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Analytical Methods

Assessment and validation of methods for the determination of γ-glutamyltransferase activity in sheep milk

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ABSTRACT

The determination of enzymatic activity in sheep milk is still today a practically unexplored field of research. This study proposes and fully validates two analytical procedures (i.e. by means of UV–vis spectrophotometry and RP-HPLC methods) for the determination of γ-glutamyltransferase activity in sheep milk. Both methods are characterised by low detection and quantification limits, excellent linearity over a wide enzymatic activity interval, very good repeatability and reproducibility, and are bias-free. The RP-HPLC method provides better sensitivity and automation capability levels than that of UV–vis, and its use is hence suggested for screening purposes. These methods have been preliminarily tested with a number of real samples of whole sheep milk, obtaining an average γ-glutamyltransferase activity value of 2.93 ± 0.50 U ml^{-1} and a range from 2.72 ± 0.10 U ml^{-1} to 3.46 ± 0.11 U ml^{-1}.

1. Introduction

Apart from bovine milk, sheep milk is without doubt the most important dairy food. Unlike the former, its production is localised in a few geographical areas, the most important of these being in the Mediterranean basin. Within this zone, Sardinia (Italy) is the most significant producer worldwide, accounting for up to 20% of the global total. More than 70% of the production of Sardinian sheep milk is transformed into three valuable PDO (Protected Designation of Origin) cheeses: Pecorino Romano, Pecorino Sardo and Fiore Sardo. Beyond intrinsic technological aspects, these products differ from each other also due to the nature of the thermal treatment that the milk undergoes before and during the transformation stage. In particular, the production technology of Pecorino Romano PDO allows the use of pasteurised milk, whereas only raw milk can be used for the production of Fiore Sardo PDO.

Thermal treatment is one of the main factors that affect milk properties. It can increase product safety and shelf-life, but usually reduces the nutritional and sensory properties of milk and milk products. For these reasons, great attention has been paid in the past to the assessment of analytical methods that aim to identify thermal treatment of dairy products (Pellegrino, Resmini, & Luf, 1995). The analytes and the matrix mainly studied were the endogenous enzymes and bovine milk, respectively. For this matrix, the inactivation of alkaline phosphatase (EC 3.1.3.1) and lactoperoxidase (EC 1.11.1.7) activities have been used as identifiers of pasteurisation and ultra-heat treatment, respectively (EEC Council, 1992). Unfortunately, the inactivation of alkaline phosphatase has been found to be reversible (Murthy, Cox, & Taylor, 1976), and the high water solubility showed by lactoperoxidase suggests that monitoring of this enzyme does not always constitute a reliable marker, at least in transformed products, such as cheese (Blel, Guingamp, Gaillard, & Humbert, 2002).

Qualitative and quantitative differences in the most important components of sheep milk in comparison to bovine milk have been highlighted by previous studies (Fox & McSweeney, 1998)). On the other hand, little attention has been paid to minor components in sheep milk and its related products. As far as research related to endogenous enzymes in sheep milk is concerned, only the contributions by Scintu, Daga, and Ledda (2000; 2001), who studied the activity and thermal inactivation process of alkaline phosphatase, are of note. The high activity values and the substantial seasonal variation in the enzyme activity of sheep milk in comparison to bovine milk made it difficult to define reliable threshold limits to permit the identification of different thermal treatments in this matrix.

γ-Glutamyltransferase (γ-GT) (EC 2.3.2.2) has also been proposed as a pasteurisation marker (Blel et al., 2002; Dos Anjos, Machado, Ferro, Otto, & Bogin, 1998; McKellar, Emmons, & Farber, 1991; Patel & Wilbey, 1994) in bovine milk. To the best of our knowledge, no data are to date available in the literature

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concerning the qualitative identification and the measurement of the activity of γ-GT in sheep milk and its related dairy products. The profound differences between sheep and bovine milk, (with regard to lipid and protein concentrations (Fox & McSweeney, 1998)), lead us to assume that this analytical task cannot be reduced to mere application of the methods developed for bovine milk. For these reasons, the aims of this work are as follows: (i) to assess and validate analytical procedures for sensitive, precise and accurate evaluation of γ-GT activity in sheep milk; (ii) to give a first evaluation of the typical average value and range of γ-GT activity in sheep milk.

2. Materials and methods

2.1. Reagents and samples

Glassware was washed prior to use with acetone and *n*-hexane (both ACS grade, Fluka, Milan, Italy) and dried at 105 °C. Ultrapure (Type 1) water (specific resistance $\geqslant$18 MΩ) was always used if not otherwise specified. HCl (ACS grade, 37%), acetic acid (99%) and Clarifying Reagent were from Fluka. Ethanol (ACS reagent), acetonitrile (HPLC grade), *p*-nitroaniline (pNA), γ-glutamyl-*p*-nitroaniline (γ-GPNA), glycylglycine (Glygly), the tetrasodium salt of ethylenediaminetetraacetic acid ($EDTANa_4$), hydroxymethyl aminomethane (TRIS) and hydroxymethyl aminomethane clorhydrate solution (TRIS–HCl), pH = 8.5, were all from Aldrich, Milan, Italy. All HPLC solvents used were previously filtered through a 0.45 μm membrane, from Millipore, Bedford, MA, to remove any impurities.

Whole bulk milk was obtained from a Sardinian sheep breed reared in the experimental farm AGRIS Sardegna (Bonassai, Sassari, Italy). After milking, the samples were kept at 4 °C (if not otherwise specified) and analysed within 4 h. Before analysis the milk was homogenised for 15 min by shaking at 40 °C.

2.2. Equipment

All UV–vis spectrophotometric determination was carried out using a double ray UV–vis spectrophotometer Varian (Palo Alto, CA) Cary 1E with a signal band width of 2 nm and 1 cm quartz cuvettes. The HPLC apparatus consisted of a Varian (mod. 9065600 E) pump system equipped with a Waters UV–vis detector (model 486, Waters Corporation, Milford, MA), a Varian autosampler (mod. 9100) and a Varian PLRP-S column (4.6 mm i.d. x250 mm, 8 μm particle size).

2.3. Procedures

2.3.1. UV–vis method

2.3.1.1. Analysis of standard solutions – Calibration plot. A number between 5 and 9 mixtures, each containing 100 μl of thermally-inactivated (100 °C for 5 min) sheep milk and 2 ml of preheated solution (2 min, 37 °C) containing 40 mM Glygly, 0.2 M Tris–HCl, pH = 8.5 and increasing amounts of pNA (between 0.020 and 0.800 μmol) were prepared in 5 ml test tubes. The mixture was quickly homogenised by gentle agitation and kept at 37 °C for 15 min; 2 ml of Clarifying Reagent® and 100 μl of $EDTANa_4$ (0.2 M) were then sequentially added. The solution was shaken vigorously and incubated for 2 min at 37 °C. The absorbance of each clarified solution was then read in triplicate at 410 nm against blank within 10 min, reported *vs*. the relevant amount of pNA and expressed in units of enzymatic activity per ml (U ml^{-1}).

2.3.1.2. Analysis of samples. Whole (or skimmed) sheep milk (100 μl) was added to 2 ml of preheated solution (2 min, 37 °C) containing 4 mM γ-GPNA, 40 mM Glygly and 0.2 M of Tris–HCl, pH = 8.5 and then underwent the same treatment as the standards. If the milk sample displayed activity that was too high (i.e., up to one unit of absorbance) then it was supplemented with known amounts of thermally-inactivated milk.

2.3.2. RP-HPLC method

Whole (or skimmed) sheep milk (20 μl) was added to 500 μl of preheated solution (2 min, 37 °C) containing 4 mM γ-GPNA, 40 mM Glygly and 0.2 M of Tris–HCl, pH = 8.5. The mixture was quickly homogenised and kept at 37 °C for 15 min; then the enzymatic reaction was stopped with the addition of 500 μl of 1 M Tris Base solution, pH = 10.0. Subsequently, 2.5 ml of ethanol was added to the solution. After shaking, the sample was centrifuged at 4500 rpm for 5 min. Fifty microlitres of clear supernatant were injected into the HPLC apparatus. Chromatographic separation was accomplished by the relevant gradient elution (water, solvent **A**, and acetonitrile, solvent **B**, both containing 1% (v/v) acetic acid, flow rate of 1 ml min^{-1}, operative wavelength of 368 nm): 0–16 min 90% **A**; 16–18 min, 58% **A**; 18–20 min, 0% **A**; 20–25 min, 90% **A**.

The quantification of pNA was calculated by interpolation of a linear calibration plot obtained from six standard solutions, each containing 20 μl of thermally-inactivated sheep milk and 500 μl of preheated solution (2 min, 37 °C) containing 40 mM Glygly, 0.2 M Tris–HCl, pH = 8.5, and increasing amounts of pNA (between 5×10^{-10} and 2×10^{-7} mol). The mixture was incubated for 15 min at 37 °C and processed in the same way as the sample. Each sample (or standard) was processed, read in triplicate and the relevant units of enzymatic activity per ml (U ml^{-1}) were calculated on the basis of absorbance.

3. Results and discussion

3.1. UV–vis spectrophotometric evaluation of γ-glutamyltransferase activity in sheep milk

UV–vis spectrophotometric determination of γ-GT activity is usually based on the quantification of *p*-nitroaniline (pNA) obtained when the enzyme transfers the γ-glutamyl group from an intentionally added precursor, γ-glutamyl-*p*-nitroaniline (γ-GPNA), to the receptor (i.e., glycylglycine) (Andrews, Anderson, & Goodenough, 1987; Baumrucker, 1979; Blel et al., 2002; Patel & Wilbey, 1994). In this case, the unit of activity (U) is defined as the quantity of enzyme that releases 1 μmol of pNA per min at 37 °C and pH 8.5. Before UV–vis measurement, a clarification step was required in order to solubilise both casein micelles and fat globules. For this purpose, alkaline mixtures of organic solvents and surfactants are commonly used. Among these, the best known is Clarifyng Reagent® (Blel et al., 2002; Humbert, Guingamp, Linden, & Gaillard, 2006; Linden, Humbert, Kouomegne, & Guingamp, 1987).

The application to sheep milk of the methods used for bovine milk provided only qualitative evidence of the presence of γ-GT. The quantitative evaluation of the activity of this enzyme in the latter matrix was hampered by the ineffectiveness of the clarification step, probably due to the higher amount of micellar casein, which characterises sheep milk, in comparison to bovine milk. In order to overcome the interference, the final pH of the solution was raised in an attempt to increase the degree of denaturation of the proteins in the sheep milk (Fox & McSweeney, 1998). Unfortunately, this change concurrently promotes alkaline hydrolysis of the γ-GPNA, with loss of specificity, due to formation of pNA not produced exclusively in an enzymatic way. Another possible approach was the addition of complexing agents of Ca^{2+} ions, which

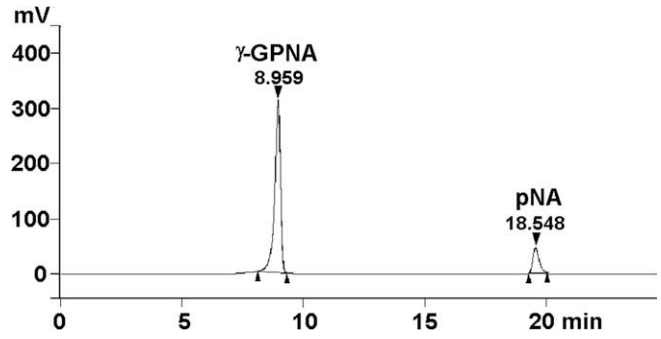

Fig. 1. RP-HPLC evaluation of γ-glutamyltransferase activity in sheep milk. Peak 1, γ-glutamyl-*p*-nitroaniline, γ-GPNA; peak 2, *p*-nitroaniline, pNA.

subtract these ions by electrostatic interaction with casein micelles, increasing micellar breakdown. Among the different complexing agents tested, a 0.2 M $EDTANa_4$ solution in water provided analytical blanks better than or similar to those obtained in the measurement of γ-GT in bovine milk.

3.2. Chromatographic evaluation of γ-glutamyltransferase activity in sheep milk

Although the pure UV–vis method for evaluating γ-GT activity in sheep milk is more than satisfactory, it appears possible that analytical performances could be improved by using an HPLC method with a UV–vis detector. In this way the clarification step also appears to be unnecessary. Moreover, to the best of our knowledge, no HPLC method has ever been assessed for the determination of γ-GT activity in milk of any origin. Hence, an RP-HPLC method was set up with this aim, as described in the materials and methods section. Fig. 1 shows a typical chromatogram obtained from a sample of sheep milk.

3.3. Evaluation of γ-glutamyltransferase activity in sheep milk samples using the proposed methods

Both methods were tested by analysing real samples of whole sheep milk collected between January and May 2007.

The range of the γ-GT activity is between 2.72 ± 0.10 U ml^{-1} and 3.46 ± 0.11 U ml^{-1} whereas the relevant average activity is 2.93 ± 0.50 U ml^{-1} (Table 1). Despite the higher amount of proteins and lipids present in sheep milk, the average value appears to be significantly lower than that measured in whole and skimmed bovine milk (4.30 ± 0.30 U ml^{-1} and 3.11 ± 0.18 U ml^{-1}, McKellar et al., 1991). Moreover, these preliminary data suggest a significant seasonal variability of the parameter, which has never been reported for bovine milk (McKellar et al., 1991; Patel & Wilbey, 1994).

3.4. Validation

3.4.1. Limit of detection (LOD) and limit of quantification (LOQ)

These parameters were calculated according to IUPAC guidelines (Currie, 1995). This model provides reliable results whenever it is possible to calculate the signal from blank solutions with good accuracy and when the slope of the calibration plot does not change significantly on lowering the concentrations of analyte. This last condition was verified by comparison of slopes of the regression plot obtained by applying both methods, with the final pNA concentration ranging from 5×10^{-6} μM to 190×10^{-6} μM and from 5×10^{-6} μM to 20×10^{-6} μM (criteria: two-tailed *t*-test, $p = 0.95$). For each method ten measurements of the blank signal were perfomed and the standard deviation, σ of this dataset was calculated. The detection limit was obtained as follows:

$$\mathrm{LOD} = 3.3\frac{\sigma}{a}$$

where a is the slope of the calibration plot in the interval of pNA concentration between 5×10^{-6} μM and 20×10^{-6} μM. The detection limit expressed in units of enzymatic activity per ml of milk was 2.8 mU ml^{-1} and 1.5 mU ml^{-1} for the UV–vis spectrophotometric and RP-HPLC methods, respectively. While the LOD value for the RP-HPLC method has no terms of comparison, our LOD value is significantly better than those previously reported in the literature for UV–vis measurements in bovine milk (Blel et al., 2002; McKellar et al., 1991). According to Currie (1995), the limit of quantification, LOQ, is equal to 3 × LOD. Hence, the LOQ values were 8.5 mU ml^{-1} and 4.6 mU ml^{-1} for the spectrophotometric and RP-HPLC methods, respectively.

3.4.2. Linearity

Excellent linear correlation coefficients were almost always observed, both in the spectrophotometric ($r^2 = 0.9995$) and in RP-HPLC ($r^2 = 0.9993$) methods in a wide γ-GT activity range (from 0.013 U ml^{-1} to 0.533 U ml^{-1} and from 0.002 U ml^{-1} to 0.667 U ml^{-1}, respectively). Table 2 presents these data.

The very low value of the *b* term in both the linear regressions reported in Table 2 appears in all cases to be not significantly different from zero (criteria: two-tailed *t*-test, $p = 0.95$). This ensures that the experimental dependence between absorbance and concentration is in complete agreement with Beer's law. In addition, graphical analysis of the residuals of the regression line for each method allowed us to exclude any "hidden" deviation from linearity in all cases. Fig. 2 reports the output of the graphical analysis relating both to the UV–vis and RP-HPLC methods.

Table 1
γ-glutamyltransferase activity in sheep milk samples measured with UV–vis spectrophotometric or RP-HPLC methods.

Sample	Month of collection	Spectrophotometric method		RP-HPLC method	
		γ-GT activity (U ml^{-1})	s[a]	γ-GT activity (U ml^{-1})	s[a]
1	January 2007	2.72	0.10	2.69	0.13
2	January 2007	2.73	0.02	2.71	0.12
3	January 2007	2.85	0.04	2.88	0.10
4	January 2007	3.44	0.13	3.36	0.11
5	February 2007	2.83	0.07	2.86	0.09
6	February 2007	3.26	0.12	3.33	0.12
7	February 2007	3.46	0.11	3.41	0.14
8	May 2007	2.76	0.08	2.72	0.18
9	May 2007	2.77	0.06	2.76	0.11
10	May 2007	2.54	0.08	2.51	0.17

[a] Standard deviation.

Table 2
Linearity parameters for UV–vis spectrophotometric and RP-HPLC methods for the determination of γ-glutamyltransferase activity in sheep milk.

Method	$Y = (a \pm s_a)X + (b \pm s_b)$				
	a	s_a	b	s_b	R^2
UV–vis[a]	2.65	0.013	−0.002	0.001	0.9995
RP-HPLC[b]	5.92×10^6	0.809×10^6	1.85×10^3	6.19×10^3	0.9993

[a] Linearity in the range from 0.013 U ml^{-1} to 0.533 U ml^{-1}.
[b] Linearity in the range from 0.002 U ml^{-1} to 0.667 U ml^{-1}.

In conclusion, the proposed methods do not suffer, within the concentration range considered, from any non-linear response behaviour.

3.4.3. Specificity

Although specificity can be an intrinsic concept in enzymatic methods, we have verified that the production of pNA from γ-GPNA could be associated exclusively with the action of γ-GT. After spiking with thermally-inactivated sheep milk, the absorbance of both reagent blanks was monitored six times for 90 min. No absorbance drift was recorded in this period (criteria: two-tailed *t*-test, $p = 0.95$, residuals analysis).

3.4.4. Precision

Precision was evaluated through repeatability and reproducibility measures.

Repeatability was evaluated from ten consecutive replicates of the complete analytical procedures, performed on sample 3 (i.e., the sample displaying γ-GT activity closest to the average value, see Table 1) and evaluated in terms of experimental coefficient of variation ($CV\%_{exp,r}$). Average repeatability was 3.5% and 2.9% for the UV–vis and RP-HPLC methods, respectively. These values are comparable to that previously reported by Blel and co-workers (3%; Blel et al., 2002). However, in order to dispel any uncertainty relating to the acceptability of the precision data, a fitness-for-purpose evaluation of these data was also performed. The approach selected was based on Horwitz's theory (Horwitz, 1982), according to which it is possible to calculate a theoretical CV% ($CV\%_H$) value on the basis of the concentration level expected. If the experimental coefficient of variation is lower than the upper confidence limit of the theoretical CV% value (equal to 1.5 $CV\%_H$) the experimental data can be deemed acceptable. Sample 3 substantiates a γ-GT activity of ca. 3 U ml^{-1}, i.e., a pNA concentration (in terms of mass ratio) between 10^{-4} and 10^{-6}, depending on the method considered. This means that, according to Horwitz's theory, the minimum theoretical repeatability value ($CV\%_{H,r}$) is equal to 4% and 1.5 $CV\%_{H,r}$ is equal to 6%. As experimental values $CV\%_{exp,r}$ are always well below 6%, the repeatability values obtained are also acceptable.

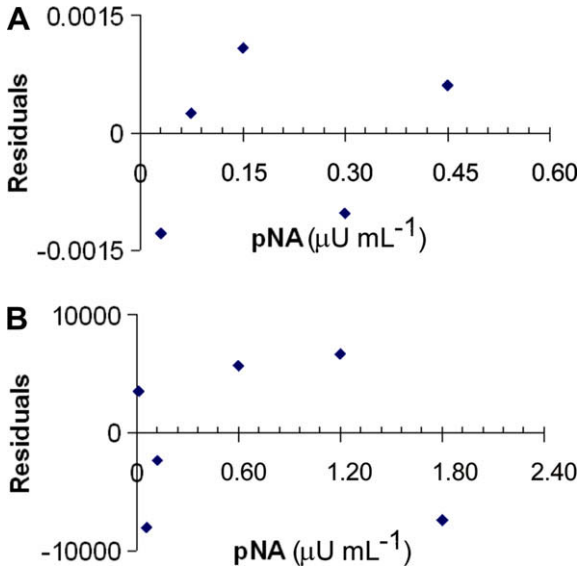

Fig. 2. Linearity in γ-glutamyltransferase activity in sheep milk. Output of the residual graphical analysis for the (A) spectrophotometric and (B) RP-HPLC methods.

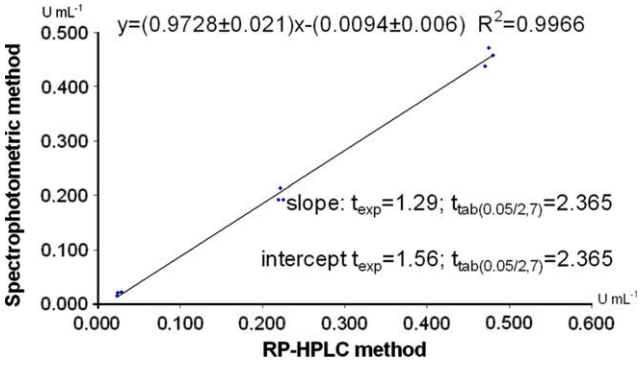

Fig. 3. Correlation between data obtained from the measurement of γ-glutamyltransferase activity in sheep milk using spectrophotometric (*y*-axis) and RP-HPLC (*x*-axis) methods.

Reproducibility was obtained as the percent coefficient of experimental variation ($CV\%_{exp,R}$) of the results of five analyses of sample 3 performed over four different analytical sessions (one every week) for the duration of one month. Within this period the sample was stored at −24 °C in order to prevent any loss of enzymatic activity. Average $CV\%_{exp,R}$ was 3.8% and 3.9% for the spectrophotometric and RP-HPLC methods, respectively. In this connection the minimum theoretical reproducibility value ($CV\%_{H,R}$) is equal to 6% whereas 1.5$CV\%_{H,R}$ is equal to 9%. This leads us to conclude that, since $CV\%_{exp,R}$ values are always less than 9%, the reproducibility parameters can also be considered acceptable.

3.4.5. Bias

The fact that Certified Reference Materials were not available led us to estimate bias by comparing datasets obtained from the independent analytical methods assessed in this study. Indeed, it is highly unlikely that two different analytical methods could be affected by the same bias. For this reason, a sample of whole sheep milk was analysed, both pure and diluted with increasing amounts of thermally-inactivated sheep milk, by means of the UV–vis and RP-HPLC methods. The average value obtained for each sample (analysed in triplicate) with these methods is reported in Fig. 3.

Comparison of enzymatic activity indicates that the two methodologies produce statistically identical results (criteria: two-tailed *t*-test, $p = 0.95$), when compared with the bias-free correlation function $y = x$. This result suggests the absence of any systematic error (bias) for both procedures.

4. Conclusions

Two analytical methods for the evaluation of γ-glutamyltransferase activity in sheep milk have been proposed. To the best of our knowledge, these are completely novel for this matrix, which is significantly different from the more extensively studied bovine milk. Assessing the UV–vis spectrophotometric method, particular attention has been paid to solving the critical problem of the clarification of the milk-containing solution before reading. An optimised addition of $EDTANa_4$ solution allowed us to overcome

interference, providing excellent values of analytical blanks. Always to the best of our knowledge, this is the first time that an RP-HPLC method is proposed for the determination of γ-glutamyltransferase activity in any milk. This approach is more sensitive and more suitable for automatic analysis than the spectrophotometric method, and can be useful for screening purposes. Very low detection and quantification limits, excellent linearity over a wide interval of enzymatic activity, good precision and absence of any bias represent the key benefits of the validation protocol for both methods. The methods were tested with authentic whole sheep milk. The average γ-GT activity value is 2.93 ± 0.50 U ml^{-1}, significantly less than values reported for bovine milk. The range of this enzymatic activity was found to be between 2.72 ± 0.10 U ml^{-1} and 3.46 ± 0.11 U ml^{-1}. Marked seasonal variability was recorded in the dataset, but the low number of samples analysed prevents us from drawing conclusions of general significance. In conclusion, it is now possible to perform a sensitive, precise and accurate evaluation of γ-glutamyltransferase activity in sheep milk with two different analytical methods. This fact could be of interest in all situations where discrimination must be made on the basis of the thermal treatment undergone by the dairy product (i.e., in compliance with PDO requirements).

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