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ARTICLE TYPE

Evaluation of macro and microelement levels for verifying the authenticity of organic eggs by using chemometric techniques

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Abstract

Elemental determination was carried out on 60 egg samples (37 organic and 23 non-organic), with the goal of identifying significant differences between the two types of eggs for classification purposes. Inductively coupled plasma-mass spectrometry was used for determination of 19 elements, As, Ba, Ca, Co, Cr, Cu, Eu, Fe, K, Mg, Mn, Na, Ni, P, Rb, Se, Tl, V and Zn. As, Co, Fe, Mn, Rb, Se, Tl and V. Levels were found to be higher in organic *versus* non-organic eggs, while Cr and P levels were higher in regular *versus* organic samples. The remaining investigated elements exhibited statistically equivalent concentration levels in the two types of eggs. Principal component analysis (PCA) and soft independent modeling of class analogy (SIMCA) statistical techniques of the elemental fingerprints were readily able to discriminate organic from regular egg samples and can be used as an alternative method for adulteration evaluation.

Keywords: Chemometrics; ICP-MS; Multi-element fingerprinting; Organic food; Quality control; Eggs.

Introduction

Health and environmental concerns as a result of the extensive use of pesticides, hormones and veterinary drugs in farming practice have triggered a significant shift of consumers' purchasing habits to organic food products in the last decade. [1] Organic farming is seen as a viable alternative that can solve many problems associated with food production, environmental contamination, animal welfare and rural developments [2]. Since retail prices of organic products are usually higher than those of their regularly grown counterparts [3], the analytical approaches for verifying the authenticity of organic food products are increasingly important.

Analytical strategies for authentication of organic products usually implement fingerprint patterns of important components that describe compositional differences between organic and regular products [4-6] Useful markers vary with the type of product and also depend on whether the product is derived from plant or animal sources [7, 8]. The present study was concerned with the authentication of organic eggs. Previously reported compositional markers include stable isotopes of light elements (^{15}N and ^{13}C) [9-11], fatty acids [12-15], carotenoids [16, 17 18], fatty acids, cholesterol, and carotenoid content of the egg yolk [19], as well as fatty acid, cholesterol, vitamin A and E [20].

Inductively coupled plasma-mass spectrometry (ICP-MS) is routinely used in many research fields such as earth, environmental, life and forensic sciences and in food, material, chemical, semiconductor and nuclear industries.[21-23] Compared to graphite furnace atomic absorption spectrometry (GF AAS) or inductively coupled plasma optical emission spectrometry (ICP OES), this technique has some distinct advantages, including simultaneous multi-element measurement capability coupled with very low detection limits.[22-24] Moreover, it offers a wider linear dynamic range which allows the determination of major and trace elements within a single sample injection.[22-24] Additionally, compared to ICP OES, ICP-MS provides simpler spectral interpretation and isotopic information.[22]

The aim of this study was to investigate comprehensive elemental fingerprints as marker patterns to distinguish organic and non-organic eggs. Elemental fingerprints have previously been used for differentiation of crops[25, 26]. The analytical approach used here was based on ICP-MS due the advantages of this technique mentioned above. To our knowledge, only one study by Giannenas et al.[3] has previously applied ICP-MS to comparison of organic and regular eggs, but organic and regular eggs were not differentiated. Our work monitored trace levels of a wide range of 19 elements and

applied multivariate analysis techniques for interpretation of the mass spectrometric data; *viz.*, principal component analysis (PCA) and soft independent modeling of class analogy (SIMCA). We demonstrate that the combination of ICP-MS and chemometric algorithms provides a robust approach for comparison of egg samples and verifying the authenticity of organic eggs.

Experimental

Instrumentation

The determination of elements in egg samples was carried out with an ICP-MS instrument ELAN DRCII, CT, USA. High-purity argon (99.999%, White Martins, Brazil) was used throughout the study. The instrumental parameters and optimized conditions are given by Batista et al.,[27] and summarized in Table 1.

Reagents

With the exception of HNO_3, all chemicals were of analytical–reagent grade. HNO_3 was purchased from Synth (Diadema, Brazil) and it was purified using a quartz sub-boiling still (Kürner Analysentechnik, Rosenheim, Germany) before use. High purity deionized water (resistivity 18.2 MΩ cm) was generated with a Milli-Q water purification system (Millipore, Bedford, MA, USA) and used throughout. Aqueous solutions (1000 mg/L) of rhodium, iron, magnesium, zinc, copper, and multi-element (10 mg/L) standard aqueous mixtures were obtained from PerkinElmer (Shelton, CT, USA). Triton® X-100 and tetramethylammonium hydroxide solution (TMAH) 25% (w/v) in water were purchased from Sigma-Aldrich (St. Louis, USA).

Sampling and analytical procedures

Certified organic (n = 37) and non organic eggs (n = 23) samples were obtained from the Brazilian retail market. All organic egg samples were certified by the Brazilian IBD-Agricultural and Food Inspections and Certifications; i.e., a government entity that is accredited by the International Federation of Organic Agriculture Movements. Aliquots of egg samples were stocked in propylene metal-free Falcon® tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and freeze-dried (−80 °C) until further use.

The ICP-MS method proposed by Batista *et al*.,[27] was used to determine the following chemical elements in egg samples: As, Ba, Ca, Co, Cu, Eu, Fe, K, Mg, Mn, Na, Ni, Rb, Se, Tl, V and Zn. A solution of rhodium at 10 μg/L was used as internal standard. A full description of the composition of the sixty-one egg samples is provided in Table S1 (see Supplementary Material).

Data analysis

PCA and SIMCA were carried out using Pirouette (Version 3.11, Infometrix, Inc., Woodinville, WA, USA). Before applying PCA and SIMCA, all variables were "autoscaled". This procedure gives all variables the same importance. Their values were autoscaled by subtracting the average value from each variable and dividing the variable by its standard deviation. F-test and T-test,[28] assuming similar and different standard deviations, were calculated from rows 64-85 of Table S1 (see Supplementary Material).

During this cross-validation test, a sample was removed from the data set. The classification model was rebuilt and the removed sample classified in this new model. All the samples of the data set were sequentially removed and reclassified.[29]

Results and Discussion

Elemental concentrations in egg samples

Table 2 shows the general results obtained for the trace elements (macro and microelements) determined in organic and regular egg samples.

The F-test illustrates that Ba, Ca, Fe, Mn, Na and V standard deviations in organic and regular eggs were not significantly different and that the standard deviations of the remaining elements were significantly different.

The T-test, assuming similar standard deviations, showed that As, Fe, Mn and V concentrations were lower in regular than in organic samples, while there was no evidence that Ca, Na and V concentrations were different. The T-test, assuming different standard deviations, demonstrated that Cr and P levels in regular samples were higher than in organic samples, Tl, Co, Se, Rb levels were lower in regular than in organic samples, while there was no evidence that Mg, Cu, Zn, K, Ni and Eu concentrations in organic samples were different from those in regular samples and vice versa.

Contrary to a previous report from Vincevica-Gaile et al. [30], who found a wide range of concentrations in hen eggs grown in Latvian organic farms, Brazilian organic and regular eggs presented elemental compositions within narrow concentration ranges.

In a previous study from our research group, de Freitas *et al.*[31] determined trace element concentrations in commercial and free-range whole chicken eggs sold in different Brazilian regions. It was found a wider range of concentrations for ordinary and free-range eggs than in the present study.

de Freitas *et al.*[31] reported As levels in Brazilian egg samples ranging from 23 $\pm$2 to 21.5 $\pm$8.5 ng/g ($\pm$ is the standard deviation, SD) for ordinary and free-range egg samples, respectively. According to Codex Alimentarius Commission[32] the acceptable limit for this toxic element in eggs and their derivatives is 500 ng/g. In the present study, we found lower As concentration in a narrow range, 16 $\pm$0.2 and 23 $\pm$0.1 ng/g for ordinary and organic egg samples, respectively.

In battery eggs, the addition of essential elements such as Mo and Se to hen feed to improve the egg's shell quality, to decrease egg losses and to increase nutritional value, is common practice.[33-35] de Freitas et al. found higher Se level in ordinary egg samples than in free-range egg samples, 0.9 and 0.6 µg/g, respectively, while they found higher Mn level in free-range egg samples than in ordinary egg

samples, 1.3 and 1.5 μg/g, respectively. In this study, we determined Se and Mo levels comparable to those found by de Freitas, and Mo and Se levels higher for organic than for ordinary egg samples.

Interestingly, organic eggs also had higher As, Mn and Se levels than ordinary samples. However, Cr and P that are used in poultry feed, were in higher concentration in ordinary than in organic samples.[36-39]

Principal component analysis (PCA)

A dataset was obtained, which consisted of 60 samples (37 organic and 23 non-organic) and 19 variables (As, Ba, Ca, Co, Cr, Cu, Eu, Fe, K, Mg, Mn, Na, Ni, P, Rb, Se, Tl, V and Zn levels). The actual measurements can be arranged in a table or a matrix of size 61 × 19; this table is shown in Table S1 in the Supplementary Material.[40]

With 60 lines and 19 columns, obtaining a proper overview of the available information within the data set was difficult. PCA is a convenient statistical technique, however, providing new variables, which better explain the variation in the entire dataset.[40]

From the data, it is obvious that some variables were measured at much larger quantities than others. For example, K was present at μg/g levels, whereas As was seen in the ng/g range. If these difference of scale are not properly handled, then PCA will only focus on high concentration numbers.[40, 41] It is always desired to model all containing variables. There is a preprocessing tool called auto-scaling, which will adjust all columns to the same 'size', giving all variables an equal opportunity of being modelled.[40] Auto-scaling means that from each variable, the mean value is subtracted and then the variable is divided by its standard deviation. Thus, our data was auto-scaled before the PCA model was build.

The variables were reduced by a projection of the 19 samples (chemical element levels) onto a smaller number of new variables termed principal components (PCs). These were orientated and the first PC described as much original variation as possible between the objects. The extent to which each of the original variables is included in the PC is described by the loadings. By plotting the loadings for the two PCs, it is possible to assess the relative importance of each of the variables. [42] Thus, Fig. 1A (the loadings plot) shows that samples placed in the northwest (NW) quadrant have higher concentrations of As, Rb and V. Samples placed in the southwest (SW) quadrant exhibit higher concentrations of K, Mg and Na. Samples placed in the northeast (NE) quadrant have higher

concentrations of Ca, Co, Fe, Mn, Se and Tl. Finally, samples placed in the southeast (SE) quadrant exhibit higher concentrations of Ba, Cu, Cr, Eu, Ni, P and Zn.

The further the variable is from the origin, the more important it is. One can also visually determine correlations between parameters. Positive correlated-variables will be located close together, while inversely correlated-variables will be at 180° from each other.[42]

Table 3 shows the coordinates of each variable in Fig. 1. According to Table 3, the most important variables in PC 1 were Zn, P, Ca, Cu and Fe and the most important variable in PC 2 were As, Co, V, Tl, Mn and Fe.

In Fig. 1, in the SW quadrant, there was a correlation between Na, Mg and K levels. In the NW quadrant, a correlation between As, V and Rb was observed. The NE quadrant demonstrated a correlation between Co, Tl and Se and Mn, Fe, Ca, whereas the SE quadrant highlighted the correlation between Ni, Ba and Eu and Cu, P and Zn. In addition, As, V and Rb levels were inversely correlated to Ba, Eu and Ni levels, while Na, Mg and K were inversely correlated to Mn, Fe and Ca.

The projection of objects onto a PC is called a score. By plotting the scores of two PCs, one is able to visually differences and similarities between objects (i.e. egg samples). For the particular case of egg samples in a score plot, the distance between two samples depicts their similarity.

Fig. 1B illustrates the score plot, showing that organic and regular egg samples were separated into two classes. In Fig. 1B, organic samples fell into the top diagram, while regular samples were located at the bottom. Thus, it was concluded that the discrimination power was in the second principal component, PC2.

Outliers are samples that are somehow disturbing or unusual;[40] for example, samples with an extreme characteristic due to at least one atypical value of the measured parameters. In a statistical sense, outliers are samples from a different population than the data majority.[43] Sometimes, outliers are wrong samples. If such an outlier sample is not either corrected or removed, the subsequent analysis is fundamentally disturbed by this outlier.[40]

Outliers could be identified using a graph of sample residual *versus* Mahalanobis distance, as shown in Fig. 2. Samples falling outside one or both of the thresholds are potential outliers. Because the sample residual threshold is based on a 95% probability limit (set internally in the Pirouette programme), 5% of *normal* samples would be expected to fall outside that cutoff. Thus, sample C3#,

T12#, P4 and I5 were taken from the sample dataset because they fell outside the cutoff value. Then, the PCA model was applied using the 19 element levels and 56 egg samples (35 organic and 21 non-organic), as shown in Fig. 3.

The loadings plot (Fig. 3A) is different from that previously obtained in Fig. 1, because the outlier samples were taken out. In Fig. 3A, samples placed in the NW quadrant have higher concentrations of As, V, Co, Tl, Rb, Mn and Se. Samples placed in the SW quadrant exhibit higher concentrations of K, Mg and Na. Samples placed in the NE quadrant show higher concentrations of Fe, Ca, Cu, Zn and P. Finally, samples placed in the SE quadrant have higher concentrations of Ba, Cr, Eu and Ni. The importance of each variable in the figure is different from those previously shown in Fig. 1A and Table 3. The coordinates of each variable are shown in Table 4. According to Table 4, for PC 1, the most important variables are P, Zn, Cu, Ni, Ca and Cr, whereas for PC 2, the most important variables are Co, As, Ca, V, Mn, Fe, Tl and Se. In addition, we observe some correlations between the pairs Na-Mg, Ba-Eu, Cr-Ni and Zn-P.

PCA was also carried out using only variables (As, Co, Cr, Fe, Mn, P, Rb, Se, Tl and V), which had shown statistical differences between organic and ordinary samples; outlier samples (I5, P4, C3# and T12#) were taken from the dataset, which resulted in a 56 line and 10 column spreadsheet (56x10). PCA of this dataset is shown in Fig. 4. The loadings plot (Fig. 4A) shows that samples placed on the left-hand side have higher levels of Cr and P and, samples placed in the right hand corner have higher levels of As, Co, Fe, Mn, Rb, Se, Tl and V. Fig. 4B demonstrates that ordinary samples are located in the left-hand corner and organic samples on the right-hand side. These observations are in accordance with the results shown in the previous section.

Soft Independent Modeling of Class Analogy (SIMCA)

This classification procedure is based on building a PCA model for each class in the training set. Unknown samples are then compared with the class models and assigned to classes according to their analogy to the training samples[44]

The threshold lines divide the plot into four quadrants. A sample in the Northwest (NW) quadrant is a member only of the x-axis class; its distance to that class is small enough for it to be considered a member of the class. A sample falling in the Southeast (SE) quadrant is a member only of the y-axis class. A sample in the Southwest (SW) quadrant could belong to either category and one in the northeast (NE) quadrant belongs to neither. These plots are decision diagrams, as described by

Coomans.[29] They present classification information visually and also draw attention to borderline cases, samples lying close to one or both thresholds.

SIMCA is a modeling technique that builds a box for each category. The centre of the box is the mean value of the objects and the orientation is defined by principal components, and a range for each component is built on the basis of the distribution of the scores.[29]

Initially, the SIMCA model was carried out using 20 element levels and 60 egg samples. It used 8 PCs for both organic and ordinary samples. The Coomans plot is shown in Fig. 5. As illustrated from the figure, recognition of the two classes (organic and ordinary) was satisfactory and SIMCA recognition correctly classified all samples; that is, with 100% prediction ability for regular and organic egg samples. None of the models admitted samples from the other category, that is, 100% specificity in all cases. However, several samples are placed in the SW quadrant. Normally, SIMCA model that have several samples placed in the SW quadrant of the Commans plot will hardly predict samples, which are outside of the calibration dataset. As shown in Fig. 5, ordinary samples were driven to the SW quadrant due to the influence of the outlier sample I5. Thus, the SIMCA model was rebuilt using 20 element levels and omitting outlier samples (I5, P4, C3# and T12#). The Commans plot of the SIMCA model is shown in Fig. 6. It also exhibits 100% prediction ability as well as 100% specificity; in addition, it admits fewer samples to the SW quadrant than the previous SIMCA model (compare Fig. 5 with Fig. 6).

Finally, SIMCA was carried out using only the variables (As, Co, Cr, Fe, Mn, P, Rb, Se, Tl and V) that had shown statistical difference between organic and ordinary samples; outlier samples (I5, P4, C3# and T12#) were omitted from the dataset. The Commans plot of this SIMCA model is shown in Fig. 7. It offers the same 100% prediction ability and 100% specificity as the previous SIMCA models, but also admitted even fewer samples in the SW quadrant than the previous SIMCA models (compare Fig. 5 and Fig. 6 to Fig. 7). Thus, we conclude that both PCA and SIMCA could differentiate ordinary and organic samples using only 10 element levels (Zn, As, Co, Cr, Fe, Mn, P, Rb, Se, Tl and V).

Conclusion

This paper describes the first application of ICP-MS data to the discrimination of organic and regular eggs. The concentration levels of 19 chemical elements (macro and microelements) were interpreted using data handling techniques such as PCA and SIMCA. Both statistical techniques provided a robust approach for the authenticity evaluation of organic egg samples. Both approaches could differentiate ordinary and organic samples using only 10 element levels (Zn, As, Co, Cr, Fe, Mn, P, Rb, Se, Tl and V), which made these approaches very simple. In Brazil, organic eggs are 3-5 times more expensive than ordinary eggs and the approaches presented could readily be implemented to assure the authenticity of these organic eggs.

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Tables

Table 1: Instrument settings for ICP-MS.

Elan DRCII (PerkinElmer SCIEX)	
Instrument	
Nebulizer	Meinhard®
Spray chamber	Cyclonic
Torch injector	Quartz (2.0 mm)
Auto lens	On
RF power (W)	1100
Gas flow rates (L min^{-1})	Nebulizer 0.56–0.98; Plasma 15; Auxiliary 1.2
Interface	Platinum cones
Sampler	1.1 mm
Skimmer	0.9 mm
q-ICP-MS (standard mode)	^{75}As, ^{138}Ba, ^{44}Ca, ^{59}Co, ^{53}Cr, ^{63}Cu, ^{166}Eu, ^{57}Fe, ^{39}K, ^{24}Mg, ^{55}Mn, ^{23}Na, ^{60}Ni, ^{31}P, ^{85}Rb, ^{82}Se, ^{205}Tl, ^{51}V, ^{64}Zn
Internal standards	^{103}Rh
Scanning mode	Peak hopping
Integration time (ms)	2000
Replicates	3
Sweeps	40
Readings	1
Dwell time (ms)	50
Lens voltage (V)	6.0
Sample uptake rate (mL min^{-1})	1.0

Table 2: Analysis of the concentration levels of 19 chemical elements in regular and organic eggs samples from Brazil. Concentrations in ng/g, except for Na, K, Ca and P, which have concentration units in μg/mg. Relative Standard Deviation, RSD.

	regular			**organic**		
	average	**median**	**RSD**	**average**	**median**	**RSD**
As	15.57	15.15	3.40	23.12	22.87	4.05
Ba	3007	2924	768	3215	3205	780
Ca	1307	1363	224	1409	1365	198
Co	5.30	5.32	0.83	8.59	8.37	1.73
Cr	2435	2432	302	2118	2120	214
Cu	3836	3872	371	3763	3745	263
Eu	0.98	0.95	0.25	0.97	0.95	0.17
Fe	82282	82481	11260	88981	88166	10684
K	5390	5152	885	5147	5312	698
Mg	492186	482472	69388	478620	470941	55958
Mn	1122	1088	264	1343	1337	310
Na	5070	5012	886	4967	4993	653
Ni	22.33	21.44	4.82	18.03	17.52	3.91
P	5267	5372	536	4972	4926	401
Rb	8363	8279	2431	13469	14030	4437
Se	669	640	96	734	748	85
Tl	1.80	1.42	1.04	2.70	3.10	1.59
V	100	101	20	119	121	17
Zn	72042	73364	9893	69516	69508	5755

Table 3: Coordination of variables in Fig. 1A.

As	-0.15	0.45
Ba	0.11	-0.04
Ca	0.33	0.17
Co	0.06	0.41
Cr	0.04	-0.26
Cu	0.29	-0.11
Eu	0.17	-0.06
Fe	0.26	0.26
K	-0.27	-0.12
Mg	-0.31	0.00
Mn	0.16	0.28
Na	-0.38	-0.04
Ni	0.12	-0.17
P	0.37	-0.10
Rb	-0.14	0.20
Se	0.06	0.18
Tl	0.05	0.32
V	-0.08	0.35
Zn	0.39	-0.07

Table 4: Coordination of variables in Fig. 3A.

As	-0.30	0.36
Ba	0.05	-0.14
Ca	0.23	0.36
Co	-0.13	0.38
Cr	0.22	-0.08
Cu	0.34	0.16
Eu	0.10	-0.16
Fe	0.05	0.30
K	-0.08	-0.12
Mg	-0.22	-0.08
Mn	-0.01	0.32
Na	-0.31	-0.14
Ni	0.27	-0.06
P	0.42	0.16
Rb	-0.16	0.16
Se	-0.02	0.21
Tl	-0.14	0.24
V	-0.21	0.33
Zn	0.41	0.17

Figure Captions

Fig. 1: Principal component analysis of elemental concentrations based on 19 element levels (As, Ba, Ca, Co, Cu, Eu, Fe, K, Mg, Mn, Na, Ni, Rb, Se, Tl, V and Zn) and 60 egg samples (37 organic and 23 non-organic). Panel A and B illustrate loadings and scores plots, respectively. Organic samples are black and also have the prefix "#"; ordinary samples are indicated in red colour.

Fig. 2: Detection of outlier sample residual vs Mahalanobis Distance.

Fig. 3: Principal component analysis of elemental concentration levels based on 19 element levels (As, Ba, Ca, Co, Cu, Eu, Fe, K, Mg, Mn, Na, Ni, Rb, Se, Tl, V and Zn) and 56 egg samples (35 organic and 21 non-organic). Panel A and B illustrate loadings and scores plots, respectively. Organic samples are black and have the prefix "#"; ordinary samples are indicated in red color.

Fig. 4: Principal component analysis of elemental concentration levels based on 10 element levels (As, Co, Cr, Fe, Mn, P, Rb, Se, Tl and V) and 56 egg samples (35 organic and 21 non-organic). Panel A and B illustrate scores and loadings plots, respectively. Organic samples are black and have the prefix "#"; ordinary samples are indicated in red color.

Fig. 5: Coomans plot of the SIMCA model carried out using 19 element levels (As, Ba, Ca, Co, Cu, Eu, Fe, K, Mg, Mn, Na, Ni, Rb, Se, Tl, V and Zn) and 56 egg samples (35 organic and 21 non-organic). CS2@8 (y axis) are ordinary samples; CS1@8 (x axis) are organic samples. Continuous lines are the critical SIMCA distance for each category. Organic samples are black and have the prefix "#"; ordinary samples are indicated in red color.

Fig. 6: Coomans plot of the SIMCA model carried out using 19 element levels (As, Ba, Ca, Co, Cu, Eu, Fe, K, Mg, Mn, Na, Ni, Rb, Se, Tl, V and Zn) and 56 egg samples (35 organic and 21 non-organic). CS2@7 (y axis) are ordinary samples; CS1@9 (x axis) are organic samples. Continuous lines are the critical SIMCA distances for each category. Organic samples are black and have the prefix "#"; ordinary samples are indicated in red color.

Fig. 7: Coomans plot of the SIMCA model carried out using 10 element levels (As, Co, Cr, Fe, Mn, P, Rb, Se, Tl and V) and 56 egg samples (35 organic and 21 non-organic). CS2@5 (y axis) are ordinary samples; CS1@5 (x axis) are organic samples. Continuous lines are the critical SIMCA distances for each category. Organic samples are black and have the prefix "#"; ordinary samples are indicated in red color.