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# Review: Authentication of grass-fed meat and dairy products from cattle and sheep

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*Meat and dairy products derived from grassland carry premium values and sensory and nutritional qualities that aroused much interest for authentication methods to guarantee grassland origin claims. This article reviews the current state of knowledge on the authentication of meat and dairy of grassland origin from food analysis in both cattle and sheep. A range of methods alone or combined, involving analysis of elemental or molecular constituents of food product and fingerprinting profiling combined with chemometrics, have been developed and proved useful to differentiate contrasted feeding regimes and authenticate grass-fed meat and dairy. Their robustness and discriminatory reliability in more complex feeding conditions, such as in the case of dietary switches or when grass only makes up part of the animal's diet, are under active investigation. Our review highlights the possibilities and limitations of these methods, the latter being chiefly posed by variations in the quantity, characteristics and composition of grassland feedstuffs consumed by animals, which are nevertheless inherent to grassland-based production systems, variations in animal responses within and across breeds, and difficulties in detecting the consumption of non-grass feedstuffs by the animal. It also highlights a number of issues for consideration, points of caution and caveats in applying these methods. Scientists agree that much of the research carried out so far has been a 'proof of concept' type and that efforts should be made in the future to develop more databases to help gain genericity and robustness.*

**Keywords:** grass-feeding, muscle, dairy products, fraud, spectroscopy

## Implications

Meat and dairy products derived from grassland carry premium values and sensory and nutritional qualities that aroused much interest for authentication methods to guarantee grassland origin claims. This article reviews the current state of knowledge on the authentication of meat and dairy of grassland origin in both cattle and sheep. It highlights the possibilities and limitations, together with a number of issues for consideration in applying these methods for authenticating grass-fed meat and dairy.

## Introduction

The authentication of grassland origin of meat and dairy products interests the actors of the food chain for several reasons. Grass feeding of animals meets consumer demands for healthy products produced in a 'natural' way. Consumers actually show growing interest in the method of production of their food and the environment, and grass feeding carries positive values in this regard. Furthermore, a number of scientific studies demonstrated the nutritional advantages of grassland-based meat and dairy products (higher content of nutritionally valuable compounds like specific fatty acids (**FAs**), vitamins and antioxidants) (Aurousseau *et al.*, 2004; Alfaia *et al.*, 2009; Butler *et al.*, 2011). Finally, farmers who are committed to complying with specific production conditions (typically through certified production standards) seek protection against abusers who may be tempted to benefit from the price premium without bearing the corresponding constraints. In view of the potential added value and the additional costs of products, farmers and consumers are thus concerned about the potential risks of fraud, which prompted the development of analytical authentication methods that go beyond on-farm inspection (self-inspection or on-farm audits by independent agencies) to guarantee that a product is effectively standards-compliant. Here we review the current scientific knowledge and methodologies for authenticating grass-fed meat and dairy in both cattle and sheep. The scientific literature regarding the authentication of grass feeding covers a range of situations, such as

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**Table 1** *Analytical methods for the authentication of grass-fed meat and dairy according to the application area*

| Application area | Analytical method | Product | References |
|---|---|---|---|
| Discrimination of grass feeding *v.* grain feeding | FAs | Meat | Aurousseau *et al.* (2004) in sheep; Alfaia *et al.* (2009) and Monahan *et al.* (2018) in cattle |
| | Volatile compounds | Meat | Priolo *et al.* (2004) in sheep; Vasta and Priolo (2006) in cattle and sheep |
| | | Dairy | Viallon *et al.* (2000) in cattle; |
| | Carotenoid pigments | Meat | Prache and Theriez (1999), Prache *et al.* (2003), Dian *et al.* (2007), Oliveira *et al.* (2012), Macari *et al.* (2017) and Devincenzi *et al.* (2019) in sheep; Monahan *et al.* (2018) in cattle |
| | | Dairy | Noziere *et al.* (2006) and Calderon *et al.* (2006 and 2007) in cattle |
| | Vitamin E stereoisomers | Meat | Monahan *et al.* (2018) in cattle |
| | Spectral methods (VIS, NIRS) | Meat | Dian *et al.* (2007 and 2008), Huang *et al.* (2015a and 2015b) and Prache *et al.* (2018) in sheep |
| | Functional genomics | Meat | Sweeney *et al.* (2016) in cattle |
| Discrimination of grass-feeding *v.* maize feeding | FAs | Dairy | Segato *et al.* (2017) and Hurtaud *et al.* (2014) in cattle |
| | Phenolic compounds | Dairy | Besle *et al.* (2010) in cattle |
| | Carotenoid pigments | Dairy | Engel *et al.* (2007) in cattle |
| | Stable isotopes | Meat | Bahar *et al.* (2005 and 2009) in cattle; Monahan *et al.* (2018) in cattle and sheep |
| | | Dairy | Auerswald *et al.* (2015), Kornexl *et al.* (1997), Segato *et al.* (2017) and Renou *et al.* (2004) in cattle |
| | Spectral methods (NIRS) | Meat | Cozzolino *et al.* (2002) in cattle |
| | | Dairy | Coppa *et al.* (2012) and Valenti *et al.* (2013) in cattle |
| | Functional genomics | Meat | Cassar-Malek *et al.* (2009) in cattle |
| Discrimination of feeding fresh grass *v.* feeding conserved grass (hay, haylage, grass silage) | FAs | Dairy | Segato *et al.* (2017) in cattle |
| | Volatile compounds | Dairy | Abilleira *et al.* (2011) in sheep; Coppa *et al.* (2011) in cattle |
| | Phenolic compounds | Dairy | Besle *et al.* (2010) and Rouge *et al.* (2013) in cattle |
| | Vitamin E stereoisomers | Dairy | Butler *et al.* (2011) in cattle |
| | Protein biomarkers | Meat | Gagaoua *et al.* (2017) in cattle |
| | Fingerprint methods (VIS, NIRS, MIR, NMR) | Dairy | Schievano *et al.* (2008), Coppa *et al.* (2012), Andueza *et al.* (2013) and Valenti *et al.* (2013) in cattle |
| Distinction of the nature of the conserved grass forage consumed | Phenolic compounds | Dairy | Besle *et al.* (2010) in cattle |
| | Protein biomarkers | Meat | Gagaoua *et al.* (2017) in cattle |
| Distinction of the nature of the grass consumed | | | |
| Legume *v.* grass | FAs | Meat | Moloney *et al.* (2018) in cattle |
| | Stable N isotopes | Meat | Prache *et al.* (2009), Devincenzi *et al.* (2014), Macari *et al.* (2017) in sheep; Moloney *et al.* (2018) in cattle |
| Cultivated *v.* permanent | FAs | Dairy | Coppa *et al.* (2015b) in cattle |
| Species-rich *v.* species poor | Volatile compounds | Dairy | Abilleira *et al.* (2011) in sheep |

VIS = visible spectroscopy; NIRS = near-infrared spectroscopy; MIR = mid-infrared spectroscopy; NMR = nuclear magnetic resonance spectroscopy.

discrimination of grass-feeding *v.* grain or maize feeding, discrimination of feeding fresh grass *v.* feeding conserved grass (hay, haylage, grass silage), distinction of the nature of the conserved forage consumed (hay *v.* haylage *v.* grass silage), distinction of the nature of the grass consumed (legume *v.* grass, cultivated *v.* permanent pasture, species-rich *v.* species-poor pasture), identification of the dietary proportion of grass and even identification of organic *v.* conventional meat and dairy. This article is an update on recent research conducted in all these situations, and Table 1 summarizes the promising analytical methods in these application areas.

## Discriminating contrasted diets

The diet fed to the animal is one of the most important factors affecting the composition of meat and dairy in cattle and sheep. This effect is due to specific compounds that either transfer directly from feed ration to food or are transformed or produced by rumen microbes or the animal's metabolism under the effect of specific diets. These compounds can thus serve as markers of an animal's dietary background. Furthermore, differences in meat or dairy product composition induce differences in their optical properties, and therefore spectral features, which can also be used to back-authenticate diet. Developments in



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authenticating grass-fed products have thus used two overarching approaches: quantification of specific elemental and molecular constituents of meat and dairy products, and more global fingerprinting profiling methods like product optical properties analysis.

*Elemental and molecular tracers*

*Fatty acids.* In lamb meat, Aurousseau *et al.* (2004) showed that FA analysis enable to 100% discriminate grass-finished lambs from lambs fed a concentrate-based diet in stall. In beef too, Alfaia *et al.* (2009) were able to 100% discriminate between animals fed a barley-based concentrate diet in a feedlot, pasture-fed animals or pasture-fed animals subsequently fed the barley-based concentrate diet for 2 or 4 months pre-slaughter. Likewise, an Irish study showed that FA composition enabled to correctly classify 92.9% beef samples from four feeding regimes: pasture-feeding for 1 year *v.* barley-based concentrate diet for 1 year *v.* grass silage for 6 months then pasture-feeding for 6 months *v.* grass silage for 6 months then pasture-feeding for 6 months with barley concentrate as 50% feed supplement (Monahan *et al.*, 2018). Miss-classified samples came from pasture-fed animals that were classified as fed on grass silage before turning out to pasture. If one had the objective of authenticating grass-fed (pasture or silage) meat, all the samples were thus correctly classified.

In dairy products, several studies have also shown that milk FA analysis enabled authenticating grass-fed milk from dairy cattle. Comparing tanker bulk milk from farms where herds were fed mainly grass (more than 75% of the total amount of forages offered) or mainly maize silage (more than 30%), Engel *et al.* (2007) showed that milk FA came out with the best discriminatory power (ahead of milk fat, protein, lactose, fat soluble vitamins, carotenoid pigments, colour and volatile compounds). In cheese, Segato *et al.* (2017) obtained similar results when comparing several feeding regimes (pasture *v.* hay *v.* maize silage). With a large number of milk samples coming from a variety of farms in Europe, Coppa *et al.* (2015a) confirmed that milk FA analysis successfully discriminated contrasted feeding regimes (diets of which more than 50% of the DM consisting a particular forage have been assigned to a specific feeding regime). However, when milk samples derived from diets comprising several forages none of which was dominant (>50% in the diet) were included in the dataset, these authors observed that FA analysis significantly loses discriminative reliability (91% *v.* 84% of samples correctly classified).

Moving beyond just grass-feeding, Moloney *et al.* (2018) used beef FA analysis to authenticate the nature of grazed forage and correctly classified 86.1% of grass-only and 80.7% of grass/clover muscle samples. In milk, several studies have shown that different types of pastures (cultivated *v.* natural) produce substantial differences in milk FA profile (Coppa *et al.*, 2015c). Nevertheless, given that forage FA profile largely varies with pasture phenological stage, botanical composition and grazing management

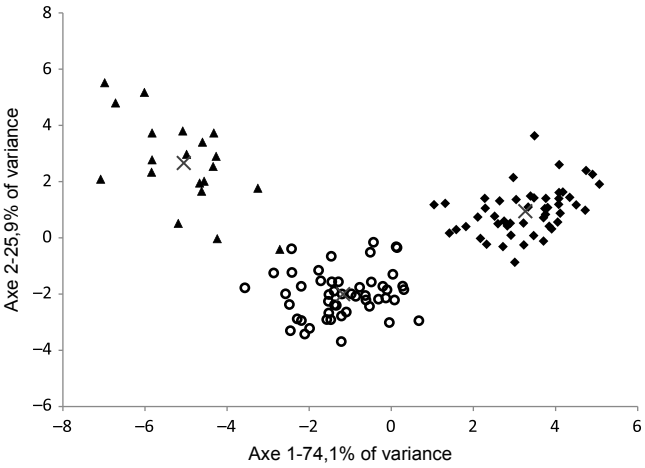


**Figure 1** Discrimination of feeding regimes in dairy cattle on the basis of discriminant analysis of fatty acids (FAs) in milk samples from animals fed grass (more than 70% of fresh herbage in the diet DM during the grazing season and more than 70% of conserved grass in the winter season) (triangles); maize silage (more than 35% maize silage in the diet DM during the grazing season and more than 60% maize silage in the diet DM in the winter season) (circles); maize silage + flaxseed: maize silage (more than 35% maize silage in the diet DM during the grazing season and more than 60% maize silage in the diet DM in the winter season, + 3% extruded flaxeeds) (diamonds) (from Hurtaud *et al.*, 2014, reprinted with permission).

(Coppa *et al.*, 2015b), it seems difficult to calibrate pasture-type discriminant methods that are year-round repeatable and large-scale reliable.

A point of caution to mention is the fact that certain non-grass sources of FA, such as flaxseed, can yield similar in-product 18:3n-3, 18:2n-6 and conjugated linoleic acid contents to grass feeding. Hurtaud et al. (2014), nevertheless, showed that even small differences in FA (chiefly in 18 : 1 isomers) were enough to discriminate milk samples from grass-fed cows *v.* cows fed maize silage and supplemented with flaxseed (Figure 1).

*Volatile compounds.* The volatile compounds in meat and dairy products are extracted using dynamic headspace technology then analysed by gas chromatography–MS. Branched-chain FA, lactones, aldehydes, indoles, 2,3-octanedione, terpenes and sulphur compounds are the meat and dairy volatiles that prove most diet-sensitive – particularly for fresh grass *v.* concentrate (Vasta and Priolo, 2006) and fresh grass *v.* conserved forages (Abilleira *et al.*, 2011). Some of these compounds – typically terpenes – are volatile feed-ration components taken up directly into animal tissue, others – like skatole and indole – are produced by animal metabolism and some – like certain sulphur compounds or lipid oxidation products – form when the meat gets cooked or when the bulk milk gets heated in-tank during heat treatment or cheesemaking. Coppa *et al.* (2011) identified eight compounds that discriminated milk from cows fed pasture *v.* hay. Through comparative analysis of four beef cattle feeding systems (pasture for 1 year *v.* barley-based concentrate for 1 year *v.* grass silage for



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6 months switched to pasture for 6 months *v.* grass silage for 6 months switched to pasture for 6 months with barley-based concentrate offered at 50% of total ration), four compounds were identified (skatole, 3-undecanone, cuminic alcohol and 2-methyl-1-butanol) that enabled discriminating animals fed pasture from animals fed concentrate (Monahan *et al.*, 2018). Germacrene D, particularly, emerged as a solid marker of grass feeding. Beyond back-tracing grass feeding, terpenes have also been proposed as markers of pasture feeding on dicotyledon-rich permanent grasslands (Abilleira *et al.*, 2011).

It should be mentioned, however, that volatile compounds deposit unevenly across different ruminant fat fractions, so discriminative performance may hinge on fat fraction selection. Furthermore, in-feed terpene profile can vary widely due to a number of factors. In pastures, the terpene profile is highly dependent on botanical composition, sward phenological stage and animal feed selection, all of which depends on grazing management (Coppa *et al.*, 2011). The resulting specificity of product terpene profiles then does not easily fit with the genericity needed for animal diet authentication models. This is an important difficulty in establishing robust, stable and generalizable relationships between an animal's feeding diet and the animal product's volatiles profile. Nevertheless, certain individual terpenes (*p*-cymene, β-caryophyllene and *trans*-cadina-1(6),4-diene) and other volatiles (toluene and several sulphur compounds) have repeatedly been identified as markers of pasture-feeding (Vasta and Priolo, 2006; Engel *et al.*, 2007). Finally, as these analyses are long and expensive, they have been performed on a small number of samples, so their real discriminant reliability still needs to be proven on a much larger number of samples. Simply observing a significant diet effect on food content of selected compounds is not sufficient for authentication purposes – the crucial part is to determine the proportion of samples correctly assigned to each diet. Skatole and indole, for example, are regularly cited as candidates for authenticating grass-fed lamb and milk (Vasta and Priolo, 2006). While some studies effectively found that these compounds were powerfully discriminant (Coppa *et al.*, 2011), other authentication studies observed that they may be also detected in stall-fed lambs (Devincenzi *et al.*, 2019) or even comparable in content between grass-fed and stall-fed lambs (Priolo *et al.*, 2004). Furthermore, there are biases to watch out for, as microbial flora produces volatiles during cheese ripening, which adds a layer of complexity, and plant extracts or essential oils rich in certain key volatiles (including terpenes) are widely used in ruminant diet or for skin application on the udder, which may limit their reliability as grass-diet tracers. There are two strong limits to these compounds: (i) the extraction techniques still carry strong limitations in terms of volatile trapping capacity and analytical repeatability, and (ii) as the candidate markers were identified on a small number of samples, there are questions hanging over their genericity and robustness across a wider range of 'real-world' contexts and conditions.

*Phenolic compounds.* Phenolic compounds are secondary metabolites found in plants that can transfer into milk or meat either directly unchanged or partially converted by rumen bacteria or host animal metabolism. These compounds are specific to each plant species, and the phenolic profile of natural grasslands varies with botanical composition (Besle *et al.*, 2010). Besle *et al.* (2010) showed that the analysis of phenolics compounds in milk enabled to clearly discriminate diets based on hay, grass silage, maize silage and grazed grass. Compounds of the carboline group were proposed as markers of silage (whatever its origin, grass or maize) feeding, whereas compounds of the glycinamide group (notably hippuric acid) were proposed as markers of pasture feeding (Rouge *et al.*, 2013). However, these studies are the first reports of such an application of phenolic compounds, and the suitability of these compounds needs to be investigated further. It should also be mentioned that the contents in phenolic compounds in the forage vary widely with botanical composition, forage phenology and conservation method, and grazing management (Besle *et al.*, 2010). Phenolic compounds thus face the same limits as volatiles compounds.

*Carotenoids.* Carotenoids form the main group of natural pigments. Lutein is the only fat-stored carotenoid in sheep, whereas cattle also (and crucially) accumulate ß-carotene. Zeaxanthin, the major carotenoid in maize kernels, is not stored in ruminants' adipose tissues. Carotenoid concentration in animal adipose tissues and animal products is directly linked to the animal's carotenoid intake level (Calderón *et al.*, 2007; Dian *et al.*, 2007). Fresh green grass is particularly rich in carotenoids, but once dried, the forage progressively loses carotenoid content, because these pigments photodegrade. Compared to initial carotenoid content measured in fresh grass, wilted silage contains 60% (at 28% DM) to 30% (at 35% DM) carotenoids *v.* around 30% in haylage and 20% in hay (Nozière *et al.*, 2006), whereas most concentrate feeds are practically devoid of lutein and ß-carotene. This is why carotenoids have been proposed to discriminate grass-fed from concentrate and/or maize silage-based diet (Prache and Thériez, 1999, for lamb; Monahan *et al.*, 2018, for beef; Engel *et al.*, 2007, for cow's milk). These studies assayed carotenoids directly by high-performance liquid chromatography or indirectly via reflectance spectrum of the body fat, milk or cheese. Prache and Thériez (1999) showed that carotenoids assayed by plasma concentration or *via* adipose-tissue reflectance spectrum can distinguish grass-fed from stall-fed lamb. They devised a spectro-colorimetric index, computed from the reflectance spectrum in the light-absorbing region of carotenoids, to quantify their 'signature' intensity and thus estimate their concentration (Prache *et al.*, 2003, Figure 2). This simple, quick and portable process was extended out to beef and dairy products (Nozière *et al.*, 2006; Monahan *et al.*, 2018), and both the results and process have since been confirmed on bigger databases covering several sheep



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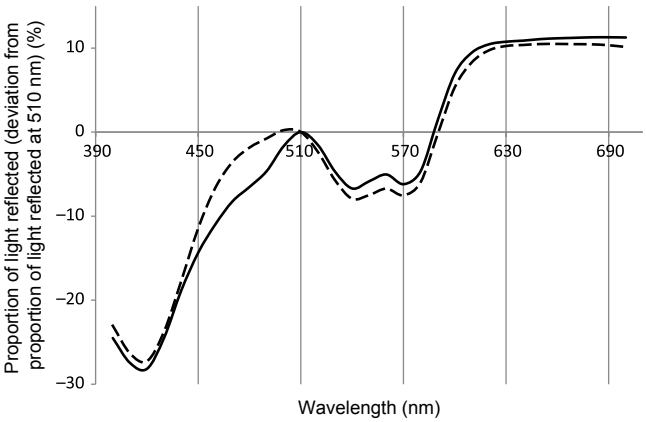


**Figure 2** Mean reflectance spectra of perirenal adipose tissue of lambs fed grass at pasture (solid line) or with a concentrate-based diet indoors (dotted line). The spectro-colorimetric index is calculated as the absolute value of the integral of the translated spectrum between 510 and 450 nm (i.e. the light-absorbing region of carotenoids). The integral is the area comprised between the curve and the x-axis in the zone 450 to 510 nm (from Prache *et al.*, 2003, reprinted with permission).

breeds (Prache *et al.*, 2018), other countries (Ireland, Spain, Italy) and other meat and dairy products. In Ireland, for example, the same reflectance spectrum approach served to discriminate beef from heifers fed grass *v.* a barley-based concentrate diet for 12 months (Monahan *et al.*, 2018).

As carotenoid enrichment differs between fat deposits, discriminant reliability is fat-site-dependent (Dian *et al.*, 2007). Furthermore, even at an identical carotenoid intake level, fat carotenoid signature intensity can still differ between breeds (Macari *et al.*, 2017). Despite these differences, a recent study on over 1000 lambs from three breeds shows that discriminative reliability between grass-fed and stall-fed lambs holds up even after pooling data from all three breeds compared to breed-specific database and analysis (Prache *et al.*, 2018). As far as milk is concerned, cow-milk carotenoid concentration varies over the grazing season (Calderon *et al.*, 2006). It decreases significantly with herbage maturity and can even drop to levels approaching that of milk from hay-fed cows, especially if the hay is barn-dried (Nozière *et al.*, 2006). It also depends on grazing management and botanical composition (Calderon *et al.*, 2006; Marino *et al.*, 2014). This significant variability creates background noise, which limits using carotenoids alone for large-scale authentication of grass-fed milk. Another point of caution to be mentioned is that carotenoids can be added to in-stall rations (e.g. by using alfalfa concentrate; Prache *et al.*, 2009; Macari *et al.*, 2017). These limits and sources of bias are, however, common to most grass-feeding marker compounds.

*Vitamin E stereoisomers.* Stereoisomeric analysis of vitamin E in animal products can back-trace natural *v.* synthetic sources of dietary vitamin E. Monahan *et al.* (2018) reported that RRR-α-tocopherol was the dominant stereoisomer in grass-fed beef (natural vitamin E), whereas all eight stereoisomers were found in concentrate-fed animals given synthetic vitamin E supplements. Likewise, grass-fed milk contains more RRR-α-tocopherol stereoisomer than milk from conserved-forages (Butler *et al.*, 2011).

*Stable isotopes.* Stable isotope ratios of the main atoms forming molecular fractions of animal tissues and products depend on the isotopic composition of the diet (feed plus water) ingested. Stable isotope proportions in a given feed – expressed as the relative abundance of two isotopes measured by isotope ratio MS – are influenced by farming practices (type and proportion of feed-ration components, type and input level of fertilizers) and geographic-environment factors (chiefly latitude, altitude and proximity to coastline). The stable isotope ratio value of an element for a given sample is conventionally compared to the standard value measured on a reference product and expressed as an index, δ.

C4 plants (e.g. maize) and C3 plants (e.g. temperate grasslands, cereals, soybean, sugar beet) have different metabolic pathways for photosynthesis, which induces differences in their stable carbon isotope ratios. The range of variation in $\delta^{13}C$ values is −14‰ to −10‰ for C4 plants *v.* −35‰ to −21‰ for C3 plants; the $\delta^{13}C$ value can thus clearly separate maize-fed *v.* pasture-fed lamb (Monahan *et al.*, 2018) and maize-fed *v.* grass silage-fed beef (Bahar *et al.*, 2005). The $\delta^{13}C$ value can also separate maize-fed *v.* grass-fed milk (Kornexl *et al.*, 1997; Auerswald *et al.*, 2015). A point of caution to be mentioned: as certain tropical grassland plants are C4 plants, feeding animals on this type of grassland could mistakenly test false-positive for feeding on a maize-based diet.

The $\delta^{18}O$ value has also been proposed to back-trace pasture feeding in cow's milk (Renou *et al.*, 2004). Engel *et al.* (2007) observed a strong increase in the $\delta^{18}O$ value of milk water when cows were switched from stall-feeding to grass-feeding. They explained this increase as related to the source of water intake, as, compared to groundwater, the plants concentrate the $^{18}O$ due to evapotranspiration (Kornexl *et al.*, 1997).

The $\delta^{15}N$ value in forage nitrogen compounds is far lower in legumes than in grasses (Devincenzi *et al.*, 2014) due to atmospheric nitrogen fixation. Devincenzi *et al.* (2014) exploited this to distinguish the meat from pasture-fed lambs that had ingested 62% alfalfa *v.* grass only. On beef, FA profile in combination with $\delta^{15}N$ correctly classified 95.7% and 86.5% of muscle samples from cattle that had grazed grass/clover *v.* grass-only pastures (Moloney *et al.*, 2018). The $\delta^{15}N$ value has also been used to authenticate grass-fed milk *v.* maize + cereal fed milk (Kornexl *et al.*, 1997).

Organic farming systems make little use of C4 plants as animal feed, and they value grassland legumes, prompting research into using C and N isotope ratios to authenticate organically farmed beef. Studies reported by Monahan *et al.* (2018) showed that $\delta^{13}C$ values were lower and less variable in organic meat, but that results for N isotopes are more variable, with $\delta^{15}N$ values sometimes lower and sometimes near-identical in organic meat.



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There are points of caution for N isotopes: (i) efficiency of atmospheric nitrogen fixation by legumes varies strongly with plant age (Devincenzi et *al.*, 2014), (ii) 'conventional' grasslands can prove legume-rich, (iii) organic nitrogen input increases plant $^{15}N$ values compared to mineral nitrogen (Boner and Forstel, 2004). Even though organic farming systems value rangeland legumes, they also use organic manure, and these two factors have antagonistic effects on $\delta^{15}N$ value in the sward and, consequently, in products derived from the animals grazing them. Furthermore, there is some degree of variability in animal response to a given diet (Devincenzi *et al.*, 2014; Macari *et al.*, 2017) due to inter-individual variability in efficiency of nitrogen utilization. This inter-individual variability generates non-diet-related 'background noise', which erodes the discriminant power. Finally, as stall-fed diets may also contain legumes (soybean, alfalfa), several tissues and methods should then be combined for authentication issues (Bahar *et al.*, 2005; Prache *et al.*, 2009). To illustrate, only a combination of muscle $\delta^{15}N$ value with perirenal-fat sesquiterpene content and plasma carotenoid content conclusively separated pasture-fed lamb from lambs fed dehydrated alfalfa and straw indoors (Prache *et al.*, 2009).

*Protein biomarkers.* Three cull-cow finishing feeding regimes (pasture *v.* haylage *v.* hay for 3 months pre-slaughter) were recently discriminated by using protein biomarkers (Gagaoua *et al.*, 2017): proportions of heavy-chain myosin isoforms – both oxidative (MyHC-IIA) and glycolytic (MyHC-IIX) – , the stress protein αβ-crystalline and the antioxidant protein superoxide dismutase, which were more abundant under pasture-finishing involving higher muscle-work exercise.

*Fingerprint methods*

The compound-specific methods covered so far in this review carry the drawback of being potentially sensitive to biases (feed sources that can 'mimic' grass). Fingerprinting methods are less bias-sensitive as they build a global product signature integrating much or most of its composition. The downside is that the reasons underlying the signature differences may be difficult to decipher. In fact, the two approaches often prove complementary.

*Spectroscopic methods.* Visible spectroscopy (**VIS**), near-infrared spectroscopy (NIRS) and mid-infrared spectroscopy (**MIR**) have proven promising approaches for authenticating grass-fed products. The product spectrum is analysed using chemometrics methods to discriminate feeding regimes and pinpoint the discriminant spectral regions-of-interest. These methods are fast, chemical-free and zero-waste.

Visible spectroscopy (400 to 700 nm) has been successfully used on lamb fat (Prache and Thériez, 1999), cow's milk (Nozière *et al.*, 2006) and cheese (Andueza *et al.*, 2013) and proved its practical relevance and robustness on lamb meat and cow's milk on significant datasets (Andueza *et al.*, 2013; Prache *et al.*, 2018). The results clearly underlined the importance of the light-absorbing region of carotenoids in the discrimination of grass-fed *v.* stall-fed lambs and suggested other compounds may be involved (Prache *et al.*, 2018). Near-infrared spectroscopy can improve discriminative performance by expanding the range of the reflectance spectrum explored (400 to 2500 nm). Using NIRS on perirenal fat, Dian *et al.* (2008) correctly assigned 97.5% of grass-fed lambs and 97.8% of stall-fed lambs (*v.* 90.8% and 98.6% for VIS). Little comparable information is available on muscle tissue. The only study using NIRS on muscle tissue is Cozzolino *et al.* (2002), who correctly assigned 82% and 79% of beef samples from cattle finished on pasture *v.* on maize silage.

Using NIRS on cow's milk, Coppa *et al.* (2012) correctly assigned 96.4%, 92.2% and 93.3% of samples when comparing (i) pasture *v.* maize silage, (ii) pasture *v.* hay and (iii) pasture *v.* grass silage. However, NIRS clearly underperformed on discriminating milk produced from different conserved-forages (maize silage *v.* conserved grass). In cheese, NIRS enabled to correctly assign 96% of samples from pasture-fed *v.* conserved-forage-fed (hay or grass silage) dairy cattle (Andueza *et al.*, 2013). In milk, MIR technology has focused attention, as it offers fast throughput (one sample every 7 s). Valenti *et al.* (2013) used MIR to successfully discriminate milk from dairy cattle fed pasture *v.* hay or pasture *v.* maize silage (>95% of samples correctly assigned), but were unable to separate milk from hay *v.* maize silage-based diets.

These spectral methods do require sophisticated mathematics, but they are fairly straightforward and field-friendly to deploy and are set to become a mainstay of authentication practice. For example, VIS measurement is fast enough to keep pace with the line speeds of carcasses in a commercial abattoir, and portable NIRS devices are emerging.

Nuclear magnetic resonance (**NMR**) spectroscopy was used successfully to discriminate Asiago d'Allevo cheese of upland extensive *v.* intensive farming systems (Schievano *et al.*, 2008). It was also used to investigate changes in metabolomic profile in beef cattle muscle and discriminate different beef production systems (Monahan *et al.*, 2018). In the latter study, discriminant analysis on urine showed good discrimination between outdoor/pasture-fed *v.* indoor/concentrate-fed cattle, and creatinine, glucose, pyruvate, phenylalanine and hippurate were identified as discriminant variables. Production system discrimination on muscle was also possible, although not as reliable as with urine, the discriminant metabolites for distinguishing concentrate-fed cattle being carnosine (higher concentration) and methylhistidine, malonate and glutamine (lower concentrations) in muscle.

*Functional genomics.* Functional genomics is one of the latest approaches to emerge. The premise is that as regulation of gene expression is modulated by various factors – including nutrients, gene expression profiles could bring pertinent information on dietary background. Analytical techniques mobilized with functional genomics



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can compare gene expression profiles (transcriptomics) or protein expression profiles (proteomics) in animal tissue samples. In steers pasture-fed or fed maize silage indoors, Cassar-Malek *et al.* (2009) found under-expression of the gene for selenoprotein W in pasture-fed animals, which complementary analyses suggested was tied more to selenium concentration/bioavailability (lower in grass than maize silage) than to greater muscle-work exercise in pasture-fed animals. Selenoprotein W expression could therefore make a gene marker of grass feeding. In a study on outdoor/pasture fed *v.* indoor/concentrate fed cattle, Sweeney *et al.* (2016) identified 26 differentially expressed genes. Some reflected FA metabolism, some correlated positively with total n-3 FA content, and expression profiles of three genes (*ALAD*, *EIF4EBP1* and *NPNT*) firmly discriminated the two feeding systems (correct assignation for 95% of outdoor/pasture fed animals and for 100% of indoor/concentrate fed animals).

## Discriminating less-contrasted diets: dose-dependent response and dietary switches

Assessing whether a method has the potential to discriminate between feeding regimes starts with comparing contrasted but time-stable feeding conditions. However, the real-world picture is rarely so clear-cut. Over the course of an animal's productive life, feeding regimes can vary with changes in feed costs and availabilities. Furthermore, a feed's tracer content and/or isotopic signature can vary with season. Furthermore, while we know how to distinguish the meat of animals grass-fed for several months pre-slaughter *v.* meat from concentrate-fed animals, we still do not know when the meat's grass-fed signature stabilizes. This ties back into the question of the time of appearance of grass markers relative to the change in diet, their dynamic from diet to meat and their persistency. Milk is often quick to show effects of diet changes (milk FA and terpene profiles change from as early as 24 to 48 h after a dietary shift; Viallon *et al.*, 2000; Coppa *et al.*, 2015b).

Feed-component plants can also show variability in tracer content, isotopic signature, and there can be added variability in ration-ingredient incorporation rate, plus further significant variability in animal intake levels and feed choices, especially on grasslands. These variations can be related to season, plant phenology, plant age and root growth and grazing management, all of which makes it necessary to know, as far as possible, the dose-dependent response to a given tracer input and the dynamics of appearance and persistency of potential tracers in animal products after a dietary switch.

### *Dose-dependent response*

Proportion of C4 plants in cows' diet can be predicted from the $\delta^{13}C$ value in the milk (Auerswald *et al.*, 2015), but with the caveat of high variability between individual animals that causes much of the estimation error. In beef cattle fed 167 days with grass silage, maize silage or a 50 : 50 mixture of both, Bahar *et al.* (2005) estimated that each 10% increment of maize in the diet should translate into a 0.9‰ to 1.0‰ increase in $\delta^{13}C$ value of muscle. They thus asserted a linear dose-dependent response, but without analysing discriminative reliability according to the dietary proportion of maize. C isotope ratio is generally used to discriminate diets that contrast on C3/C4 plants, but they have also been used to discriminate meat reared under less-contrasted diets (50% pasture + 50% barley *v.* grazed grass or grass silage, both C3-plant diets presenting only a 2‰ to 3‰ differential in $\delta^{13}C$ values; Monahan *et al.*, 2018).

Devincenzi *et al.* (2014) found that $\delta^{15}N$ value in lamb meat decreases linearly with dietary proportion of legumes. Their equation cannot, however, be considered generic as plant isotopic signatures can vary with local environment conditions. Moreover, even if global response is linear, the inter-individual variability in animal response still generates some 'noise', which erodes the discriminant power. Macari *et al.* (2017) showed largely reliable discrimination (92.9%) of meat from lambs fed 50% *v.* 0% legumes (between-diet $\delta^{15}N$ differential of 3.7‰). More accurate authentication of dietary proportion of legumes in lambs looks out of reach unless the isotopic signature differential between forages is higher.

Mean plasma carotenoid concentration increases linearly with carotenoid intake level in sheep and cattle (although with between-species differences). In dairy cattle, milk carotenoid concentration increases linearly with plasma concentration up to 5 μg/ml and then plateaus out (Calderon *et al.*, 2007). Likewise, the dose-dependent response of carotenoid signature intensity in lamb fat is curvilinear, that is, linear for low-to-moderate inputs (Dian *et al.*, 2007), then plateauing at an as-yet-unspecified threshold. Indeed, lambs grazing alfalfa only or with barley supplement (at 38% of diet) had equal-intensity fat carotenoid signatures (Devincenzi *et al.*, 2019). This method thus looks ineffective for accurately authenticating dietary proportion of grass fed to lambs.

In dairy cattle, Coppa *et al.* (2015a) proposed equations predicting herd dietary composition (dietary proportions of grazed grass, of hay, of total forage, of total grass) based on bulk tank milk FA profile. The precision of the estimation was sometimes insufficient (e.g. ±15% for proportion of grazed grass). The errors essentially stemmed from imprecision inherent to the dietary-composition information (self-reported by the farmers) and the confounding factors cited earlier, chiefly on grazing.

### *Dietary switches: dynamics of appearance and persistency of tracers in animal tissues and products*

*Elemental and molecular tracers.* Milk composition responses to diet changes are fairly rapid, but there is still a time lag before meat composition can reflect diet composition, which can prove a challenge for authentication when there is a dietary switch shortly before slaughter. The dynamic of



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appearance of isotopes in animal tissues and products is estimated from their half-life. The half-life of a compound in a tissue is its median life in that tissue, that is, the time under which over 50% of the compound is present and over which under 50% is present.

In beef cattle switched from a C3 barley-based diet to a C4 maize grain-based diet (C4) for periods of 2 up to 22 weeks pre-slaughter, the half-lives of C and N in *Longissimus thoracis et lumborum* muscle were calculated as 151 and 157 days (Bahar *et al.*, 2009). This points to a long time lag before meat isotopic signature reflected the diet switch. Half-life of isotopes varies with animal tissue turnover, which means it can vary with the tissue in a given animal (Bahar *et al.*, 2009). It also varies with animal species, and C half-life has been shown to be shorter in lamb than in beef (Harrison *et al.*, 2011). Furthermore, tissue turnover depends strongly on the animal's energy intake: C half-life in *Longissimus thoracis et lumborum* muscle was determined as 76 days for lambs on high energy allowance (average daily gain (**ADG**) 150 g/day), whereas 92 days on low energy allowance (ADG 50 g/day) (Harrison *et al.*, 2011). The time-course change of muscle $\delta^{13}C$ value after a dietary switch therefore depends not only on dietary $\delta^{13}C$ changes but also on the animal's energy intake level. Lastly, while a number of studies have clearly shown the effects of a dietary switch from C3 (e.g. grass) to a C4 (e.g. maize) or *vice versa*, on the isotopic signature of muscle, the dietary switch becomes harder to detect when the diets are isotopically closer (e.g. C3 feedstuffs like barley *v.* grass; Monahan *et al.*, 2018). There are, therefore, difficulties in using muscle isotopic signature to extract information on an animal's nutritional history, as the signature is 'integrated' over a fairly long pre-slaughter period, which means short-term changes may go undetected (Bahar *et al.*, 2009). Furthermore, if grass-fed animals are supplemented with cereals or switched to a cereal-based diet for a period of time pre-slaughter, that too may go undetected, either because animal tissue turnover is too slow to induce a muscle response or because the isotopic signature of the cereal is insufficiently different from that of the grass (Bahar *et al.*, 2009; Harrison *et al.*, 2011).

These same dietary switch challenges also concern other compounds (FA, vitamin E, volatiles). For FA in beef, Noci *et al.* (2005) showed that switching animals from grass silage with concentrate indoors to pasture grazing for periods of 40, 99 or 158 days pre-slaughter leads to a steady linear increase in conjugated linoleic acid concentration and a steady linear decrease in n-6 FA/n-3 FA ratio. Alfaia *et al.* (2009) were able to 100% discriminate four cow feeding regimes (pasture *v.* pasture followed by 2 or 4 months of stall-finishing on barley-based concentrate diet *v.* barley-based concentrate diet). The muscle FAs with strongest discriminative power were C17:1*c*9, C24:1*c*15, C14:0, C18:1*c*13, C18:3 n-3, oleic acid, C18:1*t*9, C20:1*c*11 and the *t*11, *t*13 isomer of CLA. For volatiles, Priolo *et al.* (2004) identified four terpenes enabling to distinguish pasture-fed lambs from lambs that had been stall-fed only or stall-finished after a period on pasture. They also identified 2,3-octanedione as a potential biomarker of duration of stall-finishing period after grazing.

In lambs switched from pasture-feeding to indoor concentrate finishing, the intensity of the carotenoids signature in fat tissue decreased exponentially with in-stall live weight (**LW**) gain (Huang *et al.*, 2015a) and reached a similar value to that observed in stall-fed lambs at 15.8 kg of in-stall LW gain. Given the rate of growth during stall finishing, this would equate to a response persistency of 60 days. A point of caution to be mentioned, however, is the large inter-individual variability in both ability to store carotenoid in body fat (Dian *et al.*, 2007; Macari *et al.*, 2017) and in rate of LW gain during stall-finishing (Huang *et al.*, 2015a), which creates 'noise' in the diet discrimination process. When lambs switch from carotenoid-poor to carotenoid-rich diet, the intensity of carotenoid signature in perirenal fat increases curvilinearly and then plateaus after 45 days (Oliveira *et al.*, 2012). However, the number of animals in this study was insufficient to investigate the reliability of the discrimination between the carotenoid-rich diet and the carotenoid-poor diet according to the duration of feeding on the carotenoid-rich diet.

One may take advantage of the differences between tissues and/or diet tracers in the dynamics of response time to a dietary switch. For example, in lambs finished indoors after pasture on a carotenoid-low diet, both plasma carotenoid concentration and intensity of light absorption by carotenoid pigments in the fat decrease curvilinearly with the interval from starting on the stall diet after pasture, according to a decreasing exponential model. As the deceleration parameter was much lower in fat (Huang *et al.*, 2015a) than in plasma (Prache *et al.*, 2003), the combined use of both tissues added refinement in the authentication of the animal's dietary history (Prache *et al.*, 2003).

*Fingerprint methods.* The first results obtained with VIS and NIRS in more complex feeding conditions are promising. In lamb, Huang *et al.* (2015b) discriminated three feeding regimes (grass-feeding *v.* stall-feeding *v.* stall-finished for 28 days after a pasture-feeding period) with 95.9% reliability using VIS and 99.0% using NIRS on perirenal fat. They also confirmed the spectral light-absorbing regions involved in the discriminating process between feeding regimes. These spectral methods thus remain robust when dealing with more complex feeding situations. However, they have not yet been tested in beef cattle, which can undergo more diet switches in their productive life than lambs. Coppa *et al.* (2012) showed that with NIRS, the error rate in discriminating milk from grazing *v.* non-grazing cows decreased when milk samples from cows fed less than 70% grass in their diet were removed from the grazing group. Below this threshold, discrimination of widely practised mixed-ration regimes loses reliability.



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## Conclusions

The literature shows that it is possible to discriminate contrasted feeding regimes using analytical methods that quantify specific compounds or more global fingerprinting methods, such as those based on product optical properties. However, discrimination can become performance-limited when the methods are used separately, and there are often synergies between different methods and different tissues. Results obtained in less-contrasted and diet-switched feeding regimes, which are harder to characterize, further argue for combining different tracers (and different tissues for meat products) due to their observed latency and/or persistency profile differentials. Spectral fingerprint methods, which are typically based on product optical properties – and therefore global composition – offer promising performances, even in more complex diet-switching scenarios. As these methods (MIR particularly) are already used for milk analysis (i.e. for weekly estimation of parameters linked to milk payment), it would be possible to implement authentication in routine in the short term. However, these methods do not inform precisely on the underlying reasons for differences, so research needs to push ahead on both fronts: on the single-compound analytical approaches and on the global spectral analysis-based fingerprinting approaches. Cost and ease of implementation vary among methods. Fingerprint methods, especially those based on product optical properties, are of particular interest, being fast, chemical-free and zero-waste; some of them can already be implemented online in the food industry on a large number of samples using portable devices. Other methods, such as the analysis of phenolic or volatile compounds, are much more expensive and difficult to implement; they can only be used on a small number of samples, but the possibility of them being used may deter fraud. One can also consider using these methods in stages, the easiest one on a large number of samples and the most expensive in the last resort on a much smaller number of samples. Back-authentication of animal's dietary history through animal products, and particularly authentication of grass-fed meat and dairy, faces challenges inherent to livestock farming practices, chiefly diet variations and variability over the course of an animal's productive life beyond those due to response variability between animals. Much of the research led to date is 'proof-of-concept' work, and larger databases now need to be developed to gain genericity and robustness.

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## Declaration of interest

None.

## Ethics statement

None.

## Software and data repository resources

None.

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