofuran-'[3H],9'[9H]-xanthen]-3-one) to generate products without fluorescence or react directly with the antioxidant compounds, suppressing thus the fluorescein oxidation by a hydrogen atom transfer mechanism, which preserves the fluorescence measured at 485 nm excitation and 525 nm emission (Zenbio, 2008). This behavior persists until the antioxidant is totally consumed, at which point the fluorescein probe is rapidly oxidized (Granato et al., 2010b).

The concentration of antioxidants present in the food matrix is directly proportional to the fluorescence intensity through the course of the assay (Zenbio, 2008). The data are summarized over the time and, usually, the area under the curve is integrated by software. This is then

compared to a chemical standard, usually Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid – water-soluble vitamin E analog), and is expressed as micromoles of Trolox equivalents (TE) per gram or per milliliter of sample. The advantages of this technique are: a) once fluorescein is used as a measure of oxidative damage, there is little interference of colored substances in the reading, which makes ORAC assay an important approach to measure the antioxidant capacity of high-intensity colored foods/beverages, such as wines, beers, and teas; b) the use of temperature of 37°C for both the formation of free radicals and peroxyl radical reaction between fluorescein and antioxidants; c) the use of a pH value close to that one found in humans (pH 7.1 to 7.4), mimicking thus one of the most important physiological conditions in human's metabolism; d) ORAC may be used to measure the antioxidant activity of water-soluble and also lipid-soluble compounds, pure compounds, and complex food matrices. One drawback related to the application and implementation of the ORAC method is the high cost of the automated microtiter reader, which may limit the use of such an assay.

FRAP assay: The FRAP assay is a direct method used to measure the total antioxidant capacity of many types of samples, such as food extracts, beverages, pure chemical standards, and even blood samples (Benzie & Strain, 1996). In this assay, the reduction of Fe(III), as part of the ferric tripyridyl-s-triazine (Fe(III)–TPTZ) complex to Fe(II) is measured using a spectrophotometer at 593 nm, where the antioxidant substance acts as reductant (Figure 1B). The experimental conditions favor the reduction of the complex and, thereby, color formation, provided that an antioxidant is present. In practice, it is considered a simple, accurate, reproducible, robust and direct method of assessing the antioxidant capacity of samples both in

in vitro (Schinella et al., 2009) as well as in plasma of patients subjected to clinical intervention (Benzie et al., 1999) and also in *ex vivo* experiments (Silva et al., 2008).

ABTS assay: The ABTS method measures the capacity of a compound to scavenge the dark blue/green $ABTS^{\bullet+}$, resulting in a light blue/green or even colorless product (depending on the concentration of antioxidants). The degree of discoloration can easily be followed with a spectrophotometer at a maximum wavelength of 734 nm and represents the measure for the amount of $ABTS^{\bullet+}$ scavenged by antioxidant compounds present in the food matrix (Re et al., 1999), as illustrated in Figure 1C. In this sense, the extent of discoloration, expressed as percentage inhibition of the ABTS radical, is determined as a function of concentration and time and calculated the reactivity of tea extracts is usually compared to a pure phenolic compound or even with Trolox, using the same experimental conditions (Miller et al., 2006). The method may be used to assess the antioxidant power of both water-soluble and lipid-soluble compounds, pure chemicals, food extracts, and beverages such as wine, beer, juices and teas. One drawback related to this method is that the ABTS radical cation is reactive towards most antioxidants, not only the phenolic compounds present in tea but also ascorbic acid.

One important factor that should be stressed here is that a great part of studies are limited to the determination of the total phenolic compounds according to the Folin–Ciocalteu colorimetric method (Singleton, & Rossi, 1965), which is based on the development of blue colored product due to reduction of tungstate and/or molybdate in Folin Ciocalteu's reagent (FCR) due to the presence of reductants in the medium, resulting a blue colored product (measured at 725 nm). However, as well known, other substances such as ascorbic acid may react with FCR and the results will be overestimated, not reflecting the true content of phenolic

compounds in the samples. One possible and advantageous option to overcome this experimental limitation is to assess the phenolic composition of teas by using chromatographic methods, such as high-performance liquid chromatography (HPLC) with chemiluminescence or coulometric array detection, reversed-phase LC with UV absorbance detection, capillary electrophoresis, among others. More details about these techniques may be found in a review performed by Ananingsih et al. (2012). By comparing the real concentration of individual polyphenols with the antioxidant capacity of teas is a much better approach to understand the mechanisms of action and also to provide valuable information about the bioactivity of teas.

2. Statistical approaches to evaluate the antioxidant capacity of teas

2.1 Univariate correlation between two continuous variables

Correlation is a method of analysis used to study the possible association between two continuous variables. The correlation coefficient (r) is a measure that shows the degree of association between both variables. Although correlation studies are extremely useful, they do not imply a cause-effect relationship between the variables, once other covariants may contribute to the response. One example of this situation is as follows: a researcher in China studied the antioxidant capacity measured by the ORAC assay of 130 samples and correlated to the content of (+)-catechin and total flavonoids, obtaining high and significant ($p < 0.05$) correlation coefficients – $r = 0.89$ and $r = 0.97$, respectively. If another researcher in another laboratory,

using the same experimental conditions and reagents, perform the same assays, other different correlation coefficients and significance values may be obtained.

Usually, the method for measuring the linear correlation between two quantitative variables is Pearson's *r* or product moment correlation coefficient. This parametric correlation coefficient measures the degree of linear association between the values of two variables (Y and X), and its value will be in the range ±1. The closer |*r*| is to 1, the stronger is the linear correlation between the variables (Ellison et al., 2009). The correlation between two variables is positive if higher values of one variable are associated with higher values of the other and negative if one variable tends to be lower as the other gets higher. A correlation around zero indicates that there is no linear relation between the values of two variables. A low *r* value does not necessarily mean that there is no relationship between variables, and one possible reason for this low value can be stated: there may be a non-linear correlation between these variables or there may be outliers present in one or both data sets (Altman, 1999). A simple way to check the type of correlation between two variables is performed by visual inspection of the "dispersion diagram". It is important to interpret the *r* value in conjunction with a plot of the data.

In accordance with Altman (1999), the linear correlation coefficient may be calculated for any two data sets; however, there are some important assumptions for using this coefficient: the variables involved must be random, all the observations must be independent and it is preferable that both variables present approximately normal distribution. This is essential for small samples (≤ 30); when the number of samples is high (> 30) the importance of normal distribution is decreased. This is justified by the Central Limit Theorem for multivariate distributions presented by Wichern and Johnson (1988). According to Snedecor and Cochran (1980), in practice, often

bivariate distribution of interest is far from following a normal distribution. If both variables do not follow a normal distribution, as formally tested by Shapiro-Wilk, Lillifors, Anderson-Darling or Kolmogorov-Smirnov tests, data can be transformed (root square or log transformation, for example) or a non-parametric approach, such as the Spearman´s rank correlation coefficient, can be used. A summary on how to use Spearman or Pearson correlation coefficients is demonstrated in Figure 2.

The Pearson's linear coefficient correlation between two variables X and Y is denoted by *r* and given by Equation 1:

$$r = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}} \qquad \text{(Eq. 1)}$$

where x_i and y_i are the values of X and Y for *n* observations. Consider the example: one analyst determined in triplicate the catechin content (mg/L) of a mixture of 9 types of green, black and white teas and also measured the antioxidant capacity of such products by using the ORAC assay (mmol TE/L), and obtained the following mean results:

- Catechin content = 2.55; 3.08; 4.28; 5.39; 0.25; 6.75; 8.69; 9.25; 10.08
- Antioxidant Activity = 60.37; 65.28; 72.34; 79.68; 30.36; 71.27; 90.16; 90.20; 90.30

Initially, data were checked for normality by using the Kolmogorov-Smirnov test and the p-values for the catechin content and ORAC values were 0.7696 and 0.6016, showing the results follow a normal distribution once $p > 0.05$. By using Equation 1, the analyst does not require computer software to perform the analysis and check for association between the catechin content and the antioxidant activity of teas. Indeed, the correlation coefficient was found to be

+0.9209 and, taking into account that the correlation coefficient may be comprehended between -1 and 1, the obtained value may be considered high, meaning that the higher the content of catechin, the higher the ORAC values of teas. But the correlation coefficient solely is not the only parameter to be analyzed. Rather, the significance and strength of correlation must be stated

With regard to strength of correlation, there is no consensus about the qualitative assessment of correlation coefficients. In this sense, Callegari-Jacques (2003) stated that the correlation coefficient can be assessed qualitatively as follows: if $0.00 < r < 0.30$, there is a weak linear correlation between variables; if $0.30 \leq r < 0.60$, there is a moderate linear correlation; if $0.60 \leq r < 0.90$, there is a strong linear correlation; and if $0.90 \leq r < 1.00$, there is a very strong linear correlation between both data sets. Similarly, with slight differences, Granato et al. (2010a) established to analyze the strength of correlations between variables using the following criteria: perfect ($r = \pm 1.0$), strong ($\pm 0.80 \leq r < \pm 1.0$), moderate ($\pm 0.50 \leq r < \pm 0.80$), weak ($\pm 0.10 \leq r < \pm 0.50$), and very weak correlation ($\pm 0.10 \leq r < \pm 0.01$).

As mentioned before, not only the correlation coefficient should be calculated but also its significance, which is a useful attribute that indicates how unlikely a certain correlation coefficient occurs given no relationship in the population. There is a simple test for significance of the null hypothesis of no association which is based on the *t* distribution. The null hypothesis of this test is that there is no association between variables (i.e. zero correlation), and it can be shown that the quantity $r\sqrt{\frac{n-2}{1-r^2}}$ follows a *t* distribution with n - 2 degrees of freedom. Thus the null hypothesis of no association may be tested by looking this value up in the table of the *t* distribution or by checking p-value (usually it needs to be lower than 0.05). From a practice

standpoint and experience, it is usual to obtain a strong correlation coefficient but with no significance ($p > 0.05$) when there is a small number of samples analyzed ($n < 10$). On the other hand, it is easy to achieve significance of a correlation, but the analyst should pay attention to the strength of the correlation to determine if the relationship explains the data well. Consider the example given above and taking into account that $r = 0.9209$ and $n = 9$, the calculated t would be $t = 0.9209\sqrt{\frac{9-2}{1-0.8480}} = 6.2500$, and the critical (from the Student-t table, for 7 degrees of freedom is 1.895 at a 95% of confidence. Therefore, once the calculated t is higher than the t displayed on the Student-t table, it is possible to reject the null hypothesis and state that the correlation coefficient is regarded as statistically significant. Indeed, if the analyst calculates the p-value for this example, a $p < 0.0001$ will be attained. In this situation, the analyst can confirm the positive and significant effect of catechin on the antioxidant capacity of teas.

Besides Pearson correlation, which is virtually a parametric test, non-parametric correlations, such as the Spearman correlation coefficient, should be calculated for data sets that do not present a normal distribution, when the sample size is small, or when the variables are measured at an ordinal level. Unlike Pearson's correlation coefficient, the Spearman coefficient does not require the assumption that the relationship between variables is linear, even it does not require that the variables are measured in class intervals. Spearman´s ρ (rho), which is a measure of non-parametric correlation between two data sets, evaluates an arbitrary monotonic function that can be used to describe the relationship between two variables, without making any assumptions about the statistical distribution of variables.

It is important to emphasize that rank correlations are not being interpreted at the same way as Pearson's correlation. The coefficient of Spearman ρ ranges between -1 and 1. The closer

|*r*| is to 1, the greater is the association between variables. The negative sign of the correlation means variables which vary in the opposite direction, i.e., higher categories of one variable are associated with lower categories of the other variable. A Spearman correlation close to zero indicates that there is no tendency for Y to either increase or decrease when X increases.

Spearman´s rank correlation coefficient is obtained by ranking the values of each of the two variables. The simplest way to get ρ is to calculate Pearson's *r* on the ranks of the data. There is an alternative approach which assumes that there are no ties in the data set. For each of *N* subjects being studied, the difference in the ranks, d_i, is calculated and Spearman´s rank correlation coefficient is given by Equation 2:

$$\rho = 1 - \frac{6\sum_{i=1}^{n} d_i^2}{N^3 - N} \qquad \text{(Eq. 2)}$$

where *n* = number of pairs ordination.

The null hypothesis for this test is that there is no association in the population (i.e. zero correlation), and it can be shown that for large samples ($n > 30$) the quantity $\rho\sqrt{\frac{n-2}{1-\rho^2}}$ follows a *t* distribution with n-2 degrees of freedom.

Univariate linear correlation has been used to study the relationship between polypheonlic concentation and antioxidant activity of teas, measured by different *in vitro* methods. Here we list and discuss some examples:

Kodama et al. (2010) quantified the total phenolic compounds, epigallocatechin, epicatechin gallate, epigallocatechin gallate, and gallocatechin in green tea infusions prepared using tea bags or dry leaves and the *in vitro* antioxidant activity was determined by using the

DPPH and ORAC assays, and results were subjected to linear correlation analyzes. Authors did not find a high correlation (r = 0.58; n = 13) between total phenolic content and DPPH assay, while no correlation (r = 0.06) was found between ORAC and total phenolics. When the samples were analyzed separately (teabags or ready-to-drink samples), a suitable linear correlation between DPPH and total phenolics was found for teabags (r = 0.81; n=6) and ready-to-drink samples (r = 0.97; n = 4). Authors did not perform correlation analysis between the flavonoids quantified by high performance liquid chromatography and the antioxidant capacity assays. It is important to mention that correlation analysis performed should contain, at least, 5 pairs of data in order to identify a trend (Altman, 1999).

Song et al. (2011) analyzed the linear correlation between the antioxidant activity measured by ORAC and the total phenolic content measured by the Folin-Ciocalteau method (r = 0.8840; n=5) and also between ORAC and the total content of proanhocyanidin (r = 0.9908; n=5) in yellow *Camellia* tea. These data indicate that proanthocyanidins are major compounds responsible for scavenging peroxyl radicals as measured by the ORAC technique. It is important to note that in linear correlation analysis, the probability value (p-value) for each pairwise correlation coefficient should be provided, once the significance is also dependent on the number of samples present in the data set. This is an important factor to be evaluated because, in many cases, the correlation coefficient is high (> 0.80) but the model shows a weak or no significance ($p > 0.05$).

Kerio et al. (2013) evaluated the total polyphenolic content, catequin profile and *in vitro* antioxidant capacity of black and green tee products from purple leaf coloured tea cultivars. Several correlations between the composition of the teas and their antioxidant capacity were

reported. Total polyphenolic content was significantly correlated with antioxidant activity in both green (r = 0.844; $p \leq 0.01$; n = 30) and black tea products (r = 0.797; $p \leq 0.01$; n=30). In green tea products, anthocyanins, and particularly cyanidin-3-O-glucoside, had a significant role in their antioxidant activity, as shown by the high and significant correlation coefficient between its concentration and antioxidant activity (r = 0.843; $p \leq 0.01$; n = 30). In black tea products antioxidant activity was highly correlated to the concentration of peonidin (r = 0.732; $p \leq 0.01$; n = 30), cyanidin-3-O-glucoside (r = 0.764; $p \leq 0.01$; n = 30), and cyanidin-3-O-galactoside (r = 0.748; $p \leq 0.01$; n = 30). Similarly, Luximon-Ramma et al. (2005) reported high correlation coefficients between total proanthocyanidin and antioxidant capacity measured by TEAC (r = 0.96) and FRAP (r = 0.95) in Mauritian black teas.

Jayasekara et al. (2011) reported a weak correlation (r = 0.5) between FRAP antioxidant capacity and total phenolic content (r = 0.5) for fermented and nonfermented Sri Lankan teas. However, when correlations between total phenolic content and DPPH antioxidant capacity were calculated results depended on the type of tea considered. Correlation was significant ($p < 0.05$) for fermented teas but no significant for nonfermented teas (r = 0.1). These results suggest that polyphenols were a major contributor to the ferric reducing capacity of fermented and nonfermented teas but were only a major contribution to the antioxidant capacity of fermented Sri Lankan teas. These authors also reported no significant correlation between FRAP and DPPH (r = 0.2, $p > 0.05$, n = 20), suggesting that the mechanisms involved in reduction of ferric ions and radical scavenging activity were different.

YerbaFilip et al. (2000) induced the oxidation of liposomes from egg yolk by incubation (37°C, 60 min) with 10 mM of AAPH (which generates free radicals in the aqueous

phase) in the presence or the absence of different Yerba mate aqueous extracts (*Ilex* spp), and thiobarbituric acid-reactive substances (TBARS) were quantified, while caffeoyl-derivatives were estimated by using a spectrophotometer. A significant ($p = 0.0005$) correlation was observed between the antioxidant activity and the content of caffeoyl-derivatives of the mate extracts ($n = 8$; $r = 0.94$) and authors also used linear regression analysis to propose a mathematical model to fit the results (antioxidant activity = 0.07 + 1.89 x caffeoyl-derivatives content).

Grujic et al. (2012) determined the content of chlorogenic acid, total phenolics, flavonoids, and antioxidant activity (DPPH assay) in Yerba mate tea extracts ($n = 9$) obtained by ethanol extraction and liquid carbon dioxide extraction with ethanol (co-solvent). Authors correlated, using linear regression analysis, the chemical composition with the antioxidant activity of the extracts and verified that, besides not providing any mathematical equation to explain the experimental data, a correlation coefficient of 0.85 for total phenolic compounds and a correlation coefficient of 0.74 for total flavonoids were attained. No information about correlation between DPPH and the content of chlorogenic acid was provided.

The antioxidant activity measured by the ABTS assay of aqueous extracts from 23 Bulgarian plants (including *Alchemilla vulgaris*, *Sambucus ebulus*, *Mentha spicata*, and *Fragaria vesca*) was studied in relation to their polyphenol content in comparison with mate, black tea, and honeybush (Kiselova et al., 2006). A positive correlation ($r = 0.92$) between antioxidant activity and polyphenol content was attained, corroborating the fact that polyphenols are one of the main responsible for the *in vitro* antioxidant activity of plant extracts. From this example, it is possible to observe that authors did not mention the significance, expressed by the

p-value, of the correlation. It is widely accepted that the significance of a correlation coefficient is a primary source of information about the reliability of the correlation and, therefore, but be stated with the calculated r.

The total oxyradical scavenging capacity, antiproliferative activity, total phenolic compounds, and total flavonoid compounds of seven different types of green, oolong, and black teas marketed in bags was investigated by Yang and Liu (2012). Authors expressed the correlation coefficient as R^2 (and not as *r*) of 0.9755 for the antioxidant capacity of teas and the total content of phenolics, and a R^2 = 0.9350 for the correlation between total oxyradical scavenging capacity and the flavonoid content of teas. It is important to be consistent when correlation analysis is performed, that is, the correlation coefficient, *r*, expresses the degree of association between two data sets, and the coefficient of determination, which is correctly expressed by R^2, should only be stated when regression analysis is performed.

The total content of polyphenolics, flavonoids, theaflavins, individual catechins content, metal chelating activity, and antioxidant activity measured by ORAC, LDL-oxidation, and ABTS assays of a white, two black and two green teas were assessed by Carloni et al. (2012). The metal chelating activity did not correlate with the *in vitro* antioxidant activity. On the other hand, the total phenolic compounds and total flavonoids significantly correlated with the antiradical capacity of teas measured by the ABTS method ($R^2 = 0.871$, $p = 0.000$ and $R^2 = 0.438$, $p = 0.007$, respectively). Note that authors provided the coefficient of determination instead of the correlation coefficients, *r*, which are 0.9333 and 0.6618, respectively. This is a common mistake researchers often make to express the experimental results, and therefore, attention should be paid when correlation coefficient is calculated. As previously mentioned, the

correlation coefficient represents the linear relationship between two data sets, and if this coefficient is squared, the obtained value, R^2, is called coefficient of determination. The R^2 represents the proportion of common variation in the two variables and is usually expressed in regression analysis.

In a study performed by Kim et al. (2011), the antioxidant activity of green (not fermented), oolong (partially fermented), and black (fully fermented) tea infusions was measured by ORAC and data were subjected to linear correlation analyses. The results showed that the ORAC data correlated well with the total content of phenolic substances (r = 0.79), with (-)-epigallocatechin gallate (r = 0.81), with (-)-epigallocatechin (r = 0.81), with (-)-epicatechin (r = 0.87), with (-)-epicatechin gallate (r = 0.82), and with total flavonol glycoside (r = 0.79). Authors concluded that a reduction in antioxidant activity was associated to a longer fermentation process and to differences in concentration of polyphenols present in green and black teas. Regarding this example, Multiple Linear Regression or Principal Component Analysis would be more appropriate to assess the association among all data at the same time, in case there is a distinction between independent and dependent variables.

Not only *in vitro* studies use correlation analysis to support the conclusions regarding the relationship between polyphenolic content of tea and its antioxidant capacity, but also clinical interventions. In this regard, Benzie et al. (1999) evaluated the antioxidant capacity of plasma and urine, measured by the FRAP method, after green tea ingestion in healthy adults. Authors obtained a significant correlation between urinary FRAP and urinary total phenolic concentrations, namely reducing ability (r = 0.845, $p < 0.001$). On the other hand, no significant

($p > 0.05$) correlation was found between antioxidant activity of urine and plasma in the group that consumed water.

Although linear correlation analysis is a useful tool for both checking the degree of linear dependence between two random sets of experimental results (antioxidant capacity assay *vs* phenolic composition, for example) and also for indicating a predictive relationship that can be exploited in practice, many scientists still have not widely used this statistical approach to analyze their data; that is, in many cases, authors isolate and quantify the content of individual phenolic compounds and also measure the antioxidant capacity by means of diverse methods, presenting thus a large amount of experimental results. However, in many cases, authors do not use any correlation method to verify how variables are in line with another, which would invariably support the data from chemical analyses in a statistically stand point of view and thus complementing their findings. It is more common to observe scientists concluding their work based on analysis of variance (ANOVA) followed by a *post hoc* multiple comparison mean test (Tukey, *t*-Student, Duncan, Fisher LSD, etc) within tea samples/groups rather than using correlation analysis (Naithani et al. 2006; Chan et al. 2007; Berté et al. 2011). In this case, it is highly recommended that multiple linear correlation analysis be used when more than two variables are assessed.

It is widely accepted that the antioxidant activity of food products and also teas as measured by *in vitro* methods is affected by cultivation conditions, soil chemistry, environmental conditions, variety of the plant material, pathogenesis, collection of plant materials, processing operations, packaging systems and storage conditions (Manzocco et al., 1998; Naithani et al., 2006). Once more ready-to-drink teas have been launched in the marketplace, it would be

interesting to conduct studies that check for association between the storage time and the loss in phenolic composition and also in total antioxidant capacity of tea samples. In this case, a simple linear correlation analysis, measured by the Pearson correlation coefficient, may be a suitable option when few variables (< 5) are evaluated. However, when more than 5 variables are assessed, a multivariate statistical approach is suggested to explain all experimental data simultaneously, showing not only the association among all response variables but also a multivariate comparison of all samples on a factor-plane.

2.2 Association among three or more variables: the use of unsupervised pattern recognition methods

Most studies regarding the antioxidant capacity of teas obtain data for many response variables by using simple analyses (linear correlation or ANOVA procedures) instead of using more sophisticated statistical methods. In general, it is preferable to use more advanced methods to assess the association among three or more response variables than checking separately at several parts of all data set (the case of linear correlation coefficients) (Altman, 1999). A general guide to analyze experimental data regarding phenolic composition and the antioxidant activity of teas is presented in Figures 3 and 4. Figure 3 describes the statistical methods to analyze tea samples by performing descriptive and inferential tests, while Figure 4 describes the steps to take when the analyst has more or less than 10 variables by means of a chemometric approach, such as by using Principal Components Analysis and Cluster Analysis.

Cluster analysis comprises a wide range of exploratory multivariate statistical procedures which aim at identifying homogeneous groups within a data set. It is basically an unsupervised

classification of objects or variables, according to their similarities and differences (Mardia et al., 1979). In the context of evaluating the antioxidant activity of teas this methodology is useful for identifying tea samples with similar antioxidant capacity, based on a wide range of measurements, usually results from different *in vitro* and/or *in vivo* methods for measuring antioxidant capacity. The application of this type of technique would allow the classification of teas based on their antioxidant capacity, allowing consumers to select those with the highest potential positive effect on their health.

The most popular type of cluster analysis is called agglomerative hierarchical, which aims at identifying a series of groups, named clusters, within a nested hierarchical structure (Jacobsen & Gunderson 1986). It starts by considering that each object is a separate cluster and then sequentially merges clusters according to their similarity until only one cluster is left (Burns & Burns 2009).

When working with variables in different units (as polyphenolic content and antioxidant capacity measured using different methods) the first step of the analysis is to standardize the dataset by centering the variables to mean zero and scaling to unit variance (Jacobsen & Gunderson, 1986). Then, the degree of similarity or dissimilarity between each pair of objects is calculated. Different distance measures can be used, being Euclidean distance the most common (Tan et al., 2005). If objects are considered as points in a coordinate space such and their degree of difference is estimated by calculating the geometric distance between them, using an extension of an extension of Pythagoras' theorem:

$$d_{ij} = \sqrt{\sum_{k=1}^{K} (x_{ik} - x_{jk})^2} \qquad \text{(Eq. 3)}$$

where d_{ij} is the Euclidean distance between clusters i and j, x_{ik} is the value of variable x_k for cluster i, x_{jk} is the value of variable x_k for cluster j, and K is the total number of variables in the dataset.

After the distances are calculated the clusters are merged according to the distance between them. In agglomerative hierarchical clustering every sample is considered as a cluster at the beginning of the procedure. Then, clusters are successively merged. In the first step of the procedure the two samples with the smallest distance are merged first into a cluster. Once two samples or clusters have been merged, the next step is to calculate the distance between the new and the original clusters using a clustering procedure or amalgamation rule (Jacobsen & Gunderson 1986). One of the most popular clustering procedures is average linkage, which calculates the distance between two clusters as the average distance of all possible pairs of objects from different clusters (Jacobsen & Gunderson 1986). This procedure continues until all samples belong to only one cluster.

Results of agglomerative hierarchical clustering are represented graphically in a dendrogram (Næs et al., 2010), which provides a simple visualization of the hierarchical structure of the clustering and the level at which each cluster is formed, as well as cluster membership (Jacobsen & Gunderson 1986). The main steps for performing hierarchical cluster analysis are summarized in Figure 5.

When studying the influence of domestic culinary processes on the antioxidant capacity of green tea infusions, Samaniego-Sánchez et al. (2011) applied hierarchical cluster analysis for identifying groups of samples with similar antioxidant capacity. The authors prepared the teas using different procedures, extraction temperatures and times. The author used hierarchical

cluster analysis on data from Trolox Equivalent Antioxidant Capacity (TEAC) in the ABTS assay to classify samples based on their extraction temperature and time. Two main groups were identified: one was composed of teas prepared at 70ºC for 5 min, 80ºC for 3 min and 80ºC for 5 min, which showed the highest antioxidant capacity. A second group comprised green teas prepared at 70ºC for 1 min, 80ºC for 1 min, 70ºC for 3 min, 80ºC for 3 min, 80ºC for 5 min, 90ºC for 1 min, and 90ºC for 3 min. These last preparation conditions were similar with respect to their antioxidant potential, which could be explained by a decrease in antioxidant capacity due to poor extraction during preparation (e.g. teas prepared at 70ºC for 1 min or 3 min) or to the negative effect of temperature (e.g. teas prepared at 90ºC).

Nakamura et al. (2009) used cluster analysis together with linear correlations to assess the chemical composition (triterpene saponins, methylxanthines, chlorogenic acid) and the antioxidant activity measured by the DPPH assay. These authors verified that cluster analysis separated the samples with similar characteristics while the levels of caffeine (r = 0.23; n = 8; p > 0.05) and theobromine (r = -0.01; n = 8; p > 0.05) were not significantly correlated with DPPH.

Wang et al. (2011) used high-performance liquid chromatography (HPLC) to determine a fingerprint of the polyphenolic compounds of 25 samples of oolong tea. Hierarchical cluster analysis was used on 36 peak values of the HPLC fingerprints. Samples were sorted into three main clusters according to their origin. Samples from Guangdong province were clearly different from the rest. Samples from Fujian and Taiwan were more similar and were sorted into two clusters. Samples from Fujian (southern) formed as separate cluster, whereas samples from Fujian (northern) and Taiwan were grouped.

When dealing with large datasets, as it could be the case when measuring the concentration of different polyphenolic compounds in a set of tea samples, it is usual difficult to visualize the most important relationships between variables and samples. In these situations, it is useful to apply methodologies that extract the most information and display it in a low-dimension space which is easily interpreted. Principal component analysis (PCA) is a multivariate exploratory technique which is widely-used for this purpose (Mardia et al., 1979). Through a reduction in the number of dimensions PCA allows the representation of variables and samples in simple plots with a lower number of dimensions (Piggott & Sharman, 1986). If the measured variables are highly correlated it is possible to replace them by smaller number of dimensions without losing a high amount of information (Næs et al., 2010). The new dimensions can be used to create plots in which the original variables are represented as dimensions, whereas samples are represented as points in the space. These plots allow the identification of correlations between the variables, relationships between samples and associations between samples and variables (Piggott & Sharman, 1986).

PCA is applied on a data matrix in which each column represents a variable and each row represents a sample and the intersection of a column and a row has the average value of the variable for the sample (Næs et al., 2010). Then, the center of the coordinate system is moved by subtracting the average of each variable across all samples to all values of the variable. Then, the direction with the largest variance is identified, which corresponds to the first principal component (PC1), i.e. the dimension that contains the largest amount of information. PC1 is simply a linear combination of the variables (Jonhson & Wichern, 1998; Rencher, 1998). The following step is to identify the second principal component (PC2), which is an orthogonal

dimensions to PC1 that has the largest variance. The procedure continues until all the variance of the original dataset is explained (Næs et al., 2010). The maximum number of principal components is the minimum between the number of samples and the number of variables (Piggott & Sharman, 1986). Principal components correspond to a new coordinate system in which variables and samples are projected (Næs et al., 2010).

Dominguez-Perles et al. (2011) evaluated the functional properties and chemical composition of beverages developed with organic green tea and broccoli and data were subjected to linear regression analysis and PCA. Interestingly, authors verified that the antioxidant capacity measured by the DPPH method was inversely correlated to the contents of synaptic acid derivatives ($r = -0.752$; $n = 7$; $p < 0.05$), chlorogenic acid derivatives ($r = -0.731$; $n = 7$; $p < 0.05$), total flavonoids ($r = -0.752$; $n = 7$; $p < 0.05$), and total phenolic compounds ($r = -0.758$; $n = 7$; $p < 0.05$), whereas the cathechin content and the DPPH results were positively correlated ($r = 0.733$; $n = 7$; $p < 0.05$). Principal Component Analysis showed that the first 2 principal components (PC) explained up to 90% of the total variability, and the first PC correlated positively with the total phenolic compounds, chlorogenic acid derivatives, flavonoids and sinapic acid derivatives, while the second PC was associated with differences in DPPH scavenging capacity and catechins. These variables were correlated significantly with each other (by using linear correlation) and the PCA also confirmed the correlation among all variables.

Deetae et al. (2012) evaluated the relationship between total phenolic compounds and total non-flavonoid compounds, and the antioxidant capacity measured by the ABTS and FRAP assays of different types of teas, including green, black and oolong teas, by using univariate

(Pearson linear correlation) and chemometrics (PCA and HCA). Authors verified that the antioxidant assays correlated well to the content of phenolic compounds ($r > 0.88$; $n=18$). Additionally, by using PCA, authors were able to explain up to 96% of the total variance in experimental results. In this analysis, total polyphenolic content, total flavonoid content, ABTS antioxidant capacity, FRAP antioxidant capacity and anti-advanced glycation end products were highly correlated to each other and to the first component of the PCA, suggesting that flavonoids strongly contributed to the antioxidant capacity of the teas. On the other hand, total non-flavoniod content and metal chelation capacity were mainly correlated to the second and third principal components, being not correlated to the total antioxidant capacity of the teas. Meanwhile HCA (using all response variables) was able to group the teas according to their antioxidant capacity and to characterize stevia tea in the same cluster of green/black tea, whereas sappan tea was grouped with oolong tea.

When studying the polyphenolic composition of oolong teas, Wang et al. (2011) used PCA to identify relationships between 25 samples from different sources. Sample representation in the three first PC showed that samples could be divided into three groups: samples from Fujian southern, samples from Fujian northern and Taiwan, and finally samples from Guangdong. These results were identical to that obtained using hierarchical cluster analysis.

Heck et al. (2008) used PCA and multiple linear regression to study the effect of growing location (forest or plantation), average annual rainfall, average annual temperature, elevation, country of origin and drying method (hot air or wood smoke) on phenolic concentration of 15 mate teas. The five first PCs accounted for a total of 85.4% of the total variance of the data. The first PC was correlated to growing location, whereas the second component was positively

correlated to elevation and negatively correlated to temperature. Total phenolic concentration was negatively correlated with rainfall. Using multivariate linear regression the authors showed that plantation grown mate teas exhibited higher concentration of polyphenols as compared to forest grown mate teas, which was explained by the positive effect of sunlight on the synthesis of polyphenolic compounds.

2.3 Statistical packages used in correlation analyses

In order to analyze experimental data, there are some free (the well-known is *R*, and Action for Microsoft Excel) and commercial statistical packages, such as SAS (*Statistical Analysis Software*), SPSS (*Statistical Package for Social Science*), Statistica, Statgraphics, Minitab, Design-Expert, Prisma, among others. Among these, Minitab and Statistica are the most used packages once both have friendly interface, although Statistica seems more complete and has a magnific graphics output. Action software, developed by Brazilian scientists, is also free to download. This software has also suitable graphics output and is the first statistical system that utilizes the *R* platform together with Microsoft Excel.

3. Final Remarks and Perspectives

Tea is a recognized source of antioxidant compounds, especially flavonoids and phenolic acids, and many worldwide scientific groups have studied the bioactivity of different types of teas, especially the antimutagenic, antioxidant, antimicrobial, and antidiabetic activities. In this

regard, statistical methods may be useful to help the analyst to understand how the biological activity is correlated to the phenolic composition of tea. The use of univariate linear correlation as formally determined by the Pearson and Spearman products are the most widely used and employed in all types of experimental designs. However, here we presented the importance, potential applications and examples of researchers who have associated the antioxidant capacity of teas from *Ilex paraguariensis* and *Camellia sinensis* teas with the phenolic composition (total and individual phenolics) by using chemometrics. The advantages of using Cluster Analysis and Principal Component Analysis are related to the visualization of similarities of all samples, in accordance with previously determined responses, and also to group samples with similar characteristics. These features are not displayed neither provided by using univariate statistics and, therefore, these multivariate unsupervised statistical methods should be more frequently used.

4. References

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Table 1: Summary of some statistical approaches used in Food Science and Technology

Statistical Method	Type of approach	Positive points	Limitations	Recommended number of samples and/or variables	References
Correlation analysis (Pearson's product)	Univariate	It allows the analyst determining the strength and direction of a relationship between 2 variables, and then further studies help narrowing the findings. It is easy to work out and it is easy to interpret the results.	Pearson correlation is unduly influenced by outliers, non-normality, and nonlinearity. It requires a Normal distribution for each data set, which is somewhat difficult to observe in practice. Correlation is not and cannot be taken to imply causation and therefore it cannot provide a conclusive reason for why there is a relationship.	At least 5 samples for each data set and 2 variables.	Altman (1999)
Analysis of variance (ANOVA)	Univariate	It allows the analyst to compare means from independent groups of variables.	It requires a Normal distribution with constant variance for each group, which is somewhat difficult to observe in practice. This method is related to differences among samples rather than showing correlation among variables.	At least 5 samples for each data set and 3 variables.	Calado & Montgomery (2003)
Spearman's rank correlation	Univariate	The method does not require a Normal distribution of data sets. It is very useful when the analyst admits a nonlinear monotonic underlying relationship between variables. It is less sensitive to bias beause of the effect of outliers.	A significant correlation does not necessarily mean cause and effect.	At least 5 samples for each data set and 2 variables.	Altman (1999)

Linear Regression analysis	Univariate	Enables to find a reasonable relationship between the input variable and output through empirical relationships. It is very useful for prediction, estimating parameters or fit a model to data values and make inference in the parameters.	If we are interested in the relationship of only one input variable with the response variable. It requires a Normal distribution for each data set, which is somewhat difficult to observe in practice. The mathematical relationship between variables must be linear; the error must have constant variance; not correlated and normal distribution with average = 0.	At least 5 samples for each data set and 2 variables.	Altman (1999); Ellison et al. (2009)
Multiple Regression Analysis	Univariate	The analysist may associate numerous (no more than 5, preferably) responses to explain one main variable, i.e, antioxidant capacity. This main response will be a function of multiple responses, and this can be observed in an equation $y = f(x_1, x_2, x_3,..., x_n)$, where x represents the responses.	It requires a Normal distribution of all data sets, which is somewhat difficult to observe in practice.	At least 3 variables: 1 dependent and 2 independent. At least 5 samples for each data set	Ellison et al. (2009)
Principal Component Analysis (PCA)	Multivariate	The scatter plot generated for samples is na easy way to observe how samples behave in relation to the responses. It allows the identification of patterns and assotiation among all variables and samples simultaneosly in a 2D graph. PCA is available in almost every statistical software.	The method requires a large set of samples and at least 3 response variables. When many samples are close to one another in the dispersion graph, it is hard to visualize trends and intrinsic differences.	At least five variables and five samples.	Mardia et al. (1979); Piggott & Sharman (1986)

Cluster Analysis (CA)	Multivariate	Enables to classify a set of samples or variables, according to their similarities and differences. Hierarchical CA is available in almost every statistical sofware.	CA imposes hierarchical structure on data, whether real or not, and it does not neither have a mechanism for differentiating between relevant and irrelevant variables nor depict data with multiple, independent underlying controls well. Once it is based on algorithms, solutions may be non-unique.	There is no rule about sample size necessary for CA. Small number of samples and high variable number: it is very difficult to find cluster structure in data, as data points are positioned in so many dimensions. It is recommended to use, at least, 3 variables and 2^k cases (k = number of variables), but preferably $5*2^k$.	Formann (1984); Mooi & Sarstedt (2011)

Figure 1: Example of a reaction between the DPPH radical (A), FRAP (B), and ABTS^{*+} radical (C) and a phenolic compound present in a tea extract (Modified from Rufino et al., 2006; 2007a; 2007b).

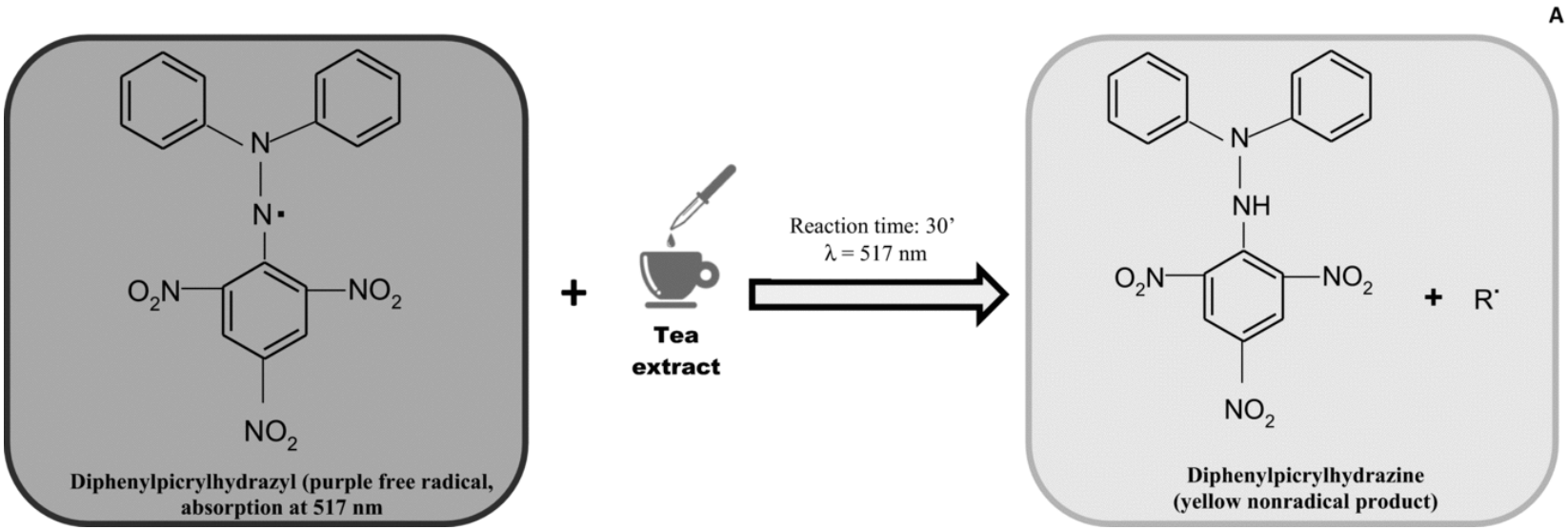

Figure 2: Steps to analyze a linear correlation between 2 response variables.

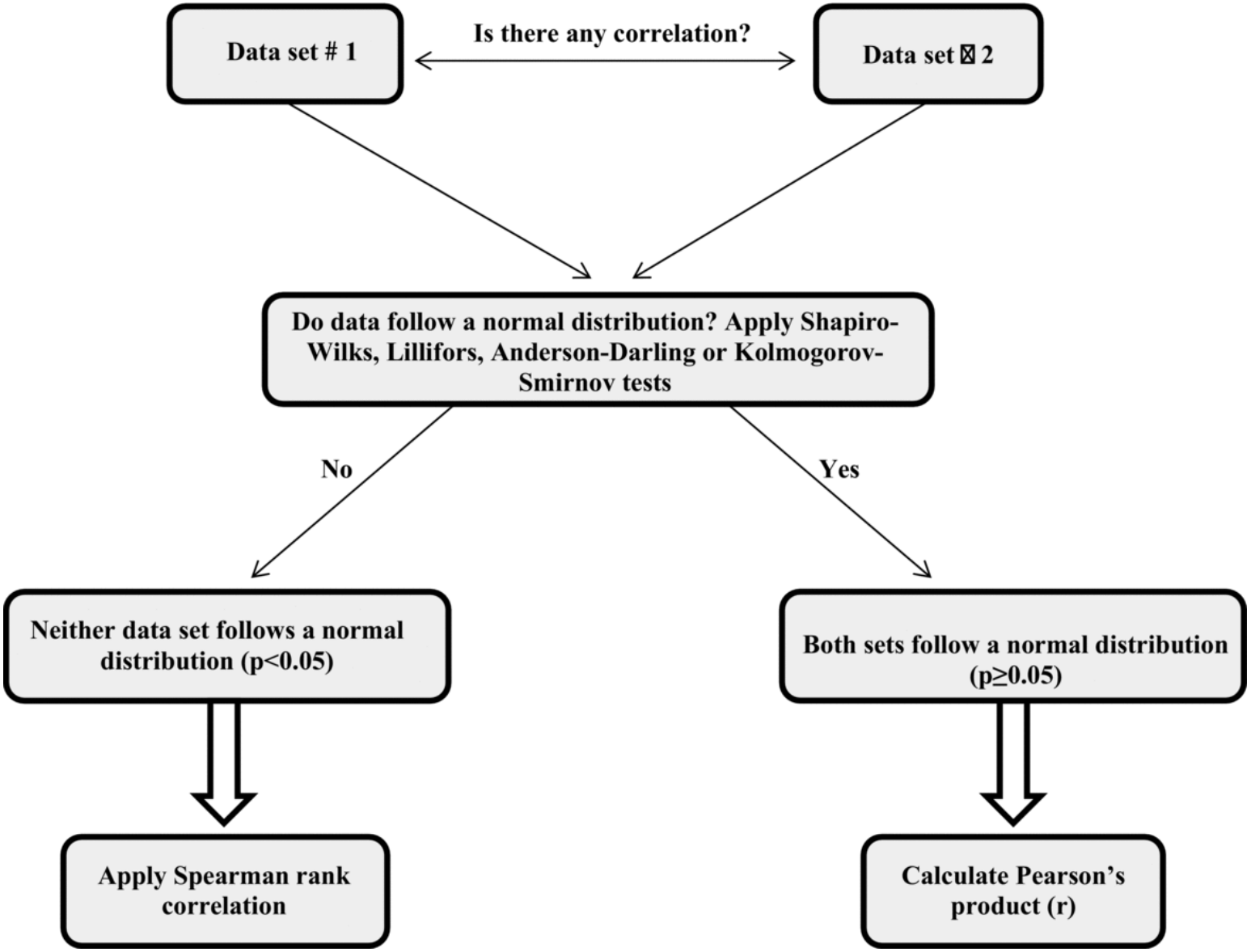

Figure 3: Descriptive and inferential statistical methods applied to analyze experiments regarding the antioxidant activity of teas.

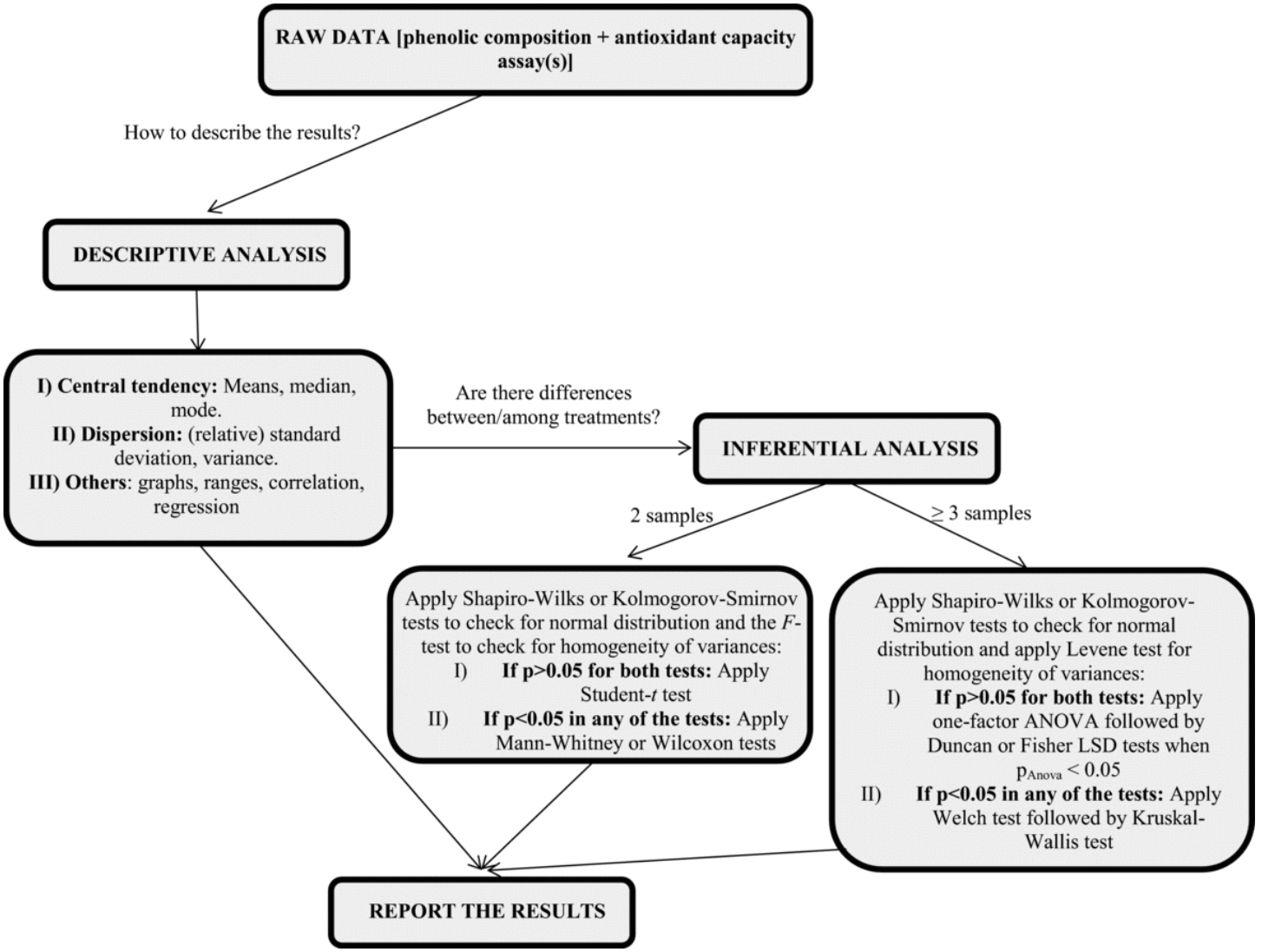

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Figure 4: Univariate and multivariate analysis of raw data.

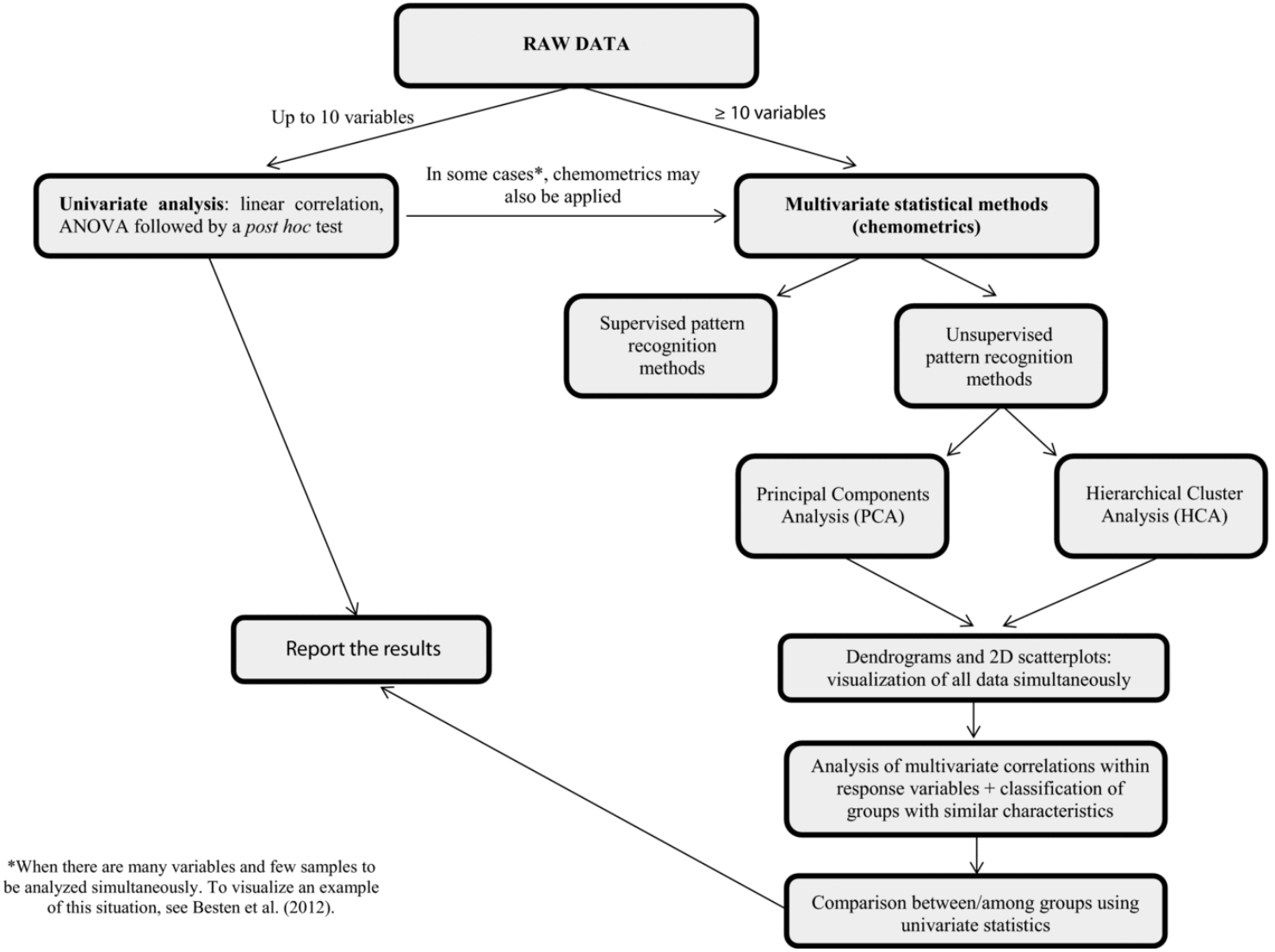

Figure 5: Recommended steps to perform Cluster Analysis. Modified from Mooi & Sarstedt (2011).