Application of Multiple Stable Isotopes to Aid Identification of the Origin of Regional and Organic Animal Products in Hesse, Germany

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Abstract

There is an increasing global demand for regional and organic produce. However, the growth of these markets depends on consumers' trust. Thus, novel methods must be developed to aid the verification of the origin of produce. We built on our previous study, to identify the geographical origin and production method of animal-derived food products. 38 samples of eggs, 99 of milk, 34 of beef and 62 of pork were collected from different regions in central Germany and analysed for their stable isotope composition. The analysis followed a single-variate authentification approach using 5 isotope signatures, $\delta^{18}O$, $\delta^{2}H$, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$. The best performing indicators for verification of the geographical origin were $\delta^{15}N$ and $\delta^{34}S$ for beef; $\delta^{18}O$, $\delta^{2}H$, and $\delta^{13}C$ for milk, and $\delta^{2}H$ and $\delta^{13}C$ for pork. These tracers indicated

statistically significant differences among regions with the exception of pork, the results recorded for eggs were inconclusive. It was possible to distinguish between production methods by means of $\delta^{15}N$ and $\delta^{34}S$ (beef); all 5 tracers (eggs), and $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ (milk). This study demonstrated how the analysis of stable isotopes can be employed to determine the geographic region of origin and production method of animal-derived products in Germany.

Keywords: food authenticity; origin of foodstuffs; analysis of stable isotopes; regional origin.

1 Introduction

In recent years, the demand for regional and organic food products has increased, partially due to the Covid-19 pandemic [1]. Studies have shown medium-term changes in daily routines and attitudes towards food, which are particularly apparent in nuclear family and senior households [1]. These changes include a preference for regional products and increased awareness of the geographical origin of food [1]. Consumers are willing to pay more for regional and/or organic food whilst relying on the information regarding the foodstuffs' authenticity provided by the manufacturer [2,3].

Thus, the fraudulent sale of non-organic/non-regional products labelled as organic/regional became profitable [4]. Numerous laws and regulations prohibit the mislabelling of foodstuffs (e.g., the European Union, EU 2018/775) but there are reported cases of mislabelling and fraud with regard to the regional and/or organic products [2,5]. Animal products, in particular, are vulnerable to mislabelling as a result of their worldwide importance to the food sector and the European food market. Furthermore, public debates on the topics of live-animal transports and animal welfare are contributing to the rising demand for e.g., regional products [3]. In line with the public interest, the European Commission has set an ambitious goal of increasing the proportion of organic agricultural land to 25% by 2030 [6,7].

The analysis of natural abundance stable isotopes has become an important tool for discrimination and verification of the origin of food products [8-15] and was shown to have potential for identification of the production method i.e., organic *vs.* conventional [10]. This methodology can be used in studies of animal-derived foods such as eggs, milk, and meat because the isotopic composition of animals reflects their diet and the region of origin of feed and drinking water, similarly to the factors that result in regional variation in plant products described in Gatzert et al. [16]. In contrast to plant products, however, the analysis of animal

63 products is more challenging because of many factors and processes that can affect the
64 discrimination of region of origin and production method. These factors can include e.g.
65 complex metabolic system which involves different isotope fractionation processes when feed
66 and water is digested and rebuild into muscles and other body parts [11,12]. Similarly, animal
67 feed is often a mixture of different feedstuffs, which can be imported (e.g., soybeans),
68 concentrated, ensiled or be derived from grazing (e.g., on local pastures) [17-19]. Coarse and
69 fresh feed typical of organic farming is often limited to the regional sources in contrast to the
70 concentrated feed commonly brought in from abroad and used in conventional farming [20].
71 Ruminants such as cattle need animal feed with a minimum amount of fibrous fodder, which is
72 not the case for pigs and chickens [21,22]. Pigs and chicken are monogastric i.e., they dependent
73 on animal feed with essential amino acids [23]. The feed of monogastric animals can consist of
74 feedstuffs such fish meal and, more recently, processed protein from farmed insects [24,25].
75 Traditionally, organic farms use a higher quantity of feed arising from their own production,
76 which is in line with one of the major principles of organic farming i.e., to have closed nutrient
77 cycles on the farm [26].

78 Another factor influencing the discrimination of animal origin and the production method
79 involves the sampling methodology. The selection of body parts designated for sampling and
80 the associated timing are important to consider because meat shows variable amino acid
81 patterns during different parts of the year as a result of seasonal feed composition and drinking
82 water intake [17,27-30]. Finally, the age of the animal can have a significant impact on the
83 isotopic composition of its tissues because a lower body size corresponds to a higher body
84 surface area in relation to body mass and thus, an increased fractional loss of oxygen via
85 respiratory air and skin is expected [31,32].

86 The aim of this study was to investigate what isotopes were best suited to differentiate the
87 geographic origin of animal-derived products i.e., eggs, milk, beef and pork, and to differentiate
88 their production methods (organic vs. conventional) to help to uncover and reduce food fraud.
89 We further hypothesised that the isotope ratios of organic products were more suitable for the
90 discrimination of origins because the absence of imported feed decreased the potential for
91 isotope values being influenced by additional sources. We compiled a dataset of four types of
92 animal products (eggs, pork, beef, and milk) from two farming regimes (organic and

conventional) across different regions within Germany (with focus on the Federal State of Hesse). We explored multiple Stable Isotopes to test whether there were systematic differences in oxygen ($\delta^{18}O$), hydrogen ($\delta^{2}H$), carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$) and sulphur ($\delta^{34}S$) isotope composition due to their region of origin (on post-code and district-levels) and production method (organic *vs.* conventional farming). For comparing production methods, the samples were grouped by their documented production method into certified organic or non-certified organic i.e., conventional. The verification of the production method was accounted for because previous studies showed that the isotope ratios might differ between organic and conventional farms [16,33,34].

2 Material and Methods

2.1 Sampling

To verify the geographic origin and the production method of selected animal products, a total of 233 samples (pork meat derived from *Sus scrofa domesticus*: N=62, beef meat derived from *Bos taurus*: N=34, milk derived from *Bos taurus*: N=99 and eggs derived from *Gallus gallus*: N=38) were collected in 2013 and 2014 as part of the Watermark project [35]. The Watermark project covered regions within the Federal State of Hesse (Germany). After collection, the samples were sorted in accordance with their origin i.e., five-digit post code regions, where the first number denotes the zone followed by the second digit representing the region. Further three digits define municipalities ordered alphabetically or according to the population size. We limited our analyses to the first two digits, using samples from the following post codes: 34xxx-37xxx and 60xxx and 65xxx. It is important to note that whereas geographical barriers such as mountain areas might better reflect the geographical circumstances of studied region, these are neither officially recognized nor documented in contrast to well-defined post code regions.

Samples from grocery stores (indicated with their origin) were bought and compared in the testing of the region of origin, after validation. An overview of all samples is given in Table 1. The sampling locations of milk, egg, pork, and beef samples within the state of Hesse are displayed in Figure 1.

A set protocol for the collection of samples was developed and carried out by trained staff. Pork and beef meat samples were obtained from slaughterhouses. Care was taken to ensure that the same muscle tissue from the same body part of an animal was sampled. Milk and egg samples were collected directly on farms with GPS references for the sampling locations recorded

124 together with postal codes and additional data comprising sampling dates, and the farming
125 method (organic or conventional). For each sample, an individual document containing farm
126 data, a photo of the collected product and the farm's address was generated.

127

128 **[Table 1 near here]**

129

130 During sampling in slaughterhouses, the samples were collected when the ear tag was still intact
131 so the origin could be traced back with a high degree of certainty. This approach, alongside our
132 standardised sampling protocol, ensured that the collected samples were authentic and have not
133 been mixed or contaminated with other sampling material from different regions of origin
134 and/or production methods.

135

136 [Figure 1 near here]

137

138 **2.2 Sample preparation and isotope analysis**

139 We analyzed the stable isotopes of oxygen, hydrogen, carbon, nitrogen, and sulphur of various
140 fractions of animal-derived products. Sample preparation and isotope analysis were carried out
141 by Agroisolab, Juelich, Germany, which specialises (> 20 years of experience) in using stable
142 isotopes for authenticity analysis (www.agroisolab.de).

143 To separate the water fraction of the beef and pork samples, between 10 to 15g of comminuted
144 muscle meat was separated into water and dry matter using freeze-drying. A modified Ritenberg
145 apparatus was developed for freeze-drying. The modified apparatus comprised spherical vessels
146 and Teflon valves which could dispense lubricating grease to guarantee a higher degree of
147 impermeability. The meat was frozen with liquid nitrogen in short piston. A vacuum of 1 mbar
148 was applied and the water trap (long piston) was inserted into the Dewar vessel filled with liquid
149 nitrogen. The samples were freeze-dried for 15 hours until complete separation of water was
150 achieved. The processing of the sample was repeated if a deviation of 3% or more was observed
151 in the total balance of dry matter and water phase. During later stages of the analysis, we noted
152 the effects of distillative fractionation (a source of error for the $\delta^{18}O$ and $\delta^{2}H$ analysis) which

were indicative of incomplete separation of water. This data was therefore not used in the analysis anymore.

The fats were extracted with dichloromethane (extraction agent) for 6 hours in Soxhlett apparatus. Dichloromethane was shown to be a good extraction agent because it was not flammable, unlike petroleum ether or diethyl ether, and it does not cause isotope fractionation in the samples. The raw protein from fat extraction was trickled in the drying oven for 2 hours at 80C and finely ground with a ball mill to homogenize the sample. The C, N and S isotope ratios were determined from the crude protein fraction (for more details, see Boner [17]), using Na_2WO_4 and the acidification protocol, following the AOAC 988.12 method [36].

We used whole eggs for processing. The shells were cleaned with water and isopropanol, and dried to determine the $^{18}O/^{16}O$ and $^{13}C/^{12}C$ isotope ratios. Extracts from the eggs' lipid fraction were analysed and might be used in future studies to determine the $^{13}C/^{12}C$ isotope ratios. For separation of the water fraction, the apparatus and process described above was used.

The three fractions (raw protein, fat, and water) were analyzed with different isotopic analytical tools (see Supplementary Information 2 and 3). Each set of samples included repeated analyses of in-house standards. Samples were analysed with two laboratory replicates and the results were accepted when the absolute difference between the measurements was ≤ 0.3 ‰ (we used the arithmetic mean for statistical analyses). If the differences between two measurements were higher than the limit (0.3 ‰), the samples were reanalyzed. The results were reported with respect to accepted international standards (see Table 2 in the Supplementary Information), according to equation 1:

$$\delta \text{ value} = \left(\frac{R_{sample} - R_{standard}}{R_{standard}}\right) * 1000 \text{ (in ‰)} \qquad \text{Eq. 1}$$

For oxygen and hydrogen, we used the Vienna Standard Mean Ocean Water (V-SMOW2) [‰] [37] as reference material. For carbon, we used the Vienna Pee Dee Belemnite standard (V-PDB). For nitrogen, we used the atmospheric air standard (AIR), and for sulphur, we used the Vienna Canyon Diablo Triolite standard (V-CDT) [38,39]. The results were described as δ^2H , $\delta^{18}O$-, $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ values. For beef and pork meat samples, hydrogen and carbon were measured from the lipid fraction (Supplementary Information Part 1). The working and calibration standards for different matrices are given in the Supplementary Information Part 2.

The criteria for quality assurance and quality control were fulfilled by Agroisolab, which takes part in annual international ring tests of the IAEA and the European Proficiency testing [35]. The following reproducibility (1σ) of the isotope measurement was achieved in routine testing: $\delta^{18}O_{(water)} \leq 0.2$ ‰, $\delta^{2}H_{(water)} \leq 1.5$ ‰, $\delta^{15}N \leq 0.2$ ‰, $\delta^{13}C \leq 0.2$ ‰, $\delta^{34}S \leq 0.3$ ‰, $\delta^{18}O_{(organic)} \leq 0.3$ ‰, $\delta^{2}H_{(organic)} \leq 2.0$ ‰.

2.3 Data and statistical analyses

Sample preparation and pooling:

The samples were pooled into different groups to allow for multiple group comparisons. For the production method comparison, we grouped samples as organic and conventional and with regard to their region of origin i.e., from the state Hesse (in-state) and outside of the state of Hesse (out-of-state). For comparisons of the regions of origin (at the post code level and the state level), the stable isotope data of each product were allocated to the post code regions. Beef, pork, and egg samples were only allocated to post code regions 3 and 6. Milk samples had a higher total number of samples and were allocated to four post code regions.

Statistical Data analysis:

Data was analysed in SPSS (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). For each group, descriptive statistics was compiled, followed by an Analysis of Variance (ANOVA). For groups of more than two regions, a post hoc test was computed to determine which regions differed significantly from each other (α=0.05). We selected the Dunnett T3 as a suitable post hoc test due to its robustness [40]. When results were not normally distributed or had heterogeneous variances, a non-parametric test was performed (e.g., Kruskal-Wallis-Test). Box-and-whisker plots were used for visualisation of descriptive statistics. Data distribution determined whether a parametric (Student's t-test) or nonparametric (Mann-Whitney-U-test) statistical test was used to compare two sample means e.g., for a comparison between organic and conventional products.

Beef samples could not be analysed with an ANOVA and box-and-whisker plots and required a polar transformation of the sulphur and nitrogen data to allow for discrimination between beef samples obtained from organic and conventional farms. Polar transformation results in a higher discrimination power in comparison to a traditional Principal Component Analysis (PCA). In

this coordinate transformation, the Cartesian coordinates (x, y) were transformed to the polar coordinates (r, θ), using the following relationship between the two:

$$r^2 = x^2 + y^2 \quad \text{Eq. 2}$$

$$\theta = tan^{-1}\left(\frac{y}{x}\right) \quad \text{Eq. 3}$$

The rotation matrix with an angle ∅ was used to orientate the polar coordinates (r, θ) to have the maximum variance of the beef samples on one axis (similar to the rotated PCA method):

$$\begin{pmatrix} cos\,(\emptyset) & -sin(\emptyset) \\ sin(\emptyset) & cos(\emptyset) \end{pmatrix} \quad \text{Eq. 4}$$

After the application of the rotated polar transformation, we could discriminate between the conventional and organic production method for beef samples with an 85% accuracy.

3 Results and Discussion

The aim of our study was to test the authenticity and credibility of animal-derived products by means of stable isotopes of hydrogen, oxygen, nitrogen, carbon and sulphur. We analysed whether their isotopic composition differed significantly with regard to their region of origin (at post code and state levels) and production method (organic *vs.* conventional). The results (with standard deviations) of the isotopic composition of animal-derived products were provided in Table 1 in the Supplementary Information.

3.1 Geographical origin

Beef

Previous studies showed that δ^2H and $\delta^{18}O$ tracers could be successfully used for distinguishing between regions that are further apart, e.g., at country-level, because hydrogen and oxygen isotopes are likely to be affected by variations in precipitation, wind direction, altitude, and topography [16]. Over smaller distances, precipitation, altitude, or topography would not be sufficiently different to allow for individual regions to be differentiated [17,20,41-43]. In our study, neither $\delta^{18}O$ nor δ^2H values could be used to distinguish between post code regions ($\delta^{18}O$: region 3= -6.2 ‰ ±0.8; region 6= -6.1 ‰ ±1.0) for beef. This might be due to the feed outweighing the effects of drinking water, especially in our study, which in contrast to other investigations, examined nearby regions. In addition, the sampling size of animal-derived

products from outside of the state of Hesse was low and the samples were collected in winter and summer (for comparisons of in-state and out-of-state meat), resulting in a possible bias. There were no statistically significant differences for the values of $\delta^{13}C$ in respect to the region of origin and the production method. This could be due to the mixing of local coarse cattle feed and imported concentrated cattle feed and its impacts on the isotopic signatures. Additional variability might be a result of homogenous feed ratios of C_3 and C_4 plants, which are known to differ in their isotopic composition [41].

In contrast, the $\delta^{15}N$ values were useful for discrimination between post code regions 3 (+5.1 ‰ ±1.1) and 6 (+5.9 ‰ ±1.2) (p=0.05). This could be due to regional differences in the feed but also resulting from different underlying physiological processes. Ten amino acids are essential and thus, must be provided to cattle via feed. The remaining 10 amino acids can be produced by cattle themselves. Therefore, the $\delta^{15}N$ values can vary between amino acids by up to 25% [17]. In the present study, the $\delta^{15}N$ values were between +4.2 ‰ and +6.6 ‰ i.e., within the expected range described in studies of beef in Japan (+7.2 ‰ to +8.1 ‰), the U.S. (+5.1 ‰ to +7.8 ‰) and Australia (+5.7 ‰ to +9.3 ‰) [44].

Muscle tissues have a high S content with 2 g kg^{-1} fresh mass compared to 0.2 g kg^{-1} for bones and 0.3 g kg^{-1} for milk [45]. However, there is limited information on the effect of sulphur isotopic composition on the metabolism of cattle with previous studies using sulphur isotopic composition to investigate the origin of animal feed [23,46]. In this work, the $\delta^{34}S$ values were used to distinguish between beef from post code regions 3 (+5.8 ‰ ±0.7) and 6 (+4.9 ‰ ±0.5) (p = 0.001) and for in-state (+5.5 ‰ ±0.7) and out-of-state samples (+3.5 ‰ ±1.4) (p = 0.05). This might be because local soil conditions are reflected in sulphur isotope values of plants which are, in turn, mirrored by the cattle that consumes local coarse feed.

Pork

Samples collected from conventional farms could be linked to their post code regions 3 and 6 (p=0.025; region 3: -255.0 ‰ ±7.4; region 6: -260.7 ‰ ±11.2) through the investigation of $\delta^{2}H$ values in lipids. However, it was not possible to determine if the feed or the drinking water had a greater impact on the $\delta^{2}H$ values of pork. We hypothesised that drinking water would be a leading factor because the animal feed in conventional farming was imported. For samples

collected from organic farms, no comparisons were made in regard to their region of origin because all six samples were collected from post code region 3.

Samples collected in-state *vs*. out-of-state were significantly different in regard to the $\delta^{13}C$ values in lipids (Hesse: -25.1 ‰ ±0.6; out of Hesse: -22.2 ‰ ±0.1) (p=0.000). We hypothesised that the ratio of maize (Zea mays), soybeans (Glycine max), and wheat (Triticum aestivum) in animal feed might differ by region and for individual farms. Since wheat and maize are grown by farmers who also keep livestock [47] and are fed to pigs in higher quantities than soybeans, the regional origin of feed could affect the animals' isotopic composition. Overall, the $\delta^{13}C$ values in our study averaged -25.1 ‰ ±0.6 (Hesse) in comparison to previously reported value of -22.5 ‰ measured for pigs on diets combining terrestrial and marine feed components [48]. Webb et al. [49] found that the percentage of marine feed in the pigs' feed correlated with the $\delta^{13}C$ values, possibly due to the increased routing of non-essential amino acids, e.g., glycine, with increasing marine components [49]. In a Polish study, the $\delta^{13}C$ values were -24.5 ‰ for conventional and -23.6 ‰ for organic pig meat [50].

The $\delta^{15}N$ is considered a poor quality indicator for animal products because the range of values is narrower than for plant-based products (+1.2 ‰ for leguminous plants and +3.3 ‰ for commercial concentrates) [12] and might depend on the feed and not the geographical origin. Furthermore, the $\delta^{13}N$ content found in animal products can reflect the level of fertilisation of plants which are fed to animals [51], with an increase of around +2.3 ‰ ±0.2 for each trophic level [52]. This enrichment (from one trophic level to another) allows for determination whether herbivores are fed with animal products such as milk or animal meal [53-57]. However, previous research indicated that $\delta^{15}N$ can be used in some applications e.g., for discrimination between local regions but not between countries [55]. Our study found no significant differences between different post code regions likely due to soybean, which constitutes a large component of pig feed and is imported from multiple countries.

Non-ruminant animals must consume enough feed comprising sulphur and sulphur-containing amino acids such as methionine which cannot be synthesized by pigs and chickens [43]. Examples of animal feed containing large quantities of sulphur include fish meal [48] and seaweed [43,51], both of which have an impact on the pigs' $\delta^{34}S$ status. In our study, the discrimination on post code regions for ^{34}S-ratios was not feasible. However, in-state (+5.2 ‰)

297 and out-of-state (+2.4 ‰, n=4) samples were shown to be significantly different in respect to
298 δ^{34}S-ratios. It is important to note that feeding practices in different countries as well as the
299 distance to sea (sea spray can influence background isotopic composition) could impact values
300 for δ^{34}S e.g., values of +10.9 ‰ and +20.7 ‰ were found for pigs with the latter recorded for
301 pigs fed with diets with marine components [48].

302 **Eggs**

303 For eggs, the δ^{2}H values tend to be considered more reliable than the δ^{18}O values because
304 previous research showed that storage time to affect the δ^{18}O ratio i.e., after 8 weeks of storage,
305 eggs were found to be enriched in ^{18}O due to diffusion of carbon dioxide through the eggshell
306 [59]. In addition, the δ^{2}H isotope values are influenced primarily by drinking water, while the
307 chickens' δ^{18}O isotope ratio can be derived from atmospheric oxygen, oxygen bound in the feed
308 and the water within the feed, each with specific δ^{18}O values [60]. In this study, we ensured
309 that eggs were stored for a limited time (< 2 days) so that the δ^{18}O ratios could not be affected
310 by atmospheric carbon dioxide diffusion. The δ^{18}O values were used to discriminate between
311 in-state (-5.4 ‰ ±0.6) and out-of-state samples (-4.2 ‰ ±0.5) (p=0.000). We note that there
312 were only seven samples from outside of Hesse and consider the sampling size a limitation of
313 this study.

314 Further significant differences in δ^{2}H values were found for samples collected from post code
315 regions 3 (-45.7 ‰ ±4.2) and 6 (-42.5 ‰ ±3.3) (p=0.018). Research in Germany indicated
316 conventional eggs to have lower variation in δ^{2}H in response to lower variation in the feed [59].
317 Other studies used δ^{2}H of feathers to track bird migration and dispersion of populations
318 throughout the year and demonstrated that the δ^{2}H values of feathers correlated strongly with
319 precipitation at breeding sites [57,61]. In contrast to wild birds, chicken receive groundwater as
320 drinking water which makes it easier to distinguish between regions over smaller distances.

321 For samples collected from organic farms, discrimination with respect to their region of origin
322 was possible by means of δ^{13}C values (region 3: -25.6 ‰ ±1.1; region 6: -26.9 ‰ ±0.8;
323 p=0.031). In organic farming, chickens have access to a wider variety of feed which could
324 contribute to their δ^{13}C ratios. For example, chickens show negative δ^{13}C values when they
325 consume insects and other small animals due to isotopic enrichment with every trophic level
326 [54]. Additionally, organic chickens are more commonly fed with locally produced and highly

variable feed. In conventional farming, feeding conditions are largely homogenous with imported feed that does not vary throughout the year [53,56,59,62].

In our study, the $\delta^{13}C$ values ranged from -22.9 ‰ to -28.2 ‰, which was comparable to studies from the Netherlands where values ranged from -22.3 ‰ to -16.3 ‰ and from New Zealand where values ranged from -19.6 ‰ to -21.3 ‰ [53]. In Poland, values for eggs ranged from -28.6 ‰ and -22.6 ‰ for yolk and from -25.0 ‰ to -20.7 ‰ for albumen [50]. In our study, only the egg albumen was analysed statistically. We hypothesise that discrimination between countries by means of $\delta^{13}C$ values is not feasible because the values of different countries overlap. However, differentiation over smaller distances might be feasible. Our results showed significant differences between regions of origin for eggs in respect to $\delta^{13}C$ values. There were no statistically significant differences between regions of origin of eggs when discriminated by means of $\delta^{34}S$ values.

Milk

For samples collected from organic farms, $\delta^{18}O$ and $\delta^{2}H$ could be used for distinguishing between post code regions. The post code regions 35/36 (-7.3 ‰ ±0.5) and 6 (-6.6 ‰ ±0.4) were distinguished at a significance level of 0.05 by means of $\delta^{18}O$ values. The post code regions 34/37 (-49.6 ‰ ±3.4) and 6 (-45.0 ‰ ±2.0) and regions 35/36 (-50.1 ‰ ±4.7) and 6 (-45.0 ‰ ±2.0) were distinguished by means of $\delta^{2}H$ values. The milk of organic cows is likely to strongly reflect the regional $\delta^{18}O$ and $\delta^{2}H$ values because the animal feed in organic farming is commonly sourced and produced locally [26] and thus, the isotopic signatures have less potential to be affected by imported concentrated feed.

For samples collected from conventional farms, no differences in $\delta^{18}O$ or $\delta^{2}H$ ratios were found. This might be due to animal feed in conventional farming commonly having a higher content of concentrated feed such as maize and soybeans, which might be imported from different parts of the world [63]. Once mixed with local feed, imported feed can affect isotopic ratios. In addition, we note that there were no samples from region 34/37 and consider this a limitation of this study.

When organic and conventional milk samples were pooled and analyzed together, it was possible to distinguish between different regions. A post hoc test indicated regions 35/36 (-7.1 ‰ ±0.6) and 6 (-6.7 ‰ ±0.5) to be significantly different ($p=0.009$), distinguished by means of

$\delta^{18}O$ values. Similarly, regions 34/37 (-49.6 ‰ ±3.4) and 6 (-45.8 ‰ ±2.8) (p=0.003) and 35/36 (-48.5‰ ±4.9) and 6 (-45.8 ‰ ±2.8) (p=0.007) were shown to be statistically different, distinguished by means of $\delta^{2}H$ values. We expect an increase in the sampling size (relative to subgroups of organic or conventional samples) to be driving the observed differences.

Statistically significant differences between the post code regions were found for milk by means of $\delta^{13}C$ (region 34/37= -27.7 ‰ ±0.8; region 35/36= -25.8 ‰ ±2.8; region 6= -23.2 ‰ ±3.3). The $\delta^{13}C$ measured in this study was comparable to previous research with values ranging from -22.9 ‰ to -31.5 ‰ for 35 German samples [64]. Other studies reported that the $\delta^{13}C$ values in milk varied seasonally [12,65], which is likely due to different feed proportions of C_3 and C_4 plants in winter and summer [27]. In this study, all samples were collected at the same time (except for additional samples that were purchased), so seasonal variations should not result in a strong bias. The proportion of C_3- and C_4- plants in animal diet is strongly reflected in the $\delta^{13}C$ value of milk [12], indicating $\delta^{13}C$ as a promising tracer for the authentication of organic vs. conventional milk. For example, milk from cows in regions dominated by grasslands is commonly characterised by negative $\delta^{13}C$ values in comparison to milk from cows in regions dominated by cropland. In the case of the latter, high quantities of maize are cultivated, resulting in animal feed containing proportionally more maize (C_4-plant) [66,67]. Furthermore, it is known, that the $\delta^{13}C$ values are higher in the protein than in the lipid fractions [41,51,55, 66,67] and the enrichment of $\delta^{13}C$ values from one trophic level to the next is low (roughly +0.5 ±0.13 ‰), but increases throughout the animals' lifespan because of the weight gains [52,68].

Statistically significant differences among post code regions were found for milk by means of $\delta^{15}N$ values. It was possible to discriminate all regions via a post hoc test 34/37 (+4.3 ‰ ±0.6) to 6 (+5.5 ‰ ±0.9), p=0.000; 34/37 (+4.3 ‰ ±0.6) to 35/36 (+4.8 ‰ ±1.0), p=0.043; and 35/36 (+4.8 ‰ ±1.0) to 6 (+5.5 ‰ ±0.9), p=0.008. Furthermore, we found significant differences between the post code regions when comparing organic and conventional samples pooled and analysed together. This is in line with previous research which successfully used $\delta^{15}N$ values (measured for butter) to discriminate between regions characterised by intense agricultural use compared to those with more extensive agriculture [27]. The $\delta^{15}N$ values in our study showed a mean value of +5.0 ‰ ±1.0. These values were comparable to $\delta^{15}N$ values from another study in Germany, which ranged from +3.9 ‰ to +6.1 ‰ for milk [68].

There were no statistically significant differences between regions of origin of milk when discriminated by means of $\delta^{34}S$ values. The $\delta^{34}S$ values are commonly considered unsuitable for differentiation between production regions of milk [69]. This is likely due to high levels of variation in plants resulting from their geographical location, fertilisation, and deposition. However, little is known about fractionation processes in cattle when testing milk samples [27,69]. We note that whereas $\delta^{34}S$ was not useful for discriminating between regions for milk samples, it was useful for beef samples. This could be due to different metabolism of muscle and milk production i.e., milk is produced daily whereas muscular hypertrophy requires longer timeframes. Different feeding practices implemented on dairy *vs.* beef farms could also be a factor influencing measured isotopic ratios.

3.2 Production method

Beef

No significant difference was found between conventional and organic samples by means of $\delta^{18}O$ and $\delta^{2}H$ ratios. Sulphur and nitrogen isotopes were useful to discriminate between organic and conventional farms but simple clustering approaches were insufficient to clearly identify the regions. There were significant differences for bulk $\delta^{15}N$ ($p=0.04$) (Figure 2a) when organic and conventional samples were compared but there were no statistically significant differences for bulk $\delta^{34}S$ ($p=0.07$) (Figure 2b). Thus, we used a polar transformation to obtain a linear separator between organic and conventional samples (see Section 2.3). The application of polar transformation allowed for discrimination between conventional and organic beef samples based on the nitrogen and sulphur isotopes ($p=0.006$) (Figure 2c).

[Figure 2 here]

Pork

There was no statistically significant difference between organic and conventionally produced pork by means of $\delta^{18}O$, $\delta^{2}H$, $\delta^{13}C$ and $\delta^{15}N$ (Figure 3a-d). The use of imported animal feed could contribute to distorted isotope ratios as both systems use at least some feed produced outside of the farm and region. However, we found significant differences between conventional samples and organic samples ($p=0.000$) by means of bulk $\delta^{34}S$ (Figure 3e). It is important to note that there were only 6 samples from organic farms (region 3 only).

[Figure 3 here]

A study on Polish pigs found that $\delta^{15}N$ values were +3.5 ‰ and +4.8 ‰ for conventional and organic pork, respectively [50]. In our study, similar values were found but no differences could be detected for conventional and organic samples (both approx. +5.1 ‰). In the UK, $\delta^{15}N$ values ranging from +2.2 ‰ to +11.6 ‰ were recorded, showing a strong dependency on soybeans and marine feed components [58]. There were no significant differences found for $\delta^{15}N$ in pork protein between organic and conventional samples.

Eggs

We found statistically significant differences between organic and conventionally produced eggs by means of all isotopes. The groups were significantly different on a 0.05-level of confidence for bulk $\delta^{18}O$ (Figure 4 a) and bulk $\delta^{15}N$ values (Figure 4 c), on a 0.005-level of confidence for bulk $\delta^{2}H$ (Figure 4 b), bulk $\delta^{13}C$ (Figure 4 d) and bulk $\delta^{34}S$ (Figure 4 e).

[Figure 4 near here]

Our findings are in line with previous research demonstrating that organic eggs could be discriminated by means of stable isotope analysis. Rogers et al. (2009) reported that organic and free-range egg components showed an enrichment of up to 4 ‰ of ^{15}N values in comparison to caged and barn-laid eggs [70]. The enrichment might be a result of diets with higher animal protein content which is characteristic of free-range chickens. The $\delta^{13}C$ values in the study by Rogers et al. (2009) was unsuitable for differentiation between organic and conventional eggs [58]. A large 2015 study used stable isotopes to verify the housing conditions of chickens (cage, barn, free range, and organic farming) in the Netherlands and New Zealand [53]. Samples of poultry feed and egg albumen collected in the Netherlands were used to determine the isotopic composition of organic and conventional feed and to determine whether there were differences in isotopic values in regard to laying regimes. Further 52 eggs samples from New Zealand were collected. In both countries, eggs collected from conventional and organic farms were discriminated by means of stable $\delta^{13}C$ and $\delta^{15}N$ isotopes. The difference between farming methods was attributed to feed composition. The authors showed stable isotopes as a promising screening tool for authentication of farming methods and proposed upper limits of 4.8 ‰ and 6.0 ‰ ($\delta^{15}N$) for eggs to be classified as organic in the Netherlands and New Zealand,

445 respectively [53]. In our study organic eggs showed higher $\delta^{15}N$ values (5 ‰) relative to
446 conventional eggs, for whole egg and not solely for egg whites (Figure 4c).

447 **Milk**

448 We found statistically significant differences between organic and conventionally produced
449 milk by means of $\delta^{13}C$ (p=0.000) (Figure 5 a), $\delta^{15}N$ (p=0.000) (Figure 5 b) and $\delta^{34}S$ values
450 (p=0.012) (Figure 5 c).

451 **[Figure 5 near here]**

452 The mean $\delta^{13}C$ ratio of organic milk in our study was higher in comparison to conventional
453 milk (Figure 5a), while the mean $\delta^{15}N$ ratio of organic milk was lower than the ratio of
454 conventional milk (Figure 5b). A study from Chung et al. (2014) found, that combining the
455 $\delta^{13}C$ and $\delta^{15}N$ ratios was more effective at distinguishing between farming methods in
456 comparison to using individual tracers [71]. These results are supported by findings from other
457 studies, where the authenticity of organic milk was determined with $\delta^{13}C$ and $\delta^{15}N$. For
458 example, Chung et al. (2020) found that $\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$, and $\delta^{34}S$ signatures of organic milk
459 could be used to distinguish among four regions in Korea. The $\delta^{13}C$ tracer was identified as the
460 most important predictor for reliable geographical discrimination (probability of wrong
461 classification < 5%) [72]. In our study $\delta^{13}C$ could be used to discriminate between both, the
462 region of origin and the production method of milk.

463 In other studies, where $\delta^{13}C$, $\delta^{15}N$, fatty acids, and vitamin E of organic and conventional milk
464 were tested with chemometric methods [73], the mean $\delta^{13}C$ and $\delta^{15}N$ were found to be lower in
465 organic milk [73,74]. This was also the case in our study (Figure 5a-b). Furthermore, previous
466 research indicated that organic and conventional milk samples differed in the total N, non-
467 protein N and milk urea content. Conventional milk was shown to have a higher level of total
468 milk urea N and a higher proportion of the total N and non-protein N fractions. Zhukova et al.
469 (2016) found that the ratio of urea N to non-protein N of milk was the most significant criterion
470 for the assessment of differences in animal diets [75]. In another study, researchers found that
471 the peptide of Thr-Ala-Val, trimethylamine *N*-oxide and D-biotin could act as metabolite
472 markers for distinguishing between organic and non-organic milk, depending on the race of the
473 studied cows [76].

4 Compilation

Here, we provide a brief summary of our findings (Table 2). For beef, significant tracers for discriminating among the regions of origin were $\delta^{15}N$ and $\delta^{34}S$ and for distinguishing between farming methods (organic vs conventional) was $\delta^{15}N$. For pork, significant tracers for discriminating among the regions of origin were $\delta^{13}C$ in lipids and $\delta^{34}S$ in lipids (for in-state vs. out-of-state samples). Distinguishing between farming methods was not possible. For eggs, $\delta^{2}H$ and $\delta^{13}C$ were the best performing tracers for distinguishing between regions of origin (organic eggs) and all isotopes could be used to aid differentiation between farming methods. For milk, $\delta^{18}O$, $\delta^{2}H$, $\delta^{13}C$ and $\delta^{15}N$ were suitable tracers to distinguish between the regions of origin (at post code level). For differentiating between organic and conventional milk, $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values could be used. We showed that verification of the origin of organic animal products was less challenging than of conventional products, likely due to the guiding principle of organic farming i.e., a closed nutrient and thus, animal feed cycles. Feed and drinking water are commonly the main drivers for differences in isotopic value with few other potential factors that might supersede the isotopic signature of the product's origin. However, when a similar feeding regime is followed by organic and conventional farms, e.g., for pigs, the stable isotope method can have a lower discriminatory power. Finally, meat was found to be more difficult to categorise in comparison to milk and eggs, likely due to the latter being produced "every day" and reflecting animals' geographic region of origin and production method quickly in comparison to meat tissue that is gained over longer timeframes and thus, has a longer reaction time. Overall, our approach showed the best results for milk samples.

[Table 2 here]

5 Conclusion

We investigated the application of multiple stable isotopes for authenticity testing of four animal-derived products (beef and pork meat, milk and eggs) in regard to their region of origin (at post code and state levels) and production method (organic vs. conventional). We showed that the stable isotope analysis could distinguish between the regions of origin and production methods of some products. However, not all products could be authenticated. Hence, the stable isotope analysis cannot replace on-site and production-process controls but rather provide supplementary information. Improvements to this study can be achieved through: (1) a more

detailed analysis of the isotopic composition of animal products collected within the scope of the "Watermark project" that provides a baseline for authenticity validation of animal products for the state of Hesse and (2) application of more complex statistical methods such as multivariate methods or machine learning. The latter is crucial because this study demonstrated that it was impossible to unequivocally assign each individual sample to a defined group using only one parameter/isotope. Finally, it is imperative to establish a German food isotope database such as e.g., the English Pork Database and the Egg Database (http://www.agroisolab.com). A major challenge in devising this type of a database will be to develop a global approach to food authenticity testing via isotope tracers as well as formulating an open data policy. Finally, research should focus on a compound-specific isotope analysis, to improve results when bulk stable isotope analysis is insufficient.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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