Optimization and validation of a chromatographic method for quantification of lysozyme in jenny milk

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Summary
Jenny milk has recently stimulated scientific interest for its attractive nutritional characteristics, particularly for geriatric and paediatric patients. Moreover, it has a high concentration of lysozyme, whose antibacterial properties are well recognized. In literature, a wide range of lysozyme concentrations in jenny milk has been reported (from 1.0 $g \cdot l^{-1}$ to 4.0 $g \cdot l^{-1}$). In this study, a step-by-step optimized protocol for jenny milk lysozyme extraction and quantification by high pressure liquid chromatography (HPLC) was developed. Moreover, a comparison between ultraviolet (UV; at 227 nm and 280 nm) and fluorescence detection was performed. All developed HPLC methods for analysis of lysozyme were validated according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines (ICH Q2(R1), 2005) for linearity, accuracy, sensitivity and precision. The protocol here presented is easy and quick to perform, with the UV detection at 280 nm providing a valid alternative to traditional fluorescence method for quantification of lysozyme in jenny milk.

Keywords
lysozyme; jenny milk; high pressure liquid chromatography; ultraviolet detection; fluorescence

Jenny milk has become increasingly important in human nutrition, as it is one of the most important milks providing nutritional benefits. Among the domestic livestock species raised for milk production, jenny provides milk that shows the closest similarity to human milk. For this reason, it is often utilized in the paediatric sphere as the best alternative to human milk in infant food. Besides that, it is used by patients who are allergic to the proteins in cows' milk [1]. The vitamin C concentration in jenny milk presents recommended daily intake for new-borns aged from 6 to 12 month [2]. In the geriatric field, it favours the prevention of cardiovascular diseases, and it is employed in patients' diet with coronary heart disease [3]. Jenny milk is also rich in bioactive compounds stimulating the scientific interest. For instance, this milk is characterized by high levels of unsaturated fatty acids (linoleic and linolenic) [4], low casein levels, high concentration of lysozyme and high concentration of lactose [5]. Moreover, jenny milk shows low levels of microbial contamination, probably because of the high amount of lysozyme [6]. This enzyme is very resistant to proteases and can play a significant role in the intestinal immune response [7].

Lysozyme is capable to degrade the polysaccharide architecture of bacterial cell walls for the purpose of protection against bacterial infection. The antibacterial activity of this enzyme is due to its ability to catalyse the hydrolysis of the β-1,4-glycosidic links between *N*-acetylmuramic acid and *N*-acetylglucosamine of polysaccharides in the Gram-positive bacterial cell wall, working in synergy with lactoferrin, lactoperoxidase and immunoglobulins [8]. Therefore, jenny milk may be used to improve the activity of the immune system in digestive tract and to reduce the effects of gas-

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trointestinal infections in children [9]. Moreover, COSENTINO et al. [10] reported that jenny milk, thanks to the high concentration of lysozyme, is effective for controlling the occurrence of late blowing defects in cheese due to *Clostridium tyrobutyricum*.

It was reported that the concentration of lysozyme in jenny milk is affected by the lactation stage and the production season [11]. The reported values of lysozyme range from 1.0 $g \cdot l^{-1}$ [12] to 4.0 $g \cdot l^{-1}$ [13], as assessed by different analytical methods [14].

The first method developed for the detection of lysozyme was based on its lytic action. Lysis of the cell wall of *Micrococcus lysodeicticus* was measured by turbidimetric analysis [15]. Enzyme-linked immunosorbent assay (ELISA) methods were described as effective for the detection of lysozyme in food [16]. However, GEORGESCU et al. [17] showed that high performance liquid chromatography (HPLC) method adapted for animal-derived foods is more suitable for quantification than ELISA. Accurate methods for lysozyme detection and quantification include fluorescence detection (FLD) [18], liquid chromatography–mass spectrometry [19] and surface-enhanced mass spectrometry [20]. PELLEGRINO and TIRELLI [21] proposed an effective protocol for lysozyme quantification in cows' milk and its derivatives. Their protocol is available as an ISO standard [22], employing HPLC-FLD to detect lysozyme in cows' milk and dairy products using a reversed-phase polymeric column. Currently, the above protocol is the only one published method for lysozyme quantification in food matrices.

Since the lysozyme concentration range in jenny milk is very broad, there is still a need to establish the exact concentration of this enzyme in this matrix. Therefore, in the present study, we applied and validated a new sensitive and accurate method for lysozyme quantification in jenny milk. This method relies on HPLC with ultraviolet (UV) detection rather than fluorescence detection, which is commonly used for lysozyme quantification in milk and dairy products, but is less versatile. Two different HPLC detectors were employed based on FLD and UV, at two different wavelengths (227 nm and 280 nm).

MATERIALS AND METHODS

The research was carried out on bulk milk of 15 pluriparous jennies (Martina Franca breed) in mid-stage of lactation (180 days after foaling), aged between 7 and 10 years. The temperature for transportation of milk samples was –20 °C, temperature of storage was –20 °C. A preliminary estimation of the lysozyme concentration in milk samples was done by a commercial ELISA kit according to manufacturer's instructions (Lysozyme ELISA Kit, SE120074; Sigma, St. Louis, Missouri, USA). According to these tests, the mean concentration of lysozyme was (1 131 ± 63) $mg \cdot l^{-1}$.

Chemicals

A freeze-dried lysozyme analytical standard (Vetranal; Aldrich, Seelze, Germany) was used. HPLC-grade acetonitrile (ACN) and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, Missouri, USA). NaCl was purchased from Carlo Erba Reagents (Milan, Italy).

Sample preparation

The following extraction method was used, based on modification of the protocol of PELLEGRINO and TIRELLI [21]. Samples were prepared by mixing 10 ml of raw jenny milk with 30 ml of 1.0 $mol \cdot l^{-1}$ NaCl. After heating under magnetic stirring at 40 °C for 10 min, the samples were stirred on rotary shaker (1.7 Hz) at room temperature for 1 h. Afterwards, some drops of 1 $mol \cdot l^{-1}$ HCl were added in order to decrease the pH value from 6.0 to 2.2 and, hence, to provide precipitation of caseins. Finally, the samples were filtered through a paper filter, and then through a 0.20 μm syringe cellulose acetate (CA) filter (Minisart NML; Sartorius, Göttingen, Germany). Given the high concentration of lysozyme (1 131 ± 63 $mg \cdot l^{-1}$) estimated by ELISA, the extracts were diluted 1:3 with water before HPLC analysis.

Liquid chromatographic separation

Chromatographic conditions were employed to optimize peak resolution and response. These included adjusting of the flow rate and modification of the solvent gradient. The following are the conditions by which the best results were obtained. The chromatographic separations were run on a Synergi MAX-RP 80 Å column (150 mm × 4.6 mm, 4 μm particle size, internal diameter 4.6 mm) from Phenomenex (Torrance, California, USA) with a MAX-RP guard column (4 mm × 2 mm, Phenomenex). Injection volume was 20 μl and flow rate was 0.8 $ml \cdot min^{-1}$. The mobile phase consisted of water (A) and acetonitrile (B), both containing 0.1 % trifluoroacetic acid (v/v). The following eluting conditions were used: 0 min 80 % A and 20 % B; 9 min 60 % A and 40 % B; 15 min 60 % A and 40 % B; 20 min 80 % A and 20 % B.

HPLC analysis was performed using a chromatographic system Agilent 1200 Series quaternary pump furnished with vacuum degasser and autoinjector (Agilent Technologies, Santa Clara, California, USA).

Detection conditions

For FLD detection, Jasco FP-2020 Plus-Intelligent fluorescence detector (Jasco, Great Dunmow, United Kindom) was employed set at 280 nm excitation wavelength and 350 nm emission wavelength. UV detection was carried out using the Agilent 1200 series diode array detector set at 227 nm and 280 nm.

Validation of methods

All developed HPLC methods for analysis of lysozyme were validated according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) guidelines [23] for linearity, detector response, sensitivity and precision.

Linearity

Seven different concentrations of lysozyme in the range of 2–150 mg·l^{-1} were used for calibration and linearity. The linearity of the method was determined by plotting the peak area versus concentration of hen egg-white (HEW)-lysozyme standard solutions. The slope (m), the intercept (b) and the coefficient of determination (r^2) were determined from the regression analysis by software Sigma Plot 11.0 (Systat Software, London, United Kingdom).

Sensitivity

Limit of detection (LOD) and limit of quantification (LOQ) were determined to assess the sensitivity of our protocol.

$$LOD = 3.3\frac{s}{m} \quad (1)$$

$$LOQ = 10\frac{s}{m} \quad (2)$$

where s is the standard deviation of the intercept and m is the slope of the calibration curve.

Recovery

The recovery (R) was measured and expressed in percent as follows:

$$R = \frac{(L_t - L_s)}{L_a} \times 100 \quad (3)$$

where Lt is the total lysozyme concentration after standard addition; Ls is the lysozyme concentration in milk sample, and La is the concentration of added lysozyme.

Precision

The precision of the system was determined by calculating the mean standard deviation (SD) and the relative standard deviation (RSD) expressed in percent. Milk samples were injected ten times per day for three days. To determine the intermediate precision (intra-day and inter-days), the milk samples were analysed in three intervals in a day for repeatability and on three successive days for reproducibility.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test was used to evaluate significant differences in lysozyme quantification among the three methods used.

Tab. 1. Comparison between Pellegrino and Tirelli's chromatographic method [21] and that one proposed in this work for lysozyme quantification by HPLC analysis.

Item	Pellegrino and Tirelli's method [21]	New proposed method
Column resin, length	Reversed-phase polymeric column PLRP-S, 25 cm	Reversed-phase C12 column, 15 cm
Eluting solvents	Stock solution I: water, 0.1 % TFA (v/v) Stock solution II: acetonitrile, 0.1 % TFA (v/v) Solvent A: Stock solution I : Stock solution II 100 : 38.4 (w/w) Solvent B: Stock solution II	Solvent A: water, 0.1 % TFA (v/v) Solvent B: acetonitrile, 0.1 % TFA (v/v)
Column temperature	45 °C	Room temperature
Flow rate	1.0 ml·min^{-1}	0.8 ml·min^{-1}
Injection volume	50 μl	20 μl
Retention time	19 min	9.5 min

TFA – trifluoroacetic acid.

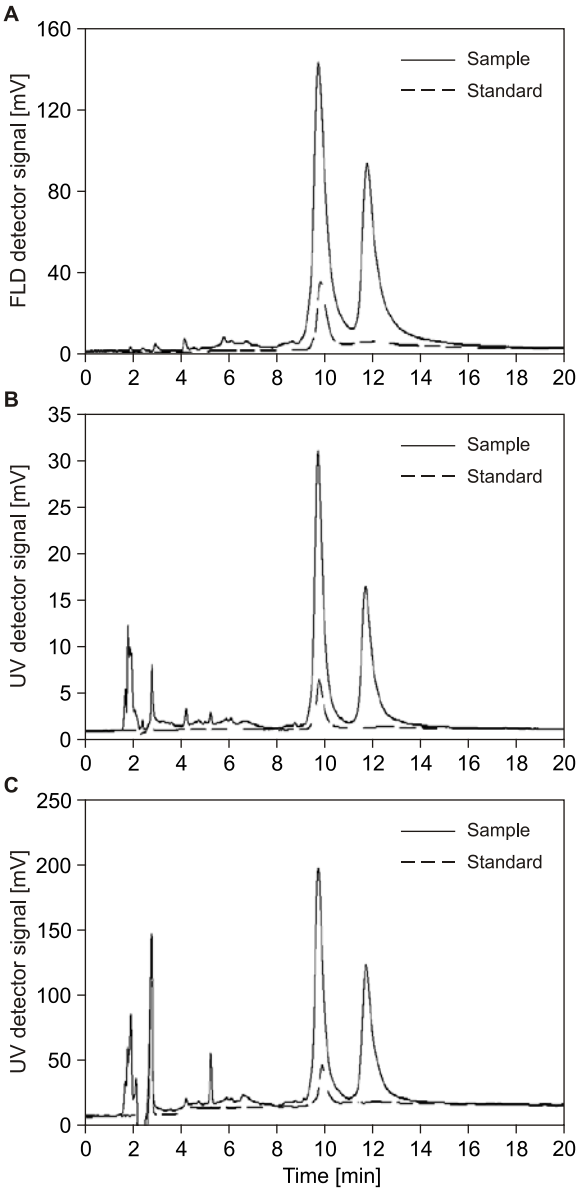

Fig. 1. Chromatograms of lysozyme extracts from raw jenny milk.

A – Fluorescence detection (excitation wavelength 280 nm, emission wavelength 350 nm), B – UV detection at a wavelength of 280 nm, C – UV detection at a wavelength of 227 nm.

RESULTS AND DISCUSSION

Amendments and changes for a new chromatographic method

The lysozyme extraction was performed according to PELLEGRINO and TIRELLI [21], with some modifications. It was not necessary to adjust the pH value with NaOH because the pH value of sample mixture was already 6. Moreover, the stirring was performed by a rotary shaker instead of a magnetic bar: in this way, more samples were stirred in the same time using only one instrument. More substantial changes were made to the chromatographic method. The main features of chromatographic conditions of Pellegrino and Tirelli's method [21] and the one proposed in this paper are shown in Tab. 1.

The chromatographic reversed-phase polymeric column (25 cm) was replaced by a shorter C12 (15 cm). The elution solvents employed were simpler and easier to prepare, preliminary preparation of stock solutions was not necessary. The column did not need heating. Eluting conditions were modified in order to reduce considerably the elution time, from 19 min (Pellegrino and Tirelli's protocol) to 9.5 min (our protocol). Both the volume injected and the flow rate were reduced.

Choice of detection method

At a first approach, a fluorescence detector was employed as proposed by PELLEGRINO and TIRELLI [21]. The HPLC separation conditions were optimized in order to obtain a good separation in a short retention time. Fig. 1A shows chromatograms at fluorescence detection of both the sample and HEW-lysozyme standard solution (50 $mg \cdot l^{-1}$).

Fluorescence detection is more sensitive than UV, but requires a less common instrument, and it is less versatile, since many compounds cannot be detected by fluorescence [24]. For this reason, many laboratories are not equipped with fluorescence detectors but only with UV detectors. In order to verify if UV detection was as valid as fluorescence detection for lysozyme quantification in jenny milk, the chromatographic analysis was repeated by replacing the fluorescence detector by UV detector. Two wavelengths were employed for lysozyme quantification by UV detection: 280 nm and 227 nm. The absorbance of a protein at 280 nm depends on the concentration of tryptophan, tyrosine and cysteine (disulfide bonds) [25]. Peptide bonds [–C(O)–NH–] exhibit a weak absorption band at 180–230 nm due to $n \rightarrow \pi^*$ transition [26]. We chose the wavelength of 227 nm as suggested by GUARINO et al. for protein detection in pasta filata cheeses [27].

Fig. 1 shows the chromatograms of jenny milk lysozyme with UV detection at 280 nm and 227 nm. The concentrations of lysozyme in jenny milk for each detection employed in this study were the following (expressed as average ± standard deviation): (1204±28) $mg \cdot l^{-1}$ with UV at 227 nm; (1087±28) $mg \cdot l^{-1}$ with UV at 280 nm; (1091±26) $mg \cdot l^{-1}$ with FLD. These results were

in line with that (1 131 mg·l^{-1} ± 63 mg·l^{-1}) obtained from ELISA.

ANOVA revealed a significant difference ($P < 0.001$) in lysozyme quantification among the three detections. The highest value was obtained by using UV detection at 227 nm (1 204 mg·l^{-1} ± 28 mg·l^{-1}), whereas there was no significant difference between the concentration values obtained by the other two detections (UV at 280 nm detection and FLD).

At 260 nm and 280 nm, the protein-related absorption is strongly dominated by the aromatic residues of tryptophan, tyrosine and phenylalanine. At 227 nm, all amino acids contribute through absorption in the peptide bond. Higher readings at around 230 nm may indicate contaminants in the sample. Strong absorbance around 230 nm may indicate the presence of organic compounds or chaotropic salts, as carbohydrates and phenolate ions absorb around 230 nm [28]. Some components of jenny milk may act just as contaminants and absorb at 227 nm. Therefore, we chose to abandon UV detection at 227 nm for the quantification of lysozyme.

Comparison between UV at 280 nm and fluorescence detections

Tab. 2 shows the comparison between validation analytical parameters obtained from lysozyme quantification in jenny milk by UV at 280 nm and fluorescence detection.

Linearity

The linearity was evaluated for lysozyme at seven concentrations in the range of 2–150 mg·l^{-1}. Calibration curve was plotted by getting an average peak area ($n = 3$) against the concentration of HEW lysozyme standard, and the result was analysed by linear regression. The coefficients of regression (0.998 and 0.999 for UV at 280 nm and FLD, respectively; Tab. 2) confirmed that the method emplying detection by UV at 280 nm was linear for the determination of lysozyme.

Analysis of variance

The concentration value obtained by fluorescence detection was higher than that obtained by using UV at 280 nm (1 087 mg·l^{-1} vs 1 091 mg·l^{-1}; Tab. 2), because the detection response value of FLD was higher than that of UV at 280 nm. Since the difference in lysozyme quantification between these two methods was not significant, it can be stated that both analytical methods have high accuracy despite the difference between the detection response values (Tab. 2).

Sensitivity

The sensitivity of the proposed method was determined by calculating the limit of detection and the limit of quantification using serial dilution of lysozyme HEW standard. *LOD* of 0.86 mg·l^{-1} and *LOQ* of 2.99 mg·l^{-1} were determined using HPLC with UV detection at 280 nm, whereas lower *LOD* and *LOQ* values were obtained using HPLC with fluorescence detection (0.49 mg·l^{-1} and 1.52 mg·l^{-1}, respectively). The *LOD* value obtained by using HPLC with FLD was lower than that (0.8 mg·l^{-1}) reported by Pellegrino and Tirelli [21] for lysozyme detection in cows' milk. Tab. 2 shows the comparison of *LOD* and *LOQ* of lysozyme by UV detection at 280 nm and FLD, with the latter showing the greater sensitivity. However, the concentration of lysozyme in jenny milk is high, much higher than the *LOD* value calculated for UV at 280 nm detection.

Accuracy

Recovery was determined to be 98.2 % ± 1.2 % for UV detection at 280 nm and 99.6 % ± 0.8 % for FLD. The low percent of standard deviation indicated the high accuracy of both proposed analytical methods (Tab. 2).

Precision

An inter-day and intra-day precision was assessed (results are summarized in Tab. 2). For intra-day precision determination, the samples were injected ten times within the same day. For

Tab. 2. Comparison between validation parameters of UV detection and fluorescence detection.

	UV	FLD
Calculated concentration [mg·l^{-1}]	1 086.89	1 090.67
Standard deviation [mg·l^{-1}]	27.95	26.46
Coefficient of determination R^2	0.998	0.999
Limit of detection [mg·l^{-1}]	0.86	0.49
Limit of quantification [mg·l^{-1}]	2.99	1.52
Recovery [%]	98.2 ± 1.2	99.6 ± 0.8
RSD intra-day [%]	2.0	1.8
RSD inter-day [%]	2.3	2.1

UV – ultraviolet detection carried out at 280 nm, FLD – fluorescence detection performed at excitation wavelength 280 nm and emission wavelength 350 nm.
Quantification of lysozyme concentration in jenny milk was determined by area under peak and linear regression equations considering the dilution factor.
RSD – relative standard deviation, *RSD* intra-day was calculated among 10 samples analysed on the same day, *RSD* inter-day was calculated among 30 samples analysed on three different days (ten per day).

the inter-day precision determination, 30 samples were injected after every day for up to three days. Satisfactory results were achieved, as demonstrated by the calculated *RSD* for both of the intra-day (2.0 % for UV) and inter-day precisions (2.3 % for UV). The low percentage of *RSD* indicated high reproducibility and repeatability in the current experimental condition.

CONCLUSIONS

In the present study, we tuned with a step-by-step approach a new method ad hoc for lysozyme quantification in jenny milk. The high concentration of lysozyme in jenny milk allowed to successfully develop a method employing a UV detector set at a wavelength of 280 nm, which was validated in terms of selectivity, linearity, accuracy, precision, stability and sensitivity. We utilized HPLC with UV detection rather than fluorescence detection, which is usually employed for lysozyme quantification in milk-dairy products, but less common and less versatile. The UV detection provided results comparable with those of the fluorescence detection. Further studies are needed to assess both the applicability of this method and to quantify the lysozyme concentration in dairy products made by adding jenny milk.

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