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Short communication

Assessing the authenticity of animal rennet using $\delta^{15}N$ analysis of chymosin

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ABSTRACT

Chymosin is a protease that curdles the milk casein. Animal rennet was the first discovered source of chymosin and its use is mandatory for the production of PDO cheeses such as Parmigiano Reggiano and Grana Padano. Of the alternatives, fermentation-produced chymosin is the most competitive because it functions in a similar way, but is much cheaper. Analytical tools are necessary in order to distinguish the 2 types of chymosin and verify the compulsory use of animal rennet in the production of PDO cheeses.

In this work, a method to analyse $^{15}N/^{14}N$ in chymosin after extraction was developed. The $\delta^{15}N$ values of animal rennet range from 5.7‰ to 8‰, whereas the $\delta^{15}N$ values of fermentation-produced chymosin are significantly lower, ranging from −5.3‰ to 2.2‰. A threshold value of 5.7‰ was defined for authentic animal rennet.

Addition of fermentation-produced chymosin to animal rennet, or its complete substitution, can be therefore detected.

1. Introduction

Rennet is a mixture of various types of proteases used during cheese making to convert liquid milk into a soft gel, usually denoted as curd.

The first discovered form of rennet, originating from the abomasal mucosa of new-born or adolescent ruminants, mainly calf and lamb vells, is called animal rennet. It contains 3 genetic variants of chymosin: A, B, C (10–90%), and pepsin, which are aspartic proteases. In suitable pH and temperature conditions, they cleave some of the peptide bonds of bovine K-casein, so that its hydrophilic C-Terminal section, caseinomacropeptide, is released into the milk serum. Due to the loss of repulsion force, casein micelles then aggregate and start to form a three-dimensional curd network.

The increasing demand for rennet, the needs of specific consumers (e.g. lactovegetarian) and the prospect of saving money have triggered the search for alternatives to animal rennet (Jaros & Rohm, 2017).

Currently, the 3 available substitutes are microbial-derived coagulants, plant-derived coagulants and fermentation-produced chymosin.

Microbial-derived coagulants refer to proteolytic enzymes produced from 3 fungal species, namely *R. miehei, Rhizomucor pusillus* and *Cryphonectria parasitica*. These proteases are less specific and induce higher proteolytic activity, resulting in lower clotting efficacy, which is then responsible for a reduced cheese yield and bitter off-flavour (Crabbe, 2004). *Cynara cardunculus* L. subsp. *Favescens* Wiklund is the most important source of plant-derived clotting enzymes. These lead to a buttery and soft texture and are therefore less suitable for producing mature cheese (Jaros & Rohm, 2017).

Fermentation-produced chymosin (or genetic chymosin) is nature-identical calf chymosin produced through fermentation by a host microorganism in which the gene for the enzyme is expressed and it is the first food-processing aid made using the recombinant DNA technique recognised by the FDA (Flamm, 1991). Fermentation-produced chymosin is therefore a product of genetically modified microorganisms (GMOs), even if it does not contain any living genetically engineered organisms. A number of suitable microorganisms, including bacilli, lactococci, yeast and mould are used to produce chymosin. Recent works have focused on recombinant camel chymosin expressed in *Aspergillus niger* var *awamori* (Kappeler et al., 2006), with a lot of advantages (reduced amount of enzymes, absence of bovine pepsin, lower bitterness, lower firmness and chewiness).

Genetic chymosin covers 80%-90% of the coagulant market in the USA and the United Kingdom (GMO Compass 2010), whereas in France

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it accounts for only 14% (Rolet-Repecaud et al., 2013).

The EU started to regulate the use of enzymes in 2009 and now the European Food Safety Authority (EFSA) is responsible for evaluation of the safety aspects of these types of products. To date, only France and Denmark have introduced national regulations allowing the use of genetic chymosin, but not vegetable coagulants (The Oxford Companion to Cheese, Oxford University Press, 25 Oct 2016, 2016).

Animal rennet is still used for the production of high-quality traditional cheeses and PDO, PGI and TSG products (The Oxford Companion to Cheese, Oxford University Press, 25 Oct 2016, 2016) and is mandatory for the production of PDO cheeses such as Parmigiano Reggiano and Grana Padano, as required in the relevant production specification (https://www.granapadano.it/public/file/DisciplinareGranaPadanoDOP17-7-18ING-31101.pdf; https://www.parmigianoreggiano.com/consortium/disciplinare_produzione_vigente_sino_agosto_2011/production_standard.aspx). However, as fermentation-produced chymosin acts in a similar way to animal chymosin, but is much cheaper, there is a suspicion that it may be used instead of animal rennet.

As the use of these alternatives is not allowed, it is necessary to develop analytical tools able to identify the origin of chymosin. Stable isotope ratio analysis of bioelements has good potential for these types of applications since it has already been used to detect the authenticity of several food matrixes and components, verifying the correspondence of the source declared on the label or allowed by law. Recent examples focus on stable isotope ratio of C to detect the source of sugar in honey (Dong, Xiao, Xian, & Wu, 2018), natural vanillin (Schipilliti, Bonaccorsi, & Mondello, 2017; Van Leeuwen, Prenzler, Ryan, Paolini, & Camin, 2018), natural flavours in citrus essential oils (Schipilliti, Bonaccorsi, Occhiuto, Dugo, & Mondello, 2018), statin in Red yeast Rice (Perini, Carbone, & Camin, 2017) and, combined with the isotope ratio of H, citric acid, in apricot liqueur (Akamatsu et al., 2017).

In the case of rennet, the most suitable isotope ratio to detect its source is that of nitrogen ($^{15}N/^{14}N$, expressed as $\delta^{15}N$). Nitrogen of animal origin indeed has high ^{15}N content, due to the fact that $\delta^{15}N$ increases approximately +3‰ per trophic level (Kurle & Worthy, 2002), whereas the ^{15}N content of recombinant chymosin is expected to be much lower, because it should not derive from animals. Regarding the use of $\delta^{15}N$ to detect the source of a product, the only example found in the literature so far was focused on the detection of animal vs vegetal contribution in the diet of organic chicken (Rogers, 2009).

In this work we considered 53 samples of authentic animal rennet and 9 samples of fermentation-produced chymosin. The samples were collected from 2013 to 2018, worldwide, including all the countries producing raw materials and considering 5 different production techniques, in order to cover the widest possible natural variability.

The aim of the study was to develop a method to extract chymosin from animal rennet, analyse its $\delta^{15}N$ and investigate whether the method can distinguish animal rennet from fermentation-produced chymosin and detect the source of chymosin used in PDO cheese production.

2. Materials and methods

2.1. Samples

53 authentic animal rennet samples and 9 samples of fermentation-produced chymosin were collected (Table 1). The samples were obtained worldwide from 2013 to 2018 in order to cover the greatest possible variability. The rennet samples were in both liquid and powder phase, from calves. The raw materials came from Italy, France, Belgium, The Netherlands, Denmark, New Zealand and Australia and were processed using 5 different technologies to obtain rennet. Fermentation-produced chymosin came from The Netherlands and Denmark. Furthermore, 11 samples were obtained by mixing animal rennet with different quantities of fermentation-produced chymosin. One sample of rennet was extracted and analysed 8 times in order to determine the repeatability of the entire analytical procedure.

Table 1
Description of the samples.

Phase	Animal	Origin
powder	calf	Australia
powder	calf	Australia
liquid	calf	Australia
powder	calf	Australia
liquid	calf	Belgium
liquid	calf	Denmark
powder	calf	Denmark
powder	calf	Denmark
liquid	calf	Denmark
powder	calf	Denmark
liquid	calf	France
liquid	calf	France
liquid	calf	France
liquid	calf	France
liquid	calf	France
liquid	calf	France
liquid	calf	Netherlands
liquid	calf	Netherlands
liquid	calf	Netherlands
liquid	buffalo	Italy
liquid	buffalo	Italy
liquid	calf	Italy
liquid	calf	Italy
powder	calf	Italy
powder	calf	Italy
liquid	calf	Italy
liquid	calf	Italy
liquid	calf	Italy
liquid	calf	Italy
liquid	calf	Italy
liquid	calf	Italy
liquid	calf	Italy
liquid	calf	Italy
liquid	calf	Italy
liquid	calf	Mix
liquid	calf	Mix
powder	calf	Mix
liquid	calf	Mix
liquid	calf	Mix
liquid	calf	Mix
liquid	calf	Mix
liquid	calf	Mix
powder	calf	Mix
powder	calf	Mix
powder	calf	New Zealand
liquid	calf	New Zealand
liquid	calf	New Zealand
powder	calf	New Zealand
powder	calf	New Zealand
liquid	calf	New Zealand
powder	calf	New Zealand
liquid	calf	New Zealand
liquid	calf	New Zealand
Fermentation-produced chymosin		
liquid		Denmark
powder		Denmark
liquid		Denmark
liquid		Denmark
liquid		Denmark
liquid		Netherlands
powder		Netherlands
liquid		Netherlands
liquid		Netherlands

2.2. Methods

2.2.1. Extraction of chymosin from animal rennet and genetic coagulant

Rennet samples and genetic coagulant were desalinated using an ultrafiltration system in order to reduce NaCl content from around 20%

to 0.5%. The residue was acidified with HCl 15%, left in the fridge at 4 °C for 24 h and filtered with a syringe (0.45 µm). The pH was then adjusted to 5.5 using NaOH. Chymosin was separated from pepsin using an anion exchange column (resin DEAE 53) and deionized water pH 5.5 as solvent. Chymosin was recovered from the column using a NaCl/water solution with conductivity of 30–33 mS/cm^2 and a pH of 5.5. The percolate was concentrated using the ultrafiltration system. NaCl (15–18%) and Na benzoate were added as preservatives to the solution containing chymosin.

Pure chymosin was obtained from 30 mL of this solution, following the method used to precipitate protein from honey (AOAC 998.12), i.e. by adding 2.0 mL of 10% $NaWO_4$ and 2.0 mL of 0.335 M H_2SO_4 in an 80 °C water bath until visible floc forms with clear supernatant. Finally, the chymosin was freeze dried.

2.2.2. IRMS analysis

The chymosin extracted from powdered animal rennet and fermentation-produced chymosin was weighed in a tin capsule using a microanalytical balance (Sartorious, Germany).

The $^{15}N/^{14}N$ ratio was measured (around 1 mg) using an isotope ratio mass spectrometer (Finnigan DELTA XP, Thermo Scientific, Germany) following total combustion in an elemental analyser (EA1112, Thermo Scientific).

The values were denoted in delta in relation to the international AIR standard following this equation:

$$\delta^{15}N = \frac{(\ ^{15}N/^{14}N \text{ sample} - ^{15}N/^{14}N \text{ ref})}{^{15}N/^{14}N \text{ ref}}$$

The delta values were multiplied by 1000 and expressed in units “per mil” (‰).

Sample analysis was carried out in duplicate. The samples were analysed using 2 working standards for normalisation, calibrated against USGS 40 (U.S. Geological Survey, Reston, VA, USA) and potassium nitrate IAEA-NO3 (IAEA, Vienna). The uncertainty (2 s) of measurements was < 0.3‰.

2.2.3. Simulated adulterated samples

In order to simulate the process of adulteration and assess the potential of $\delta^{15}N$ analysis for fraud detection, a simulation approach was applied. Bootstrap was applied to generate 10,000 mixtures of animal rennet and fermentation chymosin extracted from the experimental dataset, varying their respective contribution from 0% to 100% (step 0.05). For each mixture and each composition, the “sample” shift was calculated as the weighted average of the measured shifts. The 10,000 replicates were used to estimate the mean, median and 95 confidence intervals of the resulting $\delta^{15}N$ shifts.

2.2.4. Statistical analysis

All statistical analysis was performed in R (R R Core Team, 2018) and visualised using the tidyverse and ggplot packages (Wickham, 2017).

3. Results and discussion

3.1. Repeatability

One sample of animal rennet was subjected 8 times to the entire analytical procedure, including extraction of chymosin, precipitation and analysis.

The results are shown in Table 2.

Repeatability was very good: the standard deviation was comparable with instrumental deviation, indicating that the extraction and precipitation procedures did not cause any isotopic fractionation.

Table 2
Repeatability of $\delta^{15}N$ analysis in chymosin from animal rennet.

Repetition	$\delta^{15}N$ ‰ vs AIR
1	6.0
2	6.0
3	5.9
4	6.0
5	6.1
6	6.1
7	5.9
8	5.9
mean	6.0
std dev	0.1

3.2. $\delta^{15}N$ of chymosin

The $\delta^{15}N$ values of chymosin from animal rennet and fermentation-produced chymosin are shown in Supplementary material and summarised in Fig. 1.

The $\delta^{15}N$ values of chymosin in animal rennet were relatively homogeneous, ranging from +5.7‰ to +8‰, with an average of +6.9‰ and a standard deviation of 0.5‰. There was no significant difference either between powder and liquid animal rennet or according to geographical origin or the 5 different production technologies. On the other hand, the $\delta^{15}N$ values of fermentation-produced chymosin were significantly lower and with much higher variability, ranging from −5.3‰ to +2.2‰. The lower values and higher variability are due to the fact that the nitrogen source of fermentation-produced chymosin depends on the composition of the fermentation medium which can contain synthetic nitrogen compounds having $\delta^{15}N$ values between −6‰ to +6‰ (Bateman & Kelly, 2007). Furthermore during the fermentation atmospheric air, which has an established $\delta^{15}N$ values of ~0‰, is blown (Hellmuth & van den Brink, 2013).

Chymosin from calves showed higher $\delta^{15}N$ content, due to increased $\delta^{15}N$ of approximately +3‰ *per* trophic level (Kurle & Worthy, 2002) and to the fact that breastfed animals have further enrichment when compared to maternal values (Fuller, Fuller, Harris, & Hedges, 2006). It appears that at this trophic level, $\delta^{15}N$ variability linked to the geographical, physiological and dietary origin of animal is less evident.

On the basis of the $\delta^{15}N$ of the 2 types of chymosin, it is clear that the addition of fermentation-produced chymosin to animal rennet, or its complete substitution, affects the $\delta^{15}N$ of animal rennet, lowering the expected value.

The results of the simulations, which were run to assess the potential of the method to detect the addition of fermentation-produced chymosin to animal rennet, are summarised in Fig. 2.

The plot shows the expected range of variability of $\delta^{15}N$ shift as a function of the percentage of fermentation-produced chymosin added to the mixture. The two dashed lines represent the 95 confidence intervals, while the two continuous lines show the position of the mean and the median, respectively. To validate the results from fraud sample simulations, real mixtures starting with different samples of animal rennet and fermentation-produced chymosin were tested and analysed and their measured shifts are shown as circles in the plot (Fig. 2). Their distribution confirms the lowering of the shift as the amount of fermentation-produced chymosin. As expected, all the circles lay within the 95 confidence intervals, thus supporting the proposed simulation approach.

The set of animal samples was also used to identify a lower boundary of 5.7‰ for the shift expected for authentic rennet, which is also highlighted in Fig. 2 as a red line. Samples showing a shift lower than the proposed limit are unlikely to be composed of pure animal rennet, but Fig. 2 also shows that adulterated samples can also be characterised by higher ^{15}N shifts.

In order to better characterise the potential of $\delta^{15}N$ analysis to

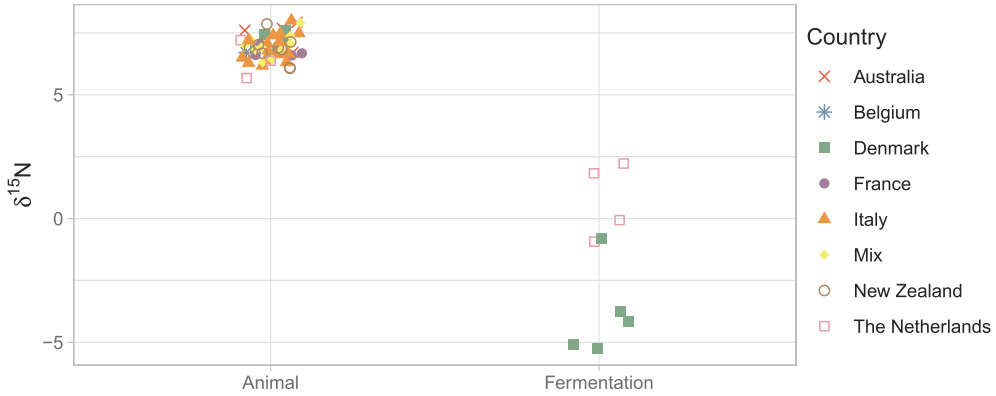

Fig. 1. $\delta^{15}N$ values of animal rennet and fermentation-produced chymosin.

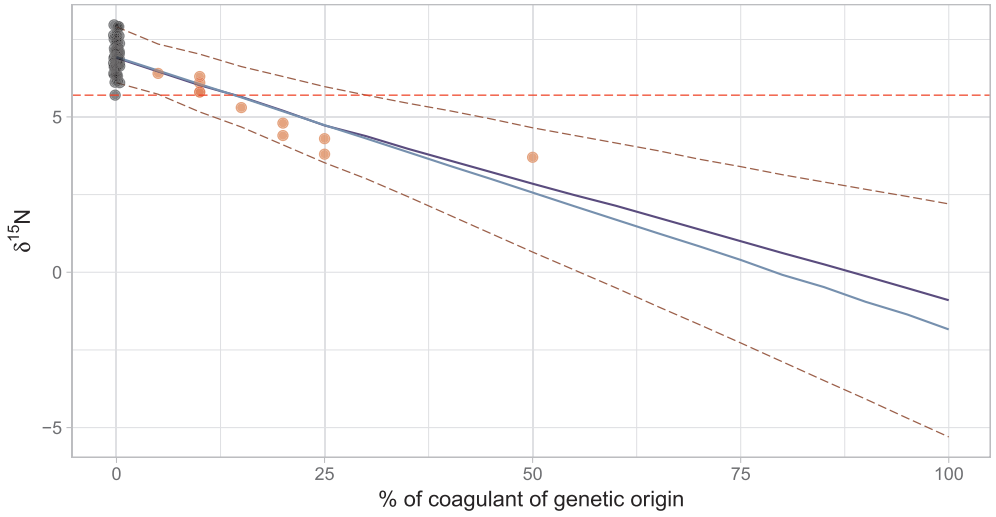

Fig. 2. $\delta^{15}N$ of animal rennet chymosin with the addition of an increasing percentage of fermentation-produced chymosin.

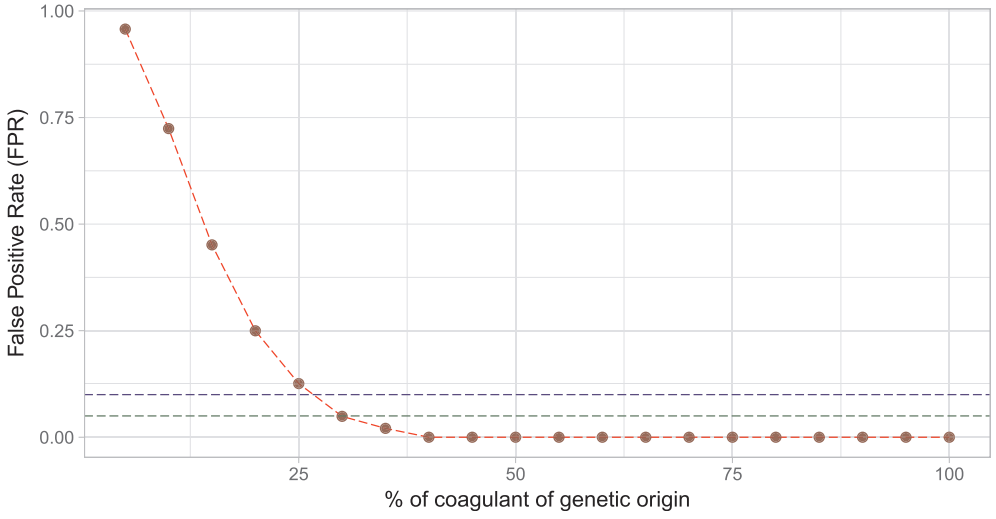

Fig. 3. False Positive Rate (FPR = False Positives/True Negative) as a function of the percentage of fermentation-produced chymosin added.

assess the authenticity of animal rennet, the limit of 5.7‰ was used to classify adulterated samples on the basis of the addition of fermentation-produced chymosin. In this classification scheme, all the samples are non-authentic (true negative), so samples showing a shift higher than 5.7‰ must be considered as False Positives. Fig. 3 shows the dependence of the False Positive Rate (FPR = False Positives/True Negative) as a function of the percentage of fermentation-produced chymosin present in the samples.

The dashed horizontal lines highlight respectively the position of the 0.05 and 0.1 limits, which in practice correspond to 5 and 10 percent error rates.

The plot clearly indicates that with a percentage of adulteration higher than 30, adulteration was correctly identified in more than 95% of cases. This decreases to 90% with a level of adulteration slightly over

25%. Below this limit, the percentage of false positives rapidly increases, reaching 95% at a 5% level of adulteration

4. Conclusions

In this study the authenticity range of $\delta^{15}N$ values of animal chymosin (5.7‰ to 8‰) and fermentation-produced chymosin (< 2.2‰) were determined for the first time. On the basis of these initial results, we can conclude that $\delta^{15}N$ chymosin is highly effective in distinguishing animal rennet from fermentation-produced chymosin. This parameter is also effective in determining the authenticity of rennet obtained by mixing animal with genetically modified chymosin.

Declaration of interests

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.04.106.

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