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Eggspectation: organic egg authentication method challenged with produce from ten different countries

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Abstract

Many consumers are willing to pay a higher price for organic eggs. Since these eggs retail at a higher price than conventional eggs and their identity is difficult to verify, they are susceptible to fraud. For the authentication of Dutch eggs RIKILT developed an analytical test method based on carotenoid profiling. In the present study, the method was challenged with eggs from 10 countries. Eggs from 94 farms (65 organic, 29 conventional) were subjected to the carotenoid High Performance Liquid Chromatography – Diode Array Detection profiling combined with *k*-nearest neighbour classification chemometrics to predict the farming management system category: organic or conventional. The eggs from 39 of the 40 EU organic farms and the eggs of 27 of the 29 EU conventional farms, as well as eggs from 17 of the 25 organic farms from outside the EU were classified correctly. The latter lower rate was mainly due to eggs from Turkey; 78% of which were misclassified. The methodology was successful in farming management prediction of the EU eggs, as well as for eggs from Canada, Israel and Norway. The identity of the eggs from Turkey was consistently incorrectly predicted and needs further research.

Keywords: carotenoids, chemometrics, fraud, profiling

1. Introduction

Carotenoids are typically bright yellow, orange, red or purple polyisoprenoid compounds, and are secondary plant metabolites that serve antioxidant functions in plant processes. They interact with chlorophyll during photosynthesis to absorb light and transfer energy, but they also quench singlet oxygen and free radicals and thereby protect plants from photooxidative damage. Furthermore, diverse biological functions in humans have been attributed to carotenoids. Carotenoids are found predominantly in photosynthesising organisms such as green plants, algae and some bacteria. They are composed of two groups of compounds: hydrocarbon carotenes and oxygenated xanthophylls (Kopsell *et al.*, 2009; Řezanka *et al.*, 2009). Egg yolks serve as a traditional source of xanthophylls, besides dark-green leafy vegetables (Ribaya-Mercado and Blumberg, 2004). They are regarded as a highly bioavailable source oflutein and zeaxanthin (Handelman *et al.*, 1999).

Eggs are composed of nutrients derived from the hen's diet. Commercial poultry feed consists of primarily pellets or mash, containing wheat, barley, maize, and soybeans. Conventional and organic laying hen feeds differ for instance considerably in their fatty acid composition (Tres and Van Ruth, 2011), which is reflected in the egg composition (Tres *et al.*, 2011). Free range and organic hens have the opportunity to forage in soils, picking up grubs, beetles, worms, grasses, and seeds as well as their commercial ration in mash or pellet form (Rogers, 2009).

In the European Union (EU), yolk colour is an important criterion for consumers' choice of eggs. Yolk colour is named at third position under egg quality traits (Hernandez *et al.*, 2000). Egg yolk colour preference varies across Europe: northern countries, with the exception of Germany, prefer weakly coloured yolks, whereas countries of southwestern Europe prefer more intensively coloured yolks. The preferences for yolk colour of consumers vary also between regions within countries (EC, 2002). Egg yolk colour directly reflects the concentration of pigments in the diets of laying hens. It is usually measured with special colour fans covering the different levels of colouration (from pale yellow to orange and red) and reflecting the different combinations of yellow and red carotenoids in the diets. The mostly used colour fan is the Roche Yolk Colour Fan displaying a scale from 1 (pale yellow) to 15 (reddish orange) (Veuilleumier, 1969).

Carotenoid pigments are common colorants of egg yolk, however, since hens do not produce carotenoids naturally, they must be supplied in feed for proper pigmentation (Borlotti *et al.*, 2003). Maize, a usual ingredient of chicken feed, is the major natural source of carotenoids pigmenting egg yolks and meat, but also alfalfa meal is known as a good source. Green pasture has also shown an increasing effect on the carotenoid content of eggs (Mugnai *et al.*, 2009). Furthermore, carotenoids and especially xanthophylls are added to conventional feeds.

In the EU, regulations permit the addition of eight xanthophylls possessing various functional groups and carbon chain lengths (C_{30}-C_{40}) to the feed of conventional laying hens in amounts of up to 80 mg/kg of feeding stuff (EC, 2003). These substances are the natural xanthophylls capsanthin (C_{40}), β-cryptoxanthin (C_{40}), lutein (C_{40}), and zeaxanthin (C_{40}), as well as the synthetic xanthophylls β-apo-8'-carotenal (C_{30}), β-apo-8'-carotenoic acid ethyl ester (C_{30}), canthaxanthin (C_{40}), and citranaxanthin (C_{33}) (Wenzel *et al.*, 2010).

Consumers have become more concerned about animal welfare recently. Eggs from caged or battery hens have become ethically less acceptable in recent times, with many consumers preferring to pay a higher price for eggs raised under more animal welfare friendly conditions. Organic agricultural practices aim to enhance biodiversity, biological cycles, and soil biological activity so as to achieve optimal natural systems that are socially, ecologically, and economically sustainable. Organic produce tends to retail at a higher price than their conventional counterparts. This premium price and occasionally demand exceeding supply makes organic produce susceptible to fraud. Analytical verification of eggs would, therefore, be an interesting tool in the track-and-trace process.

For the authentication of organic and conventional eggs an analytical approach based on carotenoid fingerprints combined with chemometrics was developed by Van Ruth *et al.* (2011). In that study models were calibrated using eggs from the Netherlands and their performance was successfully evaluated with newly collected eggs from the Netherlands and from New Zealand. In the present validation study, the methodology was challenged with eggs from ten different countries in order to evaluate its wider applicability.

2. Materials and methods

Materials

Organic and conventional eggs were collected in ten countries: within the EU in Austria, Belgium, Germany, Greece, Italy, and Portugal, and outside the EU in Canada, Israel, Norway and Turkey. Except for Belgium, Germany and Canada, eggs were collected by partners of the EU funded Network of Excellence MoniQA. The sample set included 40 EU organic farms (6 countries), 25 non-EU organic farms (4 countries), and 29 EU conventional farms (4 countries). Conventional farms included 17 barn and 12 free range farms. At the start of the study two additional organic farms (Belgian and Greek) were included, but when the eggs from these farms were consistently classified as conventional eggs, further local inspections were carried out and revealed irregularities. Therefore, these samples were removed from the sample set. The number of farms involved in the final sample set is specified per country and farming management system in Table 1.

Carotenoid fingerprinting

Egg preparation/pooling

Yolks were manually separated from the whites. Three yolks per farm were pooled and mixed. One to three samples of three yolks per farm were used in the experiments, depending on availability. In the experiments all eggs were freeze-dried prior to analysis, either prior to transport to the Netherlands or on arrival. All eggs were analysed by RIKILT (Wageningen, the Netherlands). The freeze-dried samples were analysed within 3 weeks.

Table 1. Relative carotenoid compositions based on normalised peak areas of eggs from organic and conventional farms originating from various countries (mean values (%)±SD)^{1,2}.

Country of origin (no. of farms)	Carotenoids								
	Lutein/zeaxanthin	Cantha-xanthin	*Cis*-isomer of xanthophyll (λmax = 446; 472)	Unk1 (λmax = 448; 452)	Citranta-xanthin	Unk2 (λmax = 448; 476)	Unk3 (λmax = 448; 476)	β-cryptoxanthin	β-carotene
EU organic (40)									
Austria (8)	84.6±1.3^{A}	5.2±0.3^{D}	4.8±0.8^{C}	0.0±0.0^{D}	0.0±0.0^{B}	1.3±0.3^{B}	2.0±0.5^{CDE}	1.6±0.3^{EFG}	0.6±0.2^{A}
Belgium (7)	84.1±1.3^{A}	5.8±0.5^{D}	4.8±0.8^{BC}	0.0±0.0^{D}	0.0±0.0^{B}	0.4±0.3^{DE}	2.3±0.7^{BC}	2.1±0.6^{CDEF}	0.5±0.3^{ABC}
Germany (5)	84.3±0.9^{A}	5.0±0.4^{D}	5.1±0.3^{BC}	0.0±0.0^{D}	0.0±0.0^{B}	1.2±0.3^{BC}	2.2±0.5^{CDE}	1.8±0.4^{DEFG}	0.9±0.1^{ABC}
Greece (7)	82.4±1.5^{A}	5.2±0.6^{D}	5.8±1.2^{AB}	0.0±0.0^{D}	0.0±0.0^{B}	0.0±0.0^{E}	3.7±1.2^{A}	2.9±0.6^{A}	0.0±0.0^{D}
Italy (10)	83.9±6.3^{A}	6.9±5.1^{D}	4.3±1.1^{CD}	0.3±0.9^{D}	0.0±0.0^{B}	0.4±0.4^{DE}	2.0±1.0^{CDE}	1.6±0.5^{FG}	0.6±0.6^{A}
Portugal (3)	82.5±3.0^{A}	6.9±0.7^{D}	5.1±1.5^{ABC}	0.0±0.0^{D}	0.0±0.0^{B}	0.0±0.0^{E}	2.5±1.3^{BC}	2.9±0.6^{AB}	0.1±0.2^{CD}
Non-EU organic (25)									
Canada (5)	83.4±3.0^{A}	6.2±1.9^{D}	5.2±0.5^{ABC}	0.1±0.3^{D}	0.0±0.0^{B}	0.5±0.2^{CDE}	2.2±0.4^{BCD}	2.3±0.6^{BCD}	0.1±0.2^{CD}
Israel (4)	83.4±1.4^{A}	5.1±0.5^{D}	4.8±0.3^{BC}	0.0±0.00^{D}	0.0±0.0^{B}	1.9±1.2^{A}	2.7±0.3^{BC}	2.1±0.4^{CDEF}	0.0±0.0^{D}
Norway (7)	82.9±1.4^{A}	4.8±0.8^{D}	6.2±1.3^{A}	0.0±0.00^{D}	0.0±0.0^{B}	0.6±0.5^{CD}	2.4±0.4^{BC}	2.5±0.8^{ABC}	0.6±0.1^{AB}
Turkey (9)	75.9±10.4^{AB}	12.7±8.8^{CD}	4.7±0.9^{C}	1.5±1.9^{CD}	0.0±0.0^{B}	0.0±0.0^{E}	2.8±0.5^{B}	2.1±0.4^{CDE}	0.3±0.4^{BCD}
EU conventional (29)									
Austria (8)	66.1±10.2^{C}	22.9±8.9^{B}	3.5±0.9^{DE}	3.8±1.9^{B}	0.0±0.0^{B}	0.7±0.4^{CD}	1.5±0.4^{DE}	1.2±0.3^{G}	0.3±0.3^{ABCD}
Belgium (7)	67.6±14.0^{BC}	17.6±12.7^{BC}	2.9±1.1^{E}	2.7±2.8^{BC}	5.6±14.7^{A}	0.3±0.3^{DE}	1.6±0.4^{DE}	1.6±0.9^{DEFG}	0.3±0.3^{ABCD}
Greece (8)	48.5±9.6^{D}	37.6±9.7^{A}	3.4±1.0^{DE}	6.1±1.9^{A}	0.0±0.0^{B}	0.1±0.2^{E}	2.1±0.4^{CD}	1.9±0.6^{CDEF}	0.2±0.2^{CD}
Portugal (6)	52.0±25.8^{D}	34.3±21.5^{A}	3.5±1.2^{DE}	6.4±5.1^{A}	0.0±0.0^{B}	0.8±1.4^{BCD}	1.4±0.4^{E}	1.3±0.5^{G}	0.3±0.5^{ABCD}

[1] SD was calculated over farm averages.

[2] Different superscripts in a column indicate differences between sample groups (ANOVA and post-hoc Least Significant Difference tests, *P*<0.05).

Carotenoid extraction

Freeze-dried pooled egg yolks (250 mg) were combined with 250 µl water in order to compensate for water evaporated during freeze-drying. Each sample (500 mg) of freeze-dried egg/water mixture was combined with 1,400 µl NaCl solution (5 g/100 ml), vortexed for 10 s, subsequently 1000 µl of ethanol was added, and the sample vortexed for another 20 s. After addition of 500 µl hexane, the sample was vortexed for 1 min and then centrifuged (2,700×*g*, 6 min, 20 °C) and subsequently the upper hexane layer removed. The extraction was repeated with 500 µl and 1000 µl hexane, consecutively. The hexane of the three combined fractions was evaporated under a nitrogen gas stream at room temperature for approximately 1 h until complete dryness of the sample. The residue was subsequently dissolved in 500 µl dichloromethane and 500 µl methanol. After centrifugation, the mixture was filtered (0.45 µm filter, Pall filter systems GmbH, Crailsheim, Germany) prior to High Performance Liquid Chromatography (HPLC) analysis.

Generally, single extractions were carried out on the pooled samples. Repeatability was monitored by comparison of results of duplicate extractions of each 10th sample with repeatability results obtained in a previous study (Van Ruth *et al.*, 2011). In the present study, repeatability averaged 6.0%. The same study by Van Ruth *et al.* (2011) also showed that freeze-drying hardly affected the relative carotenoid profiles of the eggs as well as the repeatability in the time period given. Differences in concentrations between fresh and freeze-dried pooled eggs were only ca. 35% of the differences observed between two samples of pooled (fresh) eggs. Thus, the variation in carotenoid composition in the eggs was considerably larger than the variation (change) caused by the freeze-drying process.

Carotenoid HPLC profiling

The carotenoid extracts were analysed on an HPLC system (Agilent 1100 series, Agilent Technologies, Amstelveen, the Netherlands) fitted with a reverse phase C18 column (Zorbax Eclipse XDB-C18, 4.6×150 mm, Agilent Technologies, Santa Clara, CA, USA) with a pre-column of the same material. A volume of 10 µl of the carotenoid extract was injected onto the column and carotenoids were detected by a G1315B Diode Array Detector (Agilent 1100 series, Agilent Technologies, Amstelveen, the Netherlands) and recorded at 445 nm. The mobile system was methanol:water (90:10, v/v, mobile phase A) and acetonitrile:2-propanol (63:37, v/v, mobile phase B). A binary gradient of A:B starting at 75:25 to 0:100 at 13 min until end time at 24 min, and a flow rate of 1 ml/min was used.

Carotenoids were previously tentatively identified using retention time and maximal wave length verification of authentic standards (Van Ruth *et al.*, 2011). The peak area data were standardised to 100% and the relative peak area data (peak area percentages) were used for further statistical analysis.

Statistical analysis

Means and standard deviations of the relative carotenoid data were calculated. They were subjected to analysis of variance (one way and two-way ANOVA) to determine significant differences between samples (two factors: farming management system, country of origin). Significance of differences was evaluated using post-hoc tests (Least Significant Difference tests). ANOVA was performed using XLSTAT (Addinsoft SARL, New York, NY, USA). The data were further explored by Principal Component Analysis (PCA) considering various data pre-treatment techniques, outliers and variation of the number of Principal Components. In the study, the category (either organic or conventional) of the samples was predicted from their carotenoid profiles using a k-nearest neighbour classification (kNN) approach described previously (Van Ruth *et al.*, 2011). kNN is a supervised learning algorithm where a sample is classified based on the category of the majority of its k-nearest neighbours. kNN models had been estimated and optimised for eggs from Dutch farms. The new egg samples from the various countries in the present study were classified by determining the majority category of the k nearest neighbours in that model. The predicted category (organic or conventional) was compared to the egg labels. PCA and kNN statistics were performed in Pirouette 4.0 (Infometrix, Bothell, WA, USA). Sensitivity and specificity were computed in this work to evaluate model performance. Sensitivity is defined as the percentage of organic egg samples identity of which is predicted as organic eggs by the model developed, using Dutch eggs as the calibration set. Specificity is the percentage of samples not belonging to the organic egg class which are correctly identified as not belonging to this class.

3. Results and discussion

Eggs from 94 farms were analysed for their carotenoid compositions, including eggs from organic and conventional farms (barn/free range) of EU and non-EU origin. An example of a chromatogram is presented in Figure 1. The relative compositions of the egg samples for nine carotenoids are presented per country and carotenoid in Table 1. Lutein/zeaxanthin make up the highest proportion of carotenoids in both categories of samples and are higher in the organic eggs (average over countries 82.7±2.5%) than in the conventional eggs (58.5±9.7%). Systematic differences are also seen in the proportion of cantaxanthin, being about 5 times higher in the conventional eggs and *cis*-xanthophyll seems to be about 1.5 times higher in the organic eggs. They are the same carotenoids as detected

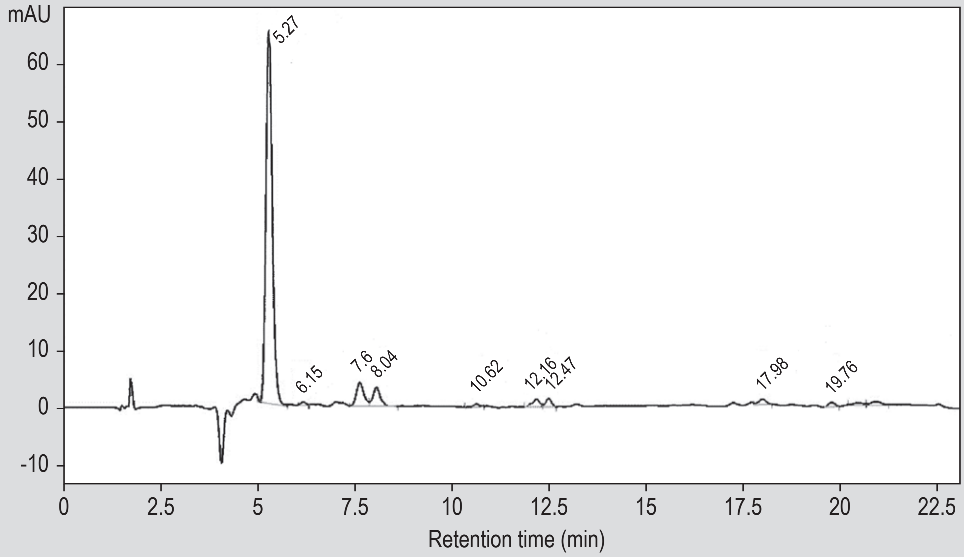

Figure 1. Chromatogram of the carotenoid profiling of organic eggs by high performance liquid chromatography.

earlier in the study on eggs from the Netherlands and New Zealand, except for β-apo-carotenal which was below detection for all samples in the present study. In organic production, carotenoids, originated from the feed and particularly maize, are the main yellow pigment source for the laying hens. The major carotenoid pigments in fresh-market sweet corn are lutein and zeaxanthin, with minor amounts of β-carotene, α-carotene, β-cryptoxanthin, and antheraxanthin (Kurilich and Juvik, 1999). Lutein, capsanthin, zeaxanthin, and β-cryptoxanthin are all natural xanthophylls legally allowed to be added to conventional laying hen feed in the EU. Three of them were identified in the present study. They occur in various plants. The other four xanthophylls allowed as colouring feed additives are β-apo-carotenal, canthaxanthin, β-apo-carotenoic acid ethyl ester, and citranaxanthin. Two of these four were determined in the present study. These xanthophylls are mostly produced by chemical synthesis (Schlatterer and Breithaupt, 2006). However, there are also natural sources of canthaxanthin. Canthaxanthin was first isolated from the edible mushroom, *Cantharellus cinnabarinus* (Haxo, 1950). Canthaxanthin is also produced in several green and blue-green algae and has been found in bacteria, crustacea and various species of fish (EC, 2002). β-carotene is the most abundant carotene in plants, but only a minor carotenoid in the egg yolks. This is due to the fact that, on the one hand carotenes are not as well absorbed as the xanthophylls in general (Na *et al.*, 2004), and on the other hand because hens convert β-carotene efficiently to vitamin A (Schlatterer and Breithaupt, 2006).

Subsequently, ANOVA was carried out on the data to evaluate the significance of the differences observed among the fourteen groups of eggs, which are listed in Table 1. The significance of the factor farming management system is shown for each carotenoid in Table 2 for all countries as well as separately for the EU countries. Six out of the nine carotenoids demonstrated highly significant differences between eggs produced in organic and conventional management systems. For the four carotenoids (lutein/zeaxanthin, the *cis*-isomer of a xanthophyll (λmax = 446; 472), Unk3 (λmax = 448; 476), and β-cryptoxanthin), significantly higher proportions were observed in the organic eggs than in the conventional eggs. For canthaxanthin and Unk1 (λmax = 448; 452), the opposite was observed. It is obvious that the balance of the carotenoids is fairly different for the organic eggs in comparison with the conventional eggs, which confirms the earlier results for eggs from the Netherlands and New Zealand (Van Ruth *et al.*, 2011).

Since the data showed great similarity with the previous results generated for eggs from the Netherlands (Van Ruth *et al.*, 2011), the carotenoid profiles were subjected to further statistical analysis. Firstly, PCA was carried out on the EU egg data. The PCA plot (Figure 2) reveals a tendency for organic samples to be grouped at the right-side of the plot in a relatively discrete cluster while the conventional farm samples form a more disperse group on the left-hand side. It illustrates the differences between the organic and conventional sample groups and the larger variability in the conventional egg samples. Organic farms generally appear to produce more similar eggs in terms of carotenoid composition than conventional farms. This is probably due to the use of artificial yolk colorants in addition to natural feed colouring agents in conventional productions, which are not allowed in organic farming. However, it should be kept in mind that variation among farms also differs from country to country. Table 3 lists the variation in carotenoid composition by country and farming management system. For the EU countries the mean coefficient of variation is 36±27% among the organic farms and 72±16% among the conventional farms. For the non-EU organic farms, the coefficient of variation is 43±26%. Within single farms, variation was 7% on average.

Table 2. Results of two way analysis of variance on the relative carotenoid compositions of eggs from organic and conventional farms originating from various countries for each carotenoid: factors farming managing system and country of origin (*P*-values for farming management system).

Carotenoid	All countries	EU countries
Lutein/zeaxanthin[1]	<0.0001	<0.0001
Canthaxanthin[2]	<0.0001	<0.0001
Cis-isomer of xanthophyll (λmax = 446; 472)[1]	<0.0001	<0.0001
Unk1 (λmax = 448; 452)[2]	<0.0001	<0.0001
Citrantaxanthin	0.144	0.211
Unk2 (λmax = 448; 476)	0.222	0.234
Unk3 (λmax = 448; 476)[1]	<0.0001	<0.0001
β-Cryptoxanthin[1]	<0.0001	0.0001
β-Carotene	0.315	0.058

[1] Significant higher proportions in organic eggs.

[2] Significant higher proportions in conventional eggs.

Using the previously developed kNN model for Dutch eggs (Van Ruth *et al.*, 2011), the farming management system category (organic or conventional) was predicted (Table 4). Out of the 40 organic farms in the EU, the eggs from 39 farms were classified as organic. Eggs from one Italian farm were classified as conventional, which was due to a considerably lower proportion of lutein/zeaxanthin (66.61%), and higher levels of canthaxanthin (21.31%) and Unk1 (λmax = 448; 452) (2.72%) in comparison to organic counterparts. The sample was not borderline organic, but its carotenoid profile showed great similarity with other conventional samples, and therefore its authenticity was considered doubtful. Out of the 29 conventional EU farms,

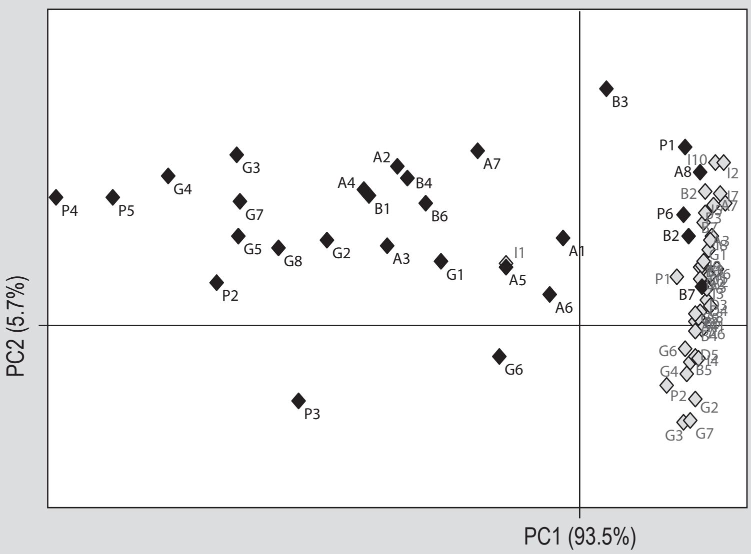

Figure 2. First two dimensions of Principal Component Analysis on relative mean-centred carotenoid fingerprint data of eggs from organic and conventional farms in the EU. Grey = organic, black = conventional; capital letters indicate EU countries: A = Austria; B = Belgium; D = Germany; G = Greece; I = Italy; P = Portugal. Samples I1 (organic), A8 and B7 (both conventional) were misclassified using the k-nearest neighbour classification model developed with Dutch eggs as calibration set.

Table 3. Variation in carotenoid composition (CV%) among organic and conventional farms inside and outside the EU.[1]

Country	Organic	Conventional
EU		
Austria	18.5	52.3
Belgium	32.2	90.3
Germany	14.8	
Greece	17.2	68.6
Italy	83.7	
Portugal	48.1	78.2
EU mean±SD	35.8±26.6	72.4±16.0
Non-EU		
Canada	70.4	
Israel	15.6	
Norway	25.5	
Turkey	59.2	
Non-EU mean±SD	42.7±26.3	

[1] CV% = Coefficients of variance averaged over individual carotenoids per country.

Table 4. Sensitivity and specificity ratings for eggs from various countries. Correct classification rates (percentages) with application of the k-nearest neighbour classification model developed using Dutch eggs as calibration set (Van Ruth *et al.*, 2011).

Country	Sensitivity (organic= organic, %)	Specificity (conventional= conventional, %)
EU		
Austria	100	88[1]
Belgium	100	86[1]
Germany	100	
Greece	100	100
Italy	90[1]	
Portugal	100	100
EU mean±SD	98.0±4.1	93.0±7.6
Non-EU		
Canada	100	
Israel	75[1]	
Norway	100	
Turkey	22[2]	
Non-EU mean±SD	72.0±36.8	

[1] One farm was misclassified.

[2] Seven out of nine farms were misclassified.

eggs from 27 farms were classified as conventional. The two misclassified farms involved an Austrian and an Belgian farm. Additional information about the two farms was collected. The misclassified eggs from the Austrian farm were labelled Weide Ei (pasture egg). The misclassified Belgian eggs were from a farming system in which antibiotics are not allowed and feed should be composed of 100% plant material. Furthermore, for pigmentation paprika powder is used. The few misclassifications for conventional eggs confirm the results of Van Ruth *et al.*, 2011, showing that eggs from Dutch farms in transition to organic production or low-input farms, produced eggs with similar carotenoid profiles as organic eggs and were therefore occasionally misclassified.

The EU farms would need to meet common specifications for organic farming. This is in contrast to the non-EU countries, and therefore, these farms were a serious challenge. The eggs from organic farms from Canada, Israel and Norway were classified correctly, except for a single Israeli farm. However, out of the nine Turkish organic farms, eggs from seven of these farms were incorrectly classified. These eggs were collected from local open markets and were not stamped like the EU eggs, and therefore without any form of identification. An additional, larger scale study would be needed to elucidate if these discrepancies are due to different organic farming management practices in Turkey. Pivotal would be egg collection at the farms to rule out any form of swapping, etc.

4. Conclusions

The carotenoid-fingerprint based methodology calibrated with Dutch eggs (Van Ruth *et al.*, 2011) was successful in predicting the farming management system of the eggs originating from EU farms (98% sensitivity; 93% specificity), as well as those from the Canadian, Israeli and Norwegian farms (94% sensitivity). The model fitted adequately for samples from the north, central, and southern part of Europe. The identity of the eggs from Turkey was consistently predicted incorrectly (22% sensitivity) and needs further research.

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