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Verification of Organic Feed Identity by Fatty Acid Fingerprinting

Alba Tres*[,†,‡] and Saskia M. van Ruth[†]

[†]RIKILT, Wageningen University and Research Centre, Post Office Box 230, 6700 AE Wageningen, The Netherlands

[‡]Department of Nutrition and Food Science, University of Barcelona, Avenida Joan XXIII s/n, 08028 Barcelona, Spain

ABSTRACT: The origin and authenticity of feed for laying hens is an important and fraud-susceptible aspect in the production of organic eggs. Chemical fingerprinting in combination with chemometric methods is increasingly used in conjunction with administrative controls to verify and safeguard the authenticity of food commodities. On the basis of fatty acid fingerprinting data of 36 organic and 60 conventional feeds, we have developed a chemometric classification model to discriminate between organic and conventional chicken feed. A two-factor partial least squares−discriminant analysis (PLS−DA) model was developed using 70% of the original data. External validation of the model with the remaining 30% of the data showed that all of the organic feeds and 90% of the conventional feeds (18 of 20) were correctly identified by the model. These results indicate that the PLS−DA model developed in this study could be routinely used to verify the identity of unknown or suspicious feed for laying hens.

KEYWORDS: Fingerprint, authenticity, organic products, feed, eggs, chemometrics

INTRODUCTION

The Food and Agricultural Organization of the United Nations[1] defines "organic agriculture" as a holistic production management system that promotes and enhances agroecosystem health, including biodiversity, biological cycles, and soil biological activity. It emphasizes the use of management practices in preference of off-farm inputs, taking into account that regional conditions require locally adapted systems. This is accomplished using, where possible, cultural, biological, and mechanical methods, as opposed to using synthetic materials, to fulfill any specific function within the system.

As a result of an increasing consumer awareness, organic farming and the organic food market are rapidly growing.[2,3] Thus far, however, there is no accepted worldwide standard for organic agriculture and production. In the European Union, the Council Regulation (EC) number 834/2007[4] defines the requirements for organic production and labeling of organic products and the Commission Regulation (EC) number 889/2008 lays down the detailed rules for organic production and labeling of organic products.[5] As with many other food products, eggs are produced in both conventional and organic farming systems. Apart from the differences in the hen housing conditions, one of the main differences between organic and conventional eggs is the feed supplied to the laying hens.[5−7] Hens laying organic eggs should be fed with organic feeds, that is to say that at least 95% of feed dry matter should come from ingredients of the organic farming.[5]

Organic products tend to sell at a higher price than their conventional counterparts, which makes organic products (such as eggs or feeds) more prone to fraud, which makes consumers require reassurance regarding their identity. Certification and verification of the authenticity of organic food is usually based on administrative controls and inspection procedures along the production and supply chain. In addition to these control mechanisms, an analytical tool to verify the organic identity of food and feed products would contribute to protection and sustainability of the long-term production of organic feed and food, would help the regulatory and inspection bodies, and would also increase the confidence of consumer in organic products. However, no systematic analytical controls on organic products are available at present because of the lack of such an analytical methodology.[2,8]

Traditional analytical strategies for guaranteeing food quality and uncovering food adulteration have largely relied on targeted analysis, in which the amount of a marker compound or compounds in a sample is determined and compared to the value(s) established for the authentic product.[9] This approach, however, fails when the natural variability of the individual compounds in the authentic product is so large that some adulterations would still go unnoticed. In such cases, application of untargeted fingerprinting techniques, which involve analysis of a wide range of compounds that are considered to be potential discriminators, is a more promising approach. Because of the large number of variables involved in chemical fingerprinting, the use of multivariate chemometric techniques is imperative to evaluate the data and provide a robust classification model. However, very few analytical methods to control some organic products are available.[2,8]

Chemical fingerprinting relies on the presence of several discriminators that allow us to discern between the authentic and non-authentic group. In the case of organic versus non-organic food products, the compounds that can be used to distinguishing between both groups often depend upon the commodity considered.[2,3] Because there are clear indications that the fatty acid (FA) profile of organic and conventional eggs is quite different,[10−12] the FA profile might be a promising discriminator for feed as well, especially because it has furthermore been shown that diet[13−15] and housing[16−18] have a strong effect on the FA composition of chicken meat and eggs.

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Table 1. FA Composition of Organic and Conventional Feeds Expressed as Peak Area Normalization as a Percentage[a]

	organic feeds (n = 36)			conventional feeds (n = 60)			
FA	average[b]	minimum[c]	maximum[c]	average[b]	minimum[c]	maximum[c]	p[d]
C8:0	0.01	0.00	0.05	0.05	0.00	0.49	0.017
C10:0	0.00	0.00	0.02	0.06	0.00	0.48	0.000
C12:0	0.03	0.00	0.27	0.28	0.01	2.98	0.001
C14:0	0.14	0.06	0.42	0.45	0.06	1.41	0.000
C15:0	0.04	0.02	0.05	0.05	0.03	0.10	0.000
C16:0	13.65	10.36	22.46	17.54	11.11	27.12	0.000
C17:0	0.09	0.08	0.12	0.12	0.08	0.27	0.000
C18:0	3.53	2.68	4.40	4.13	2.69	8.97	0.015
C20:0	0.28	0.14	0.46	0.23	0.11	0.46	0.002
C22:0	0.42	0.30	0.55	0.27	0.13	0.46	0.000
C24:0	0.27	0.23	0.35	0.19	0.10	0.29	0.000
C14:1n-5	0.00	0.00	0.00	0.03	0.00	0.11	0.000
C16:1n-9	0.04	0.03	0.05	0.10	0.03	0.25	0.000
C16:1n-7	0.11	0.09	0.20	0.70	0.10	2.53	0.000
C18:1n-9	25.72	22.97	38.80	28.62	22.71	36.91	0.000
C18:1n-7	0.91	0.70	1.52	1.30	0.74	2.56	0.000
C20:1n-9	0.28	0.22	0.44	0.38	0.22	0.84	0.000
C24:1n-9	0.02	0.01	0.05	0.02	0.01	0.07	0.029
C18:2n-6	51.01	28.55	56.05	41.92	28.70	56.83	0.000
C18:3n-6	0.00	0.00	0.01	0.02	0.00	0.07	0.000
C18:3n-3	3.36	1.60	5.00	3.01	1.51	4.53	0.047
C20:2n-6	0.04	0.03	0.05	0.08	0.02	0.29	0.000
C20:3n-6	0.00	0.00	0.01	0.02	0.00	0.06	0.000
C20:3n-3 + C20:4n-6[e]	0.00	0.00	tr	0.07	0.00	0.26	0.000
C20:5n-3	0.00	0.00	0.02	0.04	0.00	0.74	0.113
C22:4n-6	0.00	0.00	0.04	0.02	0.00	0.07	0.000
C22:5n-6	0.00	0.00	tr	0.01	0.00	0.02	0.034
C22:5n-3	0.00	0.00	0.02	0.03	0.00	0.34	0.006
C22:6n-3	0.00	0.00	0.03	0.05	0.00	0.83	0.049
18:1 *trans*	0.03	0.02	0.05	0.23	0.03	1.11	0.000
SFA	18.47	14.37	28.23	23.38	15.28	32.53	0.000
MUFA	27.08	24.30	41.01	31.14	24.23	42.65	0.000
PUFA	54.41	30.72	59.46	45.25	32.94	59.92	0.000

[a] Abbreviations used: SFA, saturated fatty acid; MUFA, *cis*-monounsaturated fatty acid; PUFA, *cis*-polyunsaturated fatty acid; tr, trace. [b] Average values shown correspond to the average of 36 different feeds for organic and the average of 60 different feeds for conventional (they are different samples and not replicates). [c] Minimum, lowest value found in each class; maximum, highest value found in each class. [d] p values were obtained from Student's t test (assuming equal variances and two-tailed distribution). [e] The FAs C20:3n-3 and C20:4n-6 are reported together because they coeluted in the chromatographic procedure.

The aim of this study is to develop and evaluate an analytical fingerprinting method to verify the organic identity of feed for laying hens that is based on FA fingerprinting in combination with chemometric classification techniques.

MATERIALS AND METHODS

Study Design. A total of 96 feeds used for laying hens were collected during 2009 and 2010 at different egg-producing farms in The Netherlands. The set of samples consisted of 36 organic feeds and 60 conventional feeds (24 for free-range, 24 for barn, and 12 for caged egg production). This sampling was conducted in the frame of a larger project, in which also models to authenticate organic eggs were developed.[8,12] Feeds collected corresponded to those supplied to the hens laying the eggs used in this project; therefore, they represent the feeds that were used in practice in laying hen farms in The Netherlands in 2009 and 2010. Farms were selected with the help of the Dutch product board for poultry and eggs (CPE) and the Dutch organic produce certification body SKAL. The sample selection was balanced with regard to the location (north, east, south, and west) and farm size (small, medium, and large) per production system (organic and conventional). The three farm size groups in each production system were defined taking into account the particular farm populations, because usually organic farms are smaller sized than conventional farms. Organic farm size groups consisted of farms <5000 hens, 5000−10 000 hens, and 10 000−20 000 hens; the conventional groups consisted of the categories 10 000−20 000 hens, 20 000−50 000 hens, and >50 000 hens.[8]

The feed samples were stored in the dark until analysis. Feeds were grinded to 0.5 mm particle size using a ZM200 Retsch ultracentrifuge mill (Retsch Benelux, Nijkerk, The Netherlands).

Reagents and Standards. Sodium methoxide (0.5 N) was purchased from Sigma-Aldrich (St. Louis, MO). Boron trifluoride methanol complex (35%) was obtained from Merck (Darmstadt, Germany). The fatty acid methyl ester (FAME) mixture of standards was supplied by Supelco (Supelco 37 Component FAME mix, Supelco, St. Louis, MO). All of the other reagents were of ACS quality grade.

FA Composition. A total of 4 g of feed was weighed, and then 4 g of anhydrous sodium sulfate was added and thoroughly mixed with the feed. Fat extraction was performed by adding 15 mL of 2:1 (v/v) chloroform/methanol, stirring the mixture at room temperature for 20 min, and then filtering the sample using filter paper (4−7 μm). Then, 10 mL of chloroform/methanol (2:1, v/v) were added to the residue, and the mixture was agitated again for 15 min and then filtered. An aliquot of the filtered phase, containing about 80−100 mg of fat, was transferred to a tube and evaporated under nitrogen.

The FAMEs were obtained as described by Guardiola et al.[14] and were determined by gas chromatography in a Varian (Palo Alto, CA) CP-3800 model gas chromatograph, fitted with a flame-ionization detector and split−splitless injector port, set at 280 and 250 °C, respectively. The split ratio was 1:30. Chromatographic separation of FAMEs was performed on a CP-Select CB for FAME capillary column (50 m × 0.25 mm inner diameter; Varian, Palo Alto, CA). Hydrogen (18 psi) was used as a carrier gas, and the oven was programmed as follows: initial temperature, 100 °C, increased at 5 °C/min to 230 °C and held for 9 min. The sample volume injected was 1 μL. Fatty acids were identified by their retention times according to those found in the FAME standard mixture.

All feed samples were analyzed in triplicate, and results were expressed as normalized peak areas (%). Data used were the average value of the three replicates of each feed.

Statistical Analysis. Principal component analysis (PCA) was used to screen the multivariate data for outliers and to explore the presence of any natural clustering in the data. PCA is a non-supervised classification technique that performs a reduction in the data dimensionality to facilitate the visualization of the data, retaining as much as possible the information present in the original data.[19] Several data preprocessing techniques were explored (none, mean centering, autoscaling, variance scale, $\log_{10}$ transformation, and combinations thereof).

Then, to develop the classification model, the 96 feed samples were divided into a training set and a validation set. The training set consisted of 70% of the feed samples in each class (organic and each of the three conventional categories), selected at random. The rest of the samples (30%) were the validation set. The training set was used to optimize a partial least squares−discriminant analysis (PLS−DA) classification model for organic feeds versus conventional feed. PLS−DA is a supervised classification technique that is often used for high-dimensional data, especially when the amount of variables greatly exceeds the number of samples. It performs a variable reduction on the data set by calculating new variables (called factors), combining the variables in the data set, to find the maximum correlation with the class variable and, thus, the maximum separation among two classes (organic versus conventional). Then, a model is developed using this reduced variable set (factors). Some preprocessing techniques were assayed (none, mean centering, autoscaling, variance scale, $\log_{10}$ transformation, and combinations thereof). Models were developed applying the orthogonal signal correction (OSC), which reduces the influence of noise variables in the model.

The PLS−DA model was optimized by a leave-10%-out cross-validation. In this cross-validation procedure, 10% of the samples are left out and the model is recalculated on the basis of the remaining 90% of samples and then used to obtain predictions for the left out samples. Next, these samples are placed back in the set, and another 10% of the samples are left out. This procedure is repeated until all of the samples have been left out once, and predictions are obtained. The number of correctly predicted samples and the standard error of cross-validation (SECV) are used to evaluate the model and to select the most successful data preprocessing technique and optimal number of factors to be included in the model.

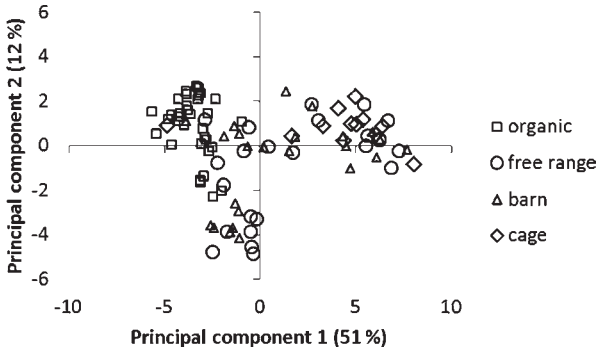

Figure 1. First two dimensions of PCA (and variance explained) on the FA profiling data of organic and conventional feeds (free range, barn, and cage): scores plot (data preprocessing: $\log_{10}$ transformation and autoscaling).

Finally, the most successful PLS−DA classification model as determined by internal cross-validation was externally validated using the remaining 30% of the samples as a validation set. To evaluate the performance (accuracy) of the model, the predictions were compared to the known class of the samples.

All multivariate statistical analyses were performed using Pirouette 4.0 software (Infometrix, Bothell, WA).

RESULTS AND DISCUSSION

PCA. The FA composition of organic (n = 36) and conventional (n = 60) feed samples is listed in Table 1. Most of FAs were found in quite wide ranges in organic and conventional feeds, because of both the high variety of available ingredients and the natural variability in the FA composition within ingredients. Those ranges were so wide that, for most of the FA, the values found for some organic feeds were in the ranges found for conventional feeds. Thus, using an univariate approach (looking at the FAs one by one), it was not possible to set up clear boundaries that allowed for the classification of new authentic feeds in organic or conventional classes, despite the statistical differences found for the individual FAs between categories. With this approach, the chances of finding both false positives (conventional feeds identified as organic) or false negatives (not being able to correctly identify an authentic organic feed) would be very high. However, in some other organic products, such as milk, thresholds for C18:3n-3, δ^{13}C, and phytanic acid have been successfully established to verify the organic identity of other food products, such as milk and dairy products.[20,21]

Instead of a univariate approach (looking at the FAs one by one), chemometrics was applied to the whole feed FA composition data to use it as a fingerprint to discriminate organic feeds from conventional feeds. The first step in the application of chemometrics is the visualization of the multidimensional data. For each feed sample, 30 variables (FAs) were available. PCA was conducted to visualize all of the 96 samples using the information of all of the FAs at once (Figure 1). As seen in Figure 1, the PCA scores plot revealed a tendency to separate organic from conventional feeds. This tendency was observed in all of the data preprocessing techniques assayed (none, mean centering, autoscaling, $\log_{10}$ transformation, and combinations among them),

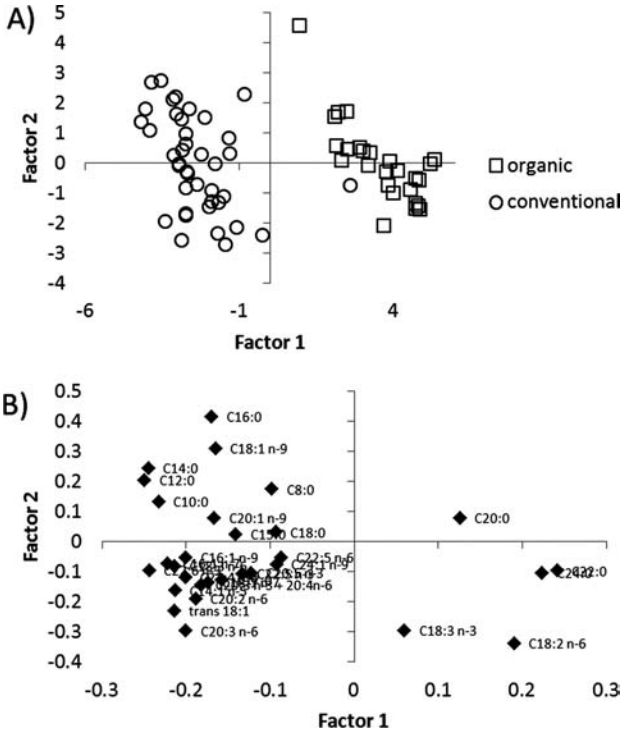

Figure 2. First two dimensions of PLS−DA on the FA profiling data of organic and conventional feeds: (A) scores and (B) loadings plot (data preprocessing: $\log_{10}$ transformation and autoscaling; OSC = 1).

Table 2. PLS−DA Classification Rates Obtained in the Internal Validation (Leave-6-out Cross-Validation) and in the External Validation of the Model Developed for Organic and Conventional Feeds Using Their FA Fingerprint

	samples classified as[a]		
type of feed	organic	conventional	percent of correct classifications (%)
	Internal Validation		
organic	26	0	100
conventional	1	39	98
	External Validation		
organic	10	0	100
conventional	2	18	90

[a] PLS−DA model on the $\log_{10}$-transformed and autoscaled FA composition (2 factors; OSC = 1).

being the combination of $\log_{10}$ transformation and autoscaling, with the option leading to a clearer separation between classes (Figure 1). Although a few organic and conventional samples were overlapped, this was a promising scenario for applying a supervised classification statistical technique, such as PLS−DA, which is designed for finding the maximum separation among classes.

On the other hand, no clustering was evident among the conventional feeds used for the production of free range, barn, or caged eggs, and these three groups were overlapped (Figure 1). Indeed, according to the regulations, the same feed might be used indistinctively for the production of any of the three conventional

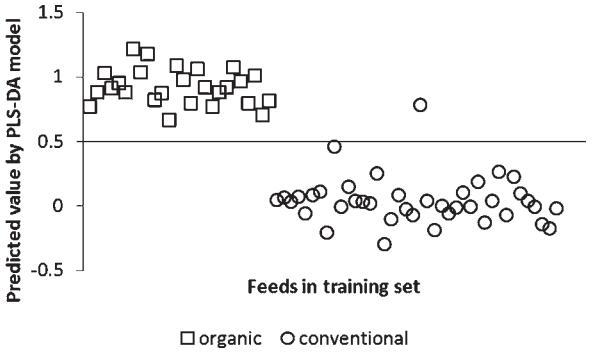

Figure 3. Internal validation: predictions obtained by the PLS−DA model for the organic and conventional feeds. Cutoff value of the model for classification into organic/conventional = 0.5 (organic class, 1; conventional class, 0).

egg categories. According to this, the identification of the three conventional feed categories was out of the scope of this study, and the three conventional categories were considered together in the conventional feed class for the binary classification of organic versus conventional feeds.

Classification Model Development: Training Set. PLS−DA models for classifying organic and conventional feeds were developed using the FA fingerprint of the samples in the training set (66 samples and 30 variables). Several data preprocessing techniques (none, mean centering, autoscaling, $\log_{10}$ transformation, and combinations among them; OSC = 1) were conducted on the FA composition data before applying the PLS−DA algorithm to find the preprocessing strategy that provided the most robust model for classifying organic and conventional feeds (Figure 2A). Cross-validation results showed that the PLS−DA models with 1 or 2 factors, on the $\log_{10}$-transformed and autoscaled data provided the best classification rates for both organic and conventional feeds (Table 2). Indeed, all organic feeds were correctly classified as organic. Only one false positive was found during cross-validation.

Figure 3 shows the predicted values obtained for samples during model cross-validation. The PLS−DA model implies that *y* values are set to 1 for one of the categories (i.e., organic feeds) and to 0 for the rest of the categories (i.e., conventional feeds). The cutoff value is set at 0.5; thus, when the predicted values are above 0.5, the sample is classified as organic, and when the predicted values are below 0.5, the samples are classified as conventional. Thus, the closer the predicted values are to either 1 or 0, the more reliable the classification. As seen, our model based on feed FA fingerprinting not only correctly classified almost all feeds into the organic or conventional classes, but also the predicted values for the organic and conventional feeds were quite close to 1 and 0, respectively. This makes the SECV of the model was very low (SECV = 0.1774) and reveals the successfulness of the model. Other preprocessing techniques assayed on the FA fingerprint, such as the $\log_{10}$ transformation or the data autoscaling alone, also provided quite successful models, but classification results were better with the PLS−DA model on the combination of $\log_{10}$ transformation and autoscaling.

Validation of Models: Validation Set. The PLS−DA model on the $\log_{10}$-transformed and autoscaled data was externally validated by predicting the identity of the feeds in the validation set. As seen in Table 2, the classification rates of the validation set were very successful, because all of the organic feeds were correctly

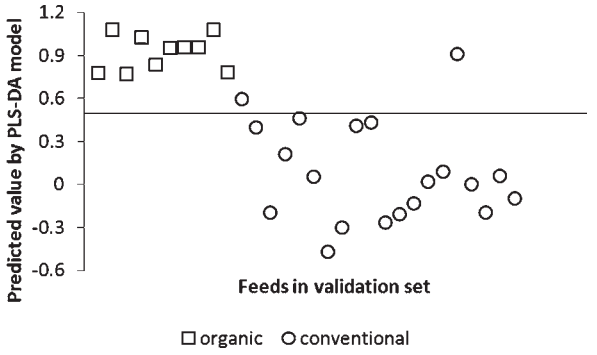

Figure 4. External validation: predictions obtained by the PLS−DA model for the organic and conventional feeds to belong to the organic class. Cutoff value of the model for classification into organic/conventional = 0.5 (organic class, 1; conventional class, 0).

identified by the model. Furthermore, only 2 false positives were encountered of the 20 conventional feeds included in the validation set (Table 2). These two samples differed from the other conventional feeds mainly in the low contents of C12:0 and C14:0. Furthermore, as seen in Figure 4, classification output of the model is quite robust, because the predicted values for the feeds in the validation set are, in general, quite separated from the cutoff value of 0.5 (1, organic; 0, conventional).

The successfulness of the external validation reveals that the PLS−DA model was not overfitted and that it is very promising for the prediction of the organic/conventional identity of new unknown feeds used for laying hens in The Netherlands. The interest of this model emerges from the fact that it can be used to verify the organic identity of feeds labeled as organic. This will certainly contribute to an improvement of the quality and reliability of the organic market, because according to our knowledge, this is the first analytical tool available to verify the organic identity of feeds used for laying hens. This analytical tool might contribute to the detection of fraud and mislabeling not only in the market of organic feeds but also in the market of organic eggs, because the verification of the organic identity at the lower levels of the food chain is crucial for the identity of the end products.

One advantage of these fingerprinting methods is that they might be updated in the future by including in the model new authentic samples. When this is performed, the possible variability on feed composition because of changes in ingredient composition, agronomic practices, feed production, or seasonal effects can be included in the model, and therefore, the model can be updated if necessary.

On the other hand, it is worth mentioning that this model has been developed using organic and conventional feeds from Dutch farms. Its application to verify the organic identity of feeds originating from other countries would first require that its performance is evaluated for non-Dutch feeds, which may show different variations in this fingerprint.

Contribution of FA to the Model. In a PLS−DA model, factors are calculated to find the maximum correlation of the variables (FAs) with the categories (organic/conventional), thus leading to the highest separation of categories. In our model, the organic and conventional feed classes are separated mainly by the first factor. The PLS−DA loadings plot (Figure 2B) reveals that there is not only one FA responsible for the differences between these feed categories; instead, several FAs participate in the model. When panels A (scores plot) and B (loadings plot) of Figure 2 are compared, it can be seen that FAs, such as C24:0, C22:0, and C18:2n-6, showed high positive contributions to factor 1 and are thus associated with the organic feed class and that FAs showing high negative loadings for factor 1 (such as C12:0, C14:0, C16:1n-9, and C16:1n-7) are associated with the conventional feed class. These variations in the FA composition might result mainly from the differences in the ingredient composition in organic and conventional feeds. Strict regulations affect the composition of organic feeds, and thus, the type and amount of ingredients available for feed production might be more restrictive for the organic than the conventional production. Furthermore, the feeds included in this study come from the real feed market (covering several feed companies producing different types of feeds), and thus, they present a wide variety of ingredients in different amounts. It is therefore difficult to attribute the differences observed in this study to the use of certain feed ingredients. As far as we are concerned, no similar surveys have been conducted assessing the FA composition in real feed samples. In some animal studies, the effect of animal feeding with organic and conventional feeds has been studied.[18] However, results on feed composition of these studies are hardly comparable to our study, because only few feeds were compared and, furthermore, they had been in-house designed for the experimental studies by maintaining the ingredient composition as similar as possible and only varying their organic and conventional origins.

On the other hand, the differences in the FA profile of organic and conventional feeds might partly explain the differences in the FA composition of organic and conventional eggs, although other factors, such as the availability of other dietary sources when hens pasture, and the metabolism of the animal, might also have an influence.[10−12]

In summary, a model to verify the organic or conventional identity of feeds used for the laying hens in The Netherlands has been developed using feed FA fingerprinting and chemometrics (PLS−DA on the $\log_{10}$-transformed and autoscaled data; OSC = 1). The model has been internally and externally validated, providing very successful results on the verification of the identity of both the organic and conventional feeds.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +31-317-48-63-44. E-mail: alba.tres@wur.nl.

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