Journal of Rare Cardiovascular Diseases
ISSN: 2299-3711 (Print) | e-ISSN: 2300-5505 (Online)
www.jrcd.eu
RESEARCH ARTICLE

QUANTIFICATION AND ANALYSIS OF OXYTOCIN RESIDUES IN BUFFALO MILK SAMPLES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC).

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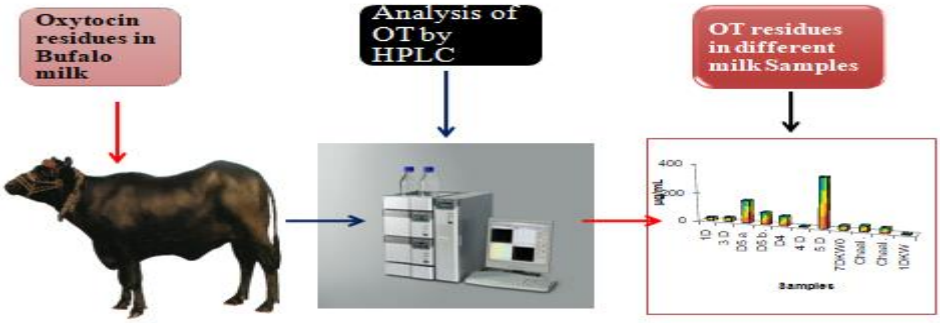
Article History
Received: 24.11.2025
Revised: 04.12.2025
Accepted: 15.12.2025
Published: 27.12.2025

Abstract: ***Background***: In developing countries including Pakistan, the unregulated administration of exogenous synthetic oxytocin to livestock is widely practiced to induce rapid milk let-down, bypassing natural maternal stimuli. This practice leaves trace peptide residues in milk, raising food safety, veterinary, and public health concerns, including early puberty in children, reproductive disorders, and hormonal imbalances.***Objective:*** To isolate, quantify, and analyze oxytocin residues in local buffalo milk using an optimized reversed-phase high-performance liquid chromatography (RP-HPLC) method.***Methodology:*** Raw milk samples (n=33) were collected from five government and private dairy farms in Sindh, Pakistan. Samples were divided into treatment (oxytocin-injected) and control groups. Sample preparation included centrifugation (10,000 × g), protein precipitation with ice-cold acetone, and lipid extraction with petroleum ether. Chromatographic separation used a Wufeng LC-20 AT system with a Purospher Star C18 column (250 mm × 4.6 mm, 5 µm). Isocratic elution used acetonitrile: 0.03 M sodium phosphate buffer (50:50 v/v, pH 3.5) at 1.0 mL/min and 40°C, with PDA detection at 220 nm.***Results:*** The method showed excellent linearity (R^2 = 0.9999), high accuracy (99.8% recovery), and precision (%RSD ≤ 2.5%). LOD and LOQ were 0.03 µg/mL and 0.1 µg/mL, respectively. Oxytocin eluted at ~5.5 min, though retention times varied from 5.11–6.98 min due to matrix effects. Quantified residues ranged from 3.9 µg/mL to 347 µg/mL across breeds (Kundhi, Thanthee, Katoo, Chaal Kundhi, ***Chaal Ghunjh).Conclusion:*** The wide variation and high concentrations confirm widespread unauthorized oxytocin use. Regulatory auditing and standard method validation are urgently needed.

Keywords:exogenous synthetic oxytocin, buffalo milk, Chromatographic separation, excellent linearity .



INTRODUCTION

Oxytocin is a mammalian nonapeptide hormone primarily synthesised in the paraventricular and supraoptic nuclei of the hypothalamus and stored in the posterior pituitary gland [1,2]. Biologically, it is essential for reproduction, social bonding, and parturiency [3,4]. Most notably, it serves as the primary chemical mediator for milk ejection (milk let-down) by inducing contraction of myoepithelial cells surrounding the alveoli in the mammary glands [5,6]. The misuse and overuse of exogenous (synthetic) oxytocin injections in livestock, as a management practice [7,8], has become common in the dairy sectors of many developing countries including Pakistan.

Farmers often inject dairy animals like cows and native buffalo breeds (Kundhi and Chaal Ghunjh) just before milking to trigger rapid and maximal milk let-down [9]. This practice circumvents the physiological maternal stimuli needed for milking but raises serious concerns in

terms of food safety, veterinary and public health [10]. This hormone administration exogenously may result in trace peptide residues in the milk supply, surviving conventional thermal processing methods such as boiling and pasteurization [11]. Milk containing residues of oxytocin is suspected of being associated with early puberty in children, hormonal imbalance and possibly adverse reproductive effects in human populations. Consequently, there is an urgent regulatory and analytical need to monitor, isolate and quantify these residue profiles in local dairy matrices [12].

Determination of trace levels of peptide hormones in complex biological fluids such as buffalo milk is a significant analytical challenge [13]. Milk is a very complex matrix, which is an emulsion of fat globules, an aqueous colloidal suspension of casein micelles, whey proteins, lactose and mineral salts [14]. Highly sensitive, selective and robust analytical methods are required to accurately quantify residual oxytocin without interference from native milk proteins and lipids [15].

Of the many analytical platforms, High-Performance Liquid Chromatography (HPLC) combined with Ultraviolet-Visible (UV-Vis) or Photodiode Array (PDA) detection has become the technique of choice for regulatory screening and academic research [16]. HPLC gives high resolution, reproducibility and precision for small peptides [17]. In most cases, the chromatographic separation is performed by using reversed phase chemistry (RP-HPLC) with a stationary phase such as an octadecylsilane (C18) column (250 mm × 4.6 mm, 5µm) [18]. The choice of this phase is justified by its capability to efficiently retain hydrophobic and moderately polar peptide fragments. The separation of oxytocin from matrix co-extractives can be optimised by careful control of variables such as mobile phase composition, wavelength selection, flow rate and temperature [19].

Mobile Phase Dynamics: Isocratic elutions with a binary system (e.g. 50:50 Acetonitrile (ACN) and an aqueous buffer) are typically used. Acetonitrile is the organic modifier that accelerates the elution of peptides. The buffer regulates the ionisation state and peak symmetry of the peptide [20].

Detection Wavelength: Photometric monitoring takes place at a wavelength of 220 nm which is in the far-UV region of the electromagnetic spectrum where peptide amide bonds (O=C-N-H) absorb the most light and therefore provide high sensitivity to detecting low amounts of analyte material [21].

System Thermostatting: The control of the column is typically a constant temperature (40ºC) through which the mobile phase has its viscosity and backpressure reduced, the kinetics of mass transfer are increased, and stabilisation of the retention time (Rt) of the target analyte is achieved, usually settling in a reproducible time frame of 5.1 to 6.3 minutes [22]. While modern C18 phases show excellent efficiency (based upon the theoretical plate count), raw milk matrix typically give rise to very complex chromatograms that contain overlapping peaks and/or significant baseline drift due to coelution of matrix components [23]. Therefore, this whole study is designed to investigate these analytical variables by comparing sample preparation methods, validating chromatographic parameters and measuring the levels of oxytocin residue in various types of buffalo milk for food safety and compliance with food regulations.

MATERIALS AND METHODS

Materials, Reagents, and Sampling Locations

All oxytocin chemical standards and high purity analytical grade reagents were obtained from Sigma-Aldrich and Fluka Chemical Co. (Karachi, Pakistan). The Table: 3 details the collection origin and specific breeds of lactating buffaloes used for the raw milk sample profiling across different urban and peri-urban dairy hubs in Lower Sindh. It associates each evaluated milk sample type, such as Kundhi, Thanthee, Katoo, Chaal Khundi, and Chaal Ghunjh with its exact extraction location, spanning commercial operations like the Atta Muhammad Khuharo dairy farm in the Kotri SITE area, the Panhwar dairy farm on Court Road in Jamshoro, and urban suppliers including Al-Hamd Dairy on Risala Road, Shah Latif Dairy in Latifabad No. 7, and Nagori Milk Dairy in Latifabad, Hyderabad. By identifying these specific breeds alongside their commercial procurement sites, the data maps out the commercial network of local dairy farms and specific livestock variations targeted for subsequent oxytocin residue quantification.

A total of Thirty-three (50 mL each) fresh milk samples were randomly collected from eleven (in triplicate, 11×3 = 33) selected milking mammals with lactating age between four to five months and in their mid-lactation period. Samples were received with injections of exogenous synthetic oxytocin to stimulate milk let-down All samples were immediately stabilized in an ice bath on-site, transported to the laboratory and kept in deep-freeze storage at -20 °C to preserve the integrity of the peptide prior to the pre-treatment and chromatographic analysis.

Analytical Method Validation

The developed reversed-phase chromatographic technique was thoroughly validated for linearity, precision, specificity, limit of detection (LOD), limit of quantification (LOQ), and system accuracy in accordance with International Council for Harmonisation (ICH) standards. The effectiveness of recovery matrices and system precision was assessed by incorporating known concentration increments of the certified oxytocin reference standard into blank control milk matrices (Figure: 1). Assessments of inter-day and intra-day precision were conducted by evaluating

standard concentrations in triplicate on one analytical day and over a period of seven consecutive days, respectively. The system demonstrated highly acceptable reproducibility benchmarks, maintaining a relative standard deviation (%RSD) reliably at or below 2.5% for all confirmed reference concentrations (Table: 2).

Matrix Pre-treatment and Extraction

A 10 mL aliquot of each raw milk sample was transferred into a 15 mL centrifuge tube and centrifuged at 10,000 × g for 30 min at 4 °C to isolate peptide targets from complex colloidal milk components, lipid fractions, and native proteins. The clarified lower whey fractions were collected by perforating the bottom of the centrifugation tubes. 50 µL aliquots of the isolated whey were directly processed with a competitive enzyme immunoassay kit for baseline comparative enzyme immunoassay profiling according to the structural guidelines provided by the manufacturer (Phoenix Pharmaceuticals, Burlingame, CA, USA).

For the enrichment of samples by liquid chromatography, 1.0 mL of the raw milk matrix was mixed with 1.0 mL of ice-cold acetone for protein precipitation. The mixture was vortexed vigorously and then centrifuged at 3,500 rpm. The isolated acetone-containing supernatant layer was then extracted with 1.0 mL of petroleum ether. The upper lipophilic ether layer with residual fats was mechanically removed following a 5 min equilibration period. The remaining lower aqueous layer was evaporated to dryness under a gentle stream of nitrogen gas and reconstituted in 0.2 mL of mobile phase buffer and filtered through a 0.45 µm syringe filter immediately before chromatographic injection.

Chromatographic Configuration and System Optimization

We separated, identified, and measured our analytes using high-performance liquid chromatography (HPLC) systems. In our case, we used a Wufeng LC-20 AT manufactured by Shanghai Wufeng Scientific Instruments Co., Ltd. in China. The HPLC was comprised of several components, such as a specialized pump, a temperature-controlled column, an autosampler to introduce the sample into the HPLC system, and a sensitive detector, all of which worked in conjunction with a software program called LC Solution. Analytes were separated using a Purospher Star C18 analytical column manufactured by Merck of Germany. Separation employed two mobile phase solvents, 50% acetonitrile, and 50% sodium phosphate buffer. The use of the solvent mixture allowed for optimal separation of analyte target.

The water buffer was adjusted carefully to a low pH of 3.5 using a weak acid to keep the peptides stable and control the state of the amino acids. The conditions for the test were kept steady with the column at a temperature of 40°C. The flow rate of the test was 1.0 mL, per minute. The amount of sample injected was 10 µL. The machine was set to monitor the light at a wavelength of 220 nm. With all these conditions working together the oxytocin being tested came out clearly at a consistent time, which was about 5.5 minutes. The oxytocin analyte had a retention time. The oxytocin came out at the time every time the test was done (Table: 1).

Table 1: Optimized RP-HPLC-PDA Parameters for Oxytocin Resolution

S.No.	Chromatographic Parameter	Operational Specification
1	**HPLC System Model**	Wufeng LC-20 AT Quaternary System
2	**Stationary Phase (Column)**	Purospher Star Hibar C18 (250 mm × 4.6 mm, 5 µm)
3	**Mobile Phase Composition**	Acetonitrile : Phosphate Buffer (50:50, v/v)
4	**Buffer Preparation**	0.03M Aqueous NaH_2PO_4 adjusted to pH 3.5 with dil. H3PO4
5	**Volumetric Flow Rate**	1.0 mL/min
6	**Detection Wavelength**	Photometric monitoring at lambda = 220 nm
7	**Column Temperature**	Thermostatted at 40 ºC
8	**Injection Loop Volume**	10 µL
9	**Target Retention Time (Rt)**	approx 5.5 minutes

Table 2 System Suitability Parameters

S.No	Parameters	Results
1	Capacity factor	0.86
2	Tailing factor	1.184
3	LOQ (µg/ml)	0.1
4	LOD (µg/ml)	0.03
5	Theoretical plates (N)	3521
6	Percentage recovery% (Accuracy)	99.8
7	Linearity range (µg/ml)	0.5-2.5
8	Resolution	6.7

Figure: 2 Different Breeds of lactating buffalos

Names of Dairy Farms

Five dairy farms were selected for the purpose of analytical work given below:

1. Atta Muhammad Khuharo dairy farm site area Kotri
2. Panhwar dairy farm court road Jamshoro
3. Al-hamd dairy risala road Hyderabad
4. Nagori milk dairy Latifabad Hyderabad
5. Shah Latif Dairy Latif abad No. 7 Hyderabad

Table: 3 Geographical and Breed-Wise Distributions of Analyzed Buffalo Milk Samples

Sample ID	Target Breed	Sampling Location / Dairy Source	District/Region
1D	Kundhi Buffalo	Atta Muhammad Khuharo Dairy Farm, SITE Area, Kotri	Jamshoro
3D	Kundhi Buffalo	Atta Muhammad Khuharo Dairy Farm, SITE Area, Kotri	Jamshoro
D5 a	Kundhi Buffalo	Atta Muhammad Khuharo Dairy Farm, SITE Area, Kotri	Jamshoro
D5 b	Thanthee Buffalo	Al-Hamd Dairy, Risala Road, Hyderabad	Hyderabad
D4	Katoo Buffalo	Shah Latif Dairy, Latifabad No. 7, Hyderabad	Hyderabad
4D	Kundhi Buffalo	Shah Latif Dairy, Latifabad No. 7, Hyderabad	Hyderabad
5D	Kundhi Buffalo	Panhwar Dairy Farm, Court Road, Jamshoro	Jamshoro
7DKW0	Kundhi Buffalo	Panhwar Dairy Farm, Court Road, Jamshoro	Jamshoro
Chaal Khundi	Chaal Kundhi Buffalo	Nagori Milk Dairy, Latifabad, Hyderabad	Hyderabad
Chaal Ghunjh	Chaal Ghunjh Buffalo	Nagori Milk Dairy, Latifabad, Hyderabad	Hyderabad
1DKW	Kundhi Buffalo	Shah Latif Dairy, Latifabad No. 7, Hyderabad	Hyderabad

RESULTS AND DISCUSSION

Quantification of Oxytocin Residues in Buffalo Milk Samples via HPLC

Through use of High-Performance Liquid Chromatography (HPLC) with UV detection, multiple buffalo milk samples collected from various parts of the Sindh Province were tested for oxytocin residue content. Chromatographic separation was achieved using a reverse phase C18 analytical column (250 mm, 4.6 mm 5 µm) maintained at a constant column temperature of 40°C. The mobile phase consisted of acetonitrile and buffer in equal volumes (isocratically) and was pumped through the system at a constant flow rate (1.0 mL per minute). The detector was calibrated to operate at an optimal wavelength (220 nm) for spectrophotometric detection. Chromatograms were compared with a chromatogram from a 10 mg oxytocin standard to determine if peaks were of the same identity (Table: 3 & Figure: 3).

Chromatographic Profiles and Peak Characteristics

The chromatographic analyses of the milk extracts revealed a variety of structural matrices depending on their origins. The retention times (Rt) of the oxytocin peaks were between 5.11 and 6.98 minutes, representing a slight shift in selectivity as a result of the matrices, which are typical for complex biolopgical samples. Table: 3 summarize the chromatographic

characteristics (e.g., retention time, peak height, peak area and column efficiency (N)) of the samples included in this study (Figure: 3 & 4).

Table 4: Consolidated Chromatographic Parameters and Quantified Oxytocin Levels in Local Buffalo Milk Samples.

Sample ID	Sample Type	Rt (min)	Oxytocin (µg/mL)
1D	Kundhi Buffalo Milk	6.270	21
3 D	Kundhi Buffalo Milk	5.667	29
D5 a	Kundhi Buffalo Milk	6.630	159
D5 b	Thanthee Buffalo Milk	6.804	83
D4	Katoo Buffalo Milk	6.977	66
4 D	Kundhi Buffalo Milk	5.675	3.9
5 D	Kundhi Buffalo Milk	6.302	347
7DKW0	Kundhi Buffalo Milk	5.354	30.4
Chaal Khundi	Chaal Khundi Buffalo Milk	6.091	40
Chaal Ghunjh	Chaal Ghunjh Buffalo Milk	5.630	36
1DKW	Khundi Buffalo Milk	5.108	4.9

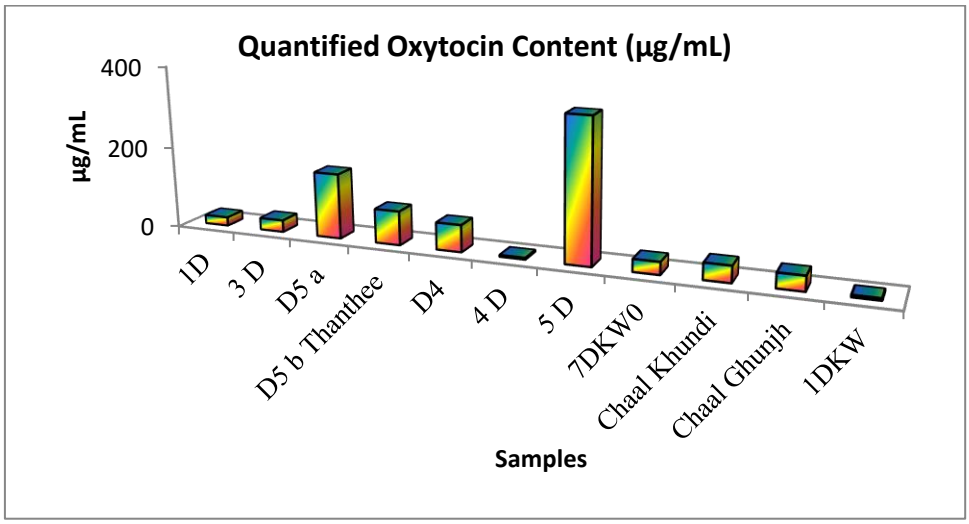
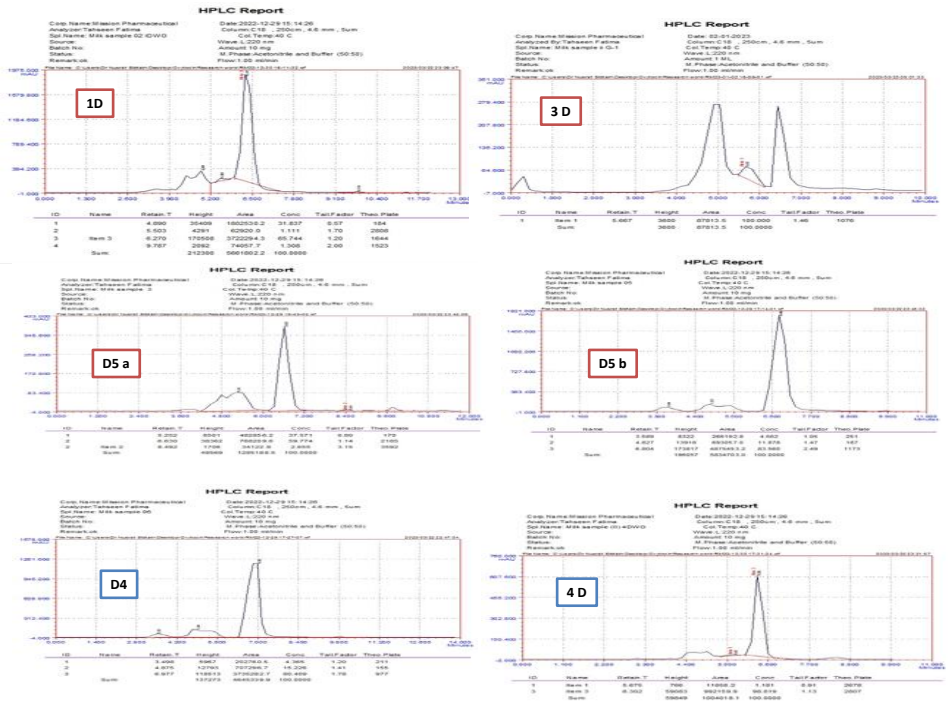


Figure: 3 Quantified Oxytocin Content (µg/mL) in different milk samples of Buffaloes



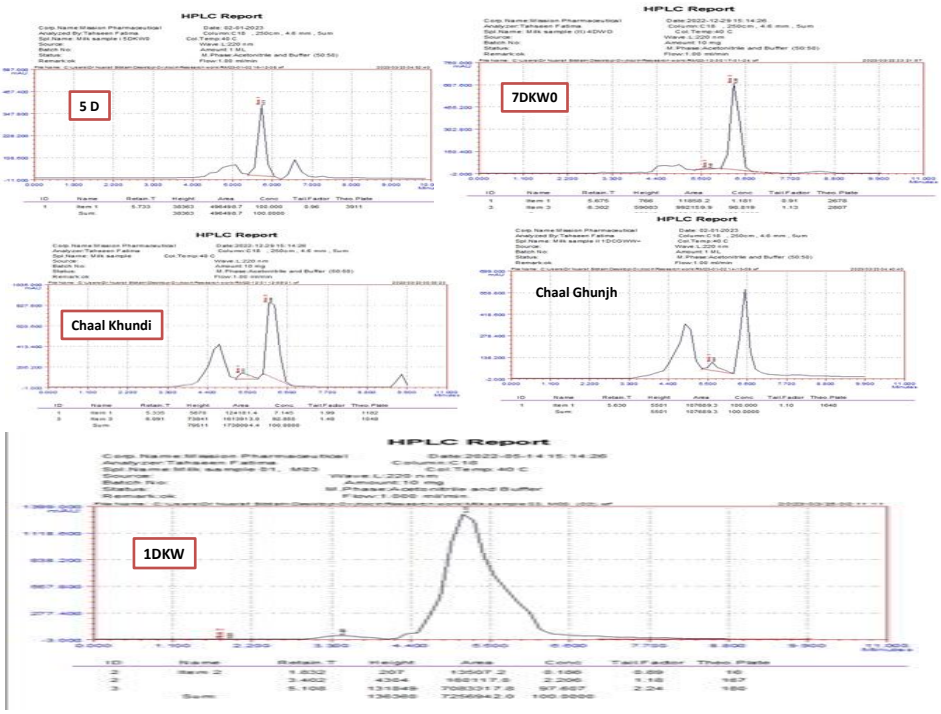


Figure: 4 Chromatograms of OT found from different samples of buffalo milk

Matrix Interference and Peak Resolution

Several secondary peaks were observed on a numerous milk samples eluting at or neighboring the target peak of oxytocin (obtained from the individual samples shown in the supplementary figures). In sample 1D (Kundhi Buffalo), there was no apparent stand-alone isolated standard geometry peak, which indicates that a combination of matrix effects and unresolved proteins have affected this particular biological sample loop. Alternatively, sample profiles such as those of sample 5D and 1DKW exhibited an improved level of chromatographic resolution as well as an increased theoretical plate count (i.e., 3,101 theoretical plates) allowing for analytical accuracy. The routine emergence of chemical peaks external to the retention spectrum of the samples indicates that the milk matrix is a biological entity containing a complex of native caseins, lipids, and water-soluble vitamins. By employing enhanced auxiliary characterization through Liquid Chromatography-Mass Spectrometry (LC-MS), it is possible to further understand the structural arrangements of these multiple compounds occurring concurrently (Figure: 4).

Quantitative Distribution and Toxicological Relevance

Oxytocin measurements were determined using peak area integration against pre-calibrated curves. Values vary greatly between as low as 3.9 µg/mL (Sample 4D) to as much as 347 µg/mL (0.347 mg/mL) (Sample 5D). This great variation and specifically high values (e.g. 159 µg/mL; 347 µg/mL) would lend themselves to significant suspicion of external misuse of unauthorized oxytocin injections to artificially lactate milking dairy buffaloes just before milking. The findings of this research illustrate the need for validation of standard methods (assessing linearity, limit of detection, specificity, and recovery metrics) for systematically evaluating dairy products being introduced into regional supply chains from a public safety perspective.

CONCLUSION

This study validated an RP-HPLC-PDA method for quantifying oxytocin residues in buffalo milk. The method demonstrated excellent linearity ($R^2 = 0.9999$), recovery (99.8%), and precision (%RSD $\leq$ 2.5%). Analysis of samples from Sindh, Pakistan, revealed highly variable oxytocin concentrations (3.9–347 µg/mL), indicating widespread unauthorized use of synthetic oxytocin before milking. These residues pose potential public health risks, including hormonal disruption and early puberty. Strict regulatory enforcement and routine farm auditing are urgently required.

Author Contribution

Tehseen Fatima Musavi conceptualized, collected samples, designed experiments, Abdul Raheem shar and Rubina Naz Mirani, collected data and prepared the draft of the article 5Tahmina Fakhr-u-Nisa Abbasi and Farzana Mangrio interpreted the data Seema Sarwar Ghumro and Rehana Keerio, performed analysis. Prof. Dr. Ghulam Qadir Shar supervised the whole work. All authors read, revised, and approved the final version of the manuscript.

Acknowledgements
The authors acknowledge the facilities provided by the Institute of Chemistry, Shah Abdul Latif University Khairpur and also technical support provided by a Mission Pharmaceuticals Pvt. Ltd. that is situated at Plot No. A-94 S.I.T.E, super highway, Karachi Pakistan.

Conflict of Interest
The authors declare no conflict of interest.

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