Assessment of the Reproducibility of ISO 20136 for the Biodegradation of Tanned Leather: Longitudinal Analysis and Statistical Validation

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Abstract

A standardized protocol to assess the biodegradability of tanned leathers using an inoculum derived from biological wastewater treatment systems is outlined in ISO 20136:2020 Leather — Determination of degradability by micro-organisms. Over a 28-day incubation period, the method enables estimation of the biodegradation potential under simulated environmental conditions. Standardized methodologies are essential for generating reproducible and representative data that approximate real-world degradation processes. However, the method's reliability depends heavily on its reproducibility, particularly in the face of biotic (e.g., inoculum activity) and abiotic (e.g., temperature, pH, humidity) variability.

In this study, 15 biodegradation experiments were analyzed, totaling approximately 11,000 test hours conducted between 2019 and 2025, using two consistent reference materials: collagen (positive control) and oxazolidine-tanned leather (internal control). The objective was to determine whether temporal and seasonal variations in inoculum properties significantly influenced biodegradation outcomes. A normalization approach was applied using collagen as a reference baseline. Statistical validation included hypothesis testing, normality assessment, variance analysis, and linear correlation.

Results showed a strong correlation between collagen and oxazolidine-tanned leather ($R^2 = 0.99$), with no significant differences in mean values after normalization. Reproducibility was confirmed through a coefficient of variation (CV) of 10.94% and a relative interquartile range of 15.11%, both within accepted thresholds for biological assays. This approach enhances the methodological robustness of ISO 20136 against experimental fluctuations, enabling more consistent and conservative interpretation of biodegradation data—critical for comparative studies and for validating biodegradable materials.

Keywords: ISO 20136:2020; Biodegradation; Tanned Leather; Reproducibility; Statistical Validation.

1. Introduction

The leather industry represents a strategic sector of the global economy, not only for its capacity to generate added value but also for its essential role in industries such as fashion, automotive,

and upholstery [1]. Beyond the supply of high-quality materials, this industry acts as a key economic driver.

In the European context, the leather and leather products sector comprises approximately 36,000 companies, directly employing around 435,000 people and generating an estimated turnover of €48 billion in 2021 [2]. Production is highly diversified: 41% of production is destined for footwear, 19% for leather goods, 17% for furniture, and 13% for the automotive industry [2], reflecting its transversal relevance across various value chains.

From a regulatory perspective, waste management in this sector has traditionally been governed by Directive 1999/31/EC on landfill and Directive 2008/98/EC on waste management [4][7].

These regulations allowed the incineration or landfilling of solid waste, such as leather trimmings or chrome-containing shavings. However, the entry into force of Directive (EU) 2018/850 on January 1st, 2025, introduces significant restrictions, explicitly prohibiting both the landfilling and incineration of industrial textile waste [6].

This measure directly impacts waste generated by the leather industry due to its compositional and environmental similarities with textile waste.

In response to this new regulatory framework, the European leather industry is transitioning toward more sustainable production models.

This includes adopting responsible manufacturing practices, reducing the use of hazardous compounds such as chromium [5][6], and promoting the development of biodegradable products that can be composted at the end of their life cycle [8].

Initiatives such as the substitution of traditional tanning agents with chromium-free alternatives, coupled with more sustainable finishing treatments, represent a turning point for the sector [8]. Likewise, the implementation of modern manufacturing guidelines—such as the use of renewable-source materials—encourages cleaner and more traceable processes [9].

This transformation aligns with the European Green Deal and, in particular, with the EU Strategy for Sustainable and Circular Textiles [3], which outlines a roadmap to reshape the sector toward a resilient, resource-efficient, and competitive economy. This strategy fosters technological innovation, ecodesign, and material circularity, including leather.

Within this context, the integration of environmental criteria into product design, the improvement of tanning processes, and the traceability of industrial waste flows become key pillars to ensure regulatory compliance and long-term sustainability of the sector.

Leather is a durable and flexible material derived from animal hides or skins. It is primarily composed of collagen fibers, accounting for approximately 90% of its protein content [10].

The remaining components include elastin, keratin, various lipids, minerals, and water. The unique three-dimensional network of interwoven collagen fibers provides leather with its strength, flexibility, and resistance to tearing [11].

The tanning process chemically modifies the collagen structure to improve its stability and confer desired properties [12]. Various tanning agents are used to stabilize and preserve leather through different chemical mechanisms [13].

Although tanning significantly enhances the durability and lifespan of leather products, it also affects their biodegradability and environmental impact [14]. Leather decomposition is a complex biological process influenced by several microbial groups, with bacteria playing a key role [15].

These microorganisms produce specialized enzymes, such as proteases and lipases, which enable the breakdown of proteins, lipids, and tanning agents present in leather [16].

Through these enzymatic activities, bacteria initiate a cascade of biochemical reactions that gradually degrade the leather structure and alter its physical properties [17].

The biodegradation rate of leather is influenced by several factors, including the type of tanning agent used, the tanning process itself, and environmental conditions (biotic and abiotic factors).

This makes the development of more sustainable leathers, that meet regulatory demands and exhibit higher degradation rates at end-of-life, a relevant technical challenge for the sector.

To support this goal, laboratory testing methods are required that allow for the assessment of the degradative behavior of new materials without requiring extended periods or field trials that may last years.

Most standardized biodegradability evaluation methods were originally designed for plastic materials [18][19][20], requiring testing periods of 90 to 180 days. In 2017, standard ISO 20136:2017 “Leather — Determination of degradability by micro-organisms”, specifically for leather, was published and later updated in 2020 [21].

This methodology uses a complex microbial consortium derived from urban and/or tannery wastewater as an inoculum in a liquid medium, evaluating biodegradation though quantification of CO_2 generation. One of its main advantages is its relatively short test duration, around 30 days, allowing for rapid assessment of the degradative potential of newly developed leathers.

However, this rapid response must be supported by methodological reliability and reproducibility of results over time. Given the use of complex microbial inocula, such as waters derived from biological tanks of tanneries, municipal plants, or both, rigorous microbial standardization is not feasible.

Therefore, ensuring the reproducibility of results, regardless of the geographic origin of the inoculum or the season in which the wastewater was collected, is essential for scientifically validating the biodegradability of a leather material.

This study aimed to evaluate the reproducibility of the leather biodegradation test using the standardized ISO 20136:2020 method. To ensure traceability and minimize experimental variability, all tests were performed using a single representative sample of the same model material: bovine leather tanned with oxazolidine.

Additionally, the influence of microbial inocula collected in different seasons (spring, summer, autumn, and winter) over a five-year period (2020–2025) was assessed.

Due to the 30-day duration of the test, a total of 15 independent trials were conducted. In each trial, pure collagen was used as a positive control, as specified in ISO 20136:2020.

The leather biodegradation percentage was calculated relative to the value obtained for collagen, which was considered as the 100% reference. From this value, the relative biodegradation of the tanned leather sample was calculated.

This methodology allows for systematic comparison of the degradability of the tested leather against the behavior of collagen under identical experimental conditions, thereby ensuring the consistency and comparability of results.

2. Material and Method

2.1. Equipment

The tests were carried out using equipment developed by INESCOP, specifically to comply with ISO 20136:2020. INESCOP's system (Figure 1) is based on Method B of the standard, in which biodegradation is determined by quantifying the CO_2 produced during collagen degradation. This is achieved through direct infrared detection and continuous CO_2 concentration monitoring.

The equipment is designed to measure CO_2 levels from multiple samples placed in separate reactors.

The CO_2 generated during the microbial degradation process is detected by an infrared sensor. Airflow from each reactor is directed through a multiplexed system comprising a rotating drum, which consists of multiple inlet channels. Each reactor's air outlet is connected to one of these inlets.

The drum has a single outlet that is connected to a flow detector and then to a sealed measurement cell containing the infrared CO_2 sensor.

The system automatically records the resulting data on airflow, CO_2 concentration, atmospheric pressure, and temperature using a data acquisition system connected to a computer.

This innovative setup enables the assessment of leather biodegradation potential within a relatively short period. In 30 days, it can determine the degradation behavior of leather that would otherwise take years under natural environmental conditions. INESCOP currently operates two such systems, with capacities for 16 and 20 reactors, respectively.

Figure 1. Biodegradation equipment at INESCOP

2.2. Description and Principle of the ISO 20136:2020 Method

The aerobic biodegradability of the samples was assessed in accordance with ISO 20136:2020, which provides a standardized method for evaluating leather degradation through the action of aerobic microorganisms.

This procedure is applicable to both tanned and untanned leather, with the aim of evaluating their end-of-life behavior from an environmental perspective.

The principle of the method is based on the measurement of carbon dioxide (CO_2) produced during the microbial decomposition of the sample's organic matter, primarily collagen. The CO_2 generated results directly from the metabolic activity of an active microbial inoculum, typically obtained from tannery wastewater, municipal wastewater, or a mixture of both.

The accumulated CO_2 is used to calculate the degree of biodegradation, expressed as the percentage of total carbon in the sample converted into CO_2.

This value is then compared to the theoretical carbon content, previously determined by elemental analysis, enabling an accurate quantification of the material's biodegradability.

2.3. Sample

To evaluate the reproducibility of ISO 20136:2020, two types of reference materials were used. Pure type I collagen (Sigma®) served as the positive control, as specified in the standard. The second material was oxazolidine-tanned leather, which demonstrated a high biodegradation potential, with a relative biodegradation exceeding 75% compared to pure collagen, meeting the standard's defined criteria.

This high degradability makes oxazolidine-tanned leather a suitable internal control material, particularly useful for interlaboratory studies and methodological validation. In this study, a single oxazolidine-tanned hide was used to minimize variability associated with potential differences in tanning-agent concentration, which could otherwise influence biodegradation outcomes.

Oxazolidine is a synthetic organic tanning agent used in the leather industry as an alternative or complement to traditional tanning agents, such as chromium salts, vegetable tannins, or aldehyde-based compounds.

Its use is especially widespread in tanning and retanning processes. Oxazolidine functions by forming covalent bonds with collagen's functional groups, particularly amino groups, thereby stabilizing the protein structure of the tanned material [22].

2.4. Statistical analysis

Various statistical tools were employed to evaluate the reproducibility of the biodegradation tests. Because the duration of each test varied, the time scale was normalized to validate reproducibility as a plausible hypothesis.

For each experiment, the initial and final times were set to 0 and 1, respectively. Within this time frame, data were further normalized into percentage intervals, divided into 5% increments from 0% to 100%.

This approach was considered the most appropriate to evaluate reproducibility, as direct comparison of degradation values across 15 tests with durations ranging from 400 to 900 hours would be inconsistent.

This normalization minimized potential bias that could arise when comparing results at a fixed time point (e.g., 200 hours) between tests of differing total durations.

The statistical techniques applied included coefficients of variation, Pearson correlation coefficient, Lin's Concordance Correlation Coefficient (CCC), Kolmogorov test, Bland–Altman plots, and Quality Control Charts (QCCs).

The coefficient of variation indicates relative variability, i.e., the degree of dispersion relative to the mean. Pearson's correlation coefficient evaluates the linear relationship between two experimental curves. Lin's coefficient assesses both the shape and magnitude agreement of curves.

The Kolmogorov test, a non-parametric test, determines whether two samples derive from the same distribution. Bland–Altman plots display mean differences between two experiments over time, defining thresholds to assess statistical significance.

QCCs are statistical control charts used to evaluate whether a process behaves consistently and stably across different trials.

3. Results and Discussion

As previously described, the absolute biodegradation values for both collagen and oxazolidine-tanned leather were normalized to ensure consistency across results. This allowed for a systematic comparison of the degradability of the tested leather relative to the behavior of collagen under identical experimental conditions, thereby ensuring coherence and comparability.

The biodegradation percentage of leather was calculated relative to the value obtained for collagen, which was considered the 100% reference. Based on this, the relative biodegradation of oxazolidine-tanned leather was determined.

The analysis of the 15 tests revealed that all degradation curves were highly similar for both collagen and oxazolidine-tanned leather. The shaded areas representing standard deviation and 95% confidence intervals in the figures were narrow, indicating low variability among trials (Figure 2).

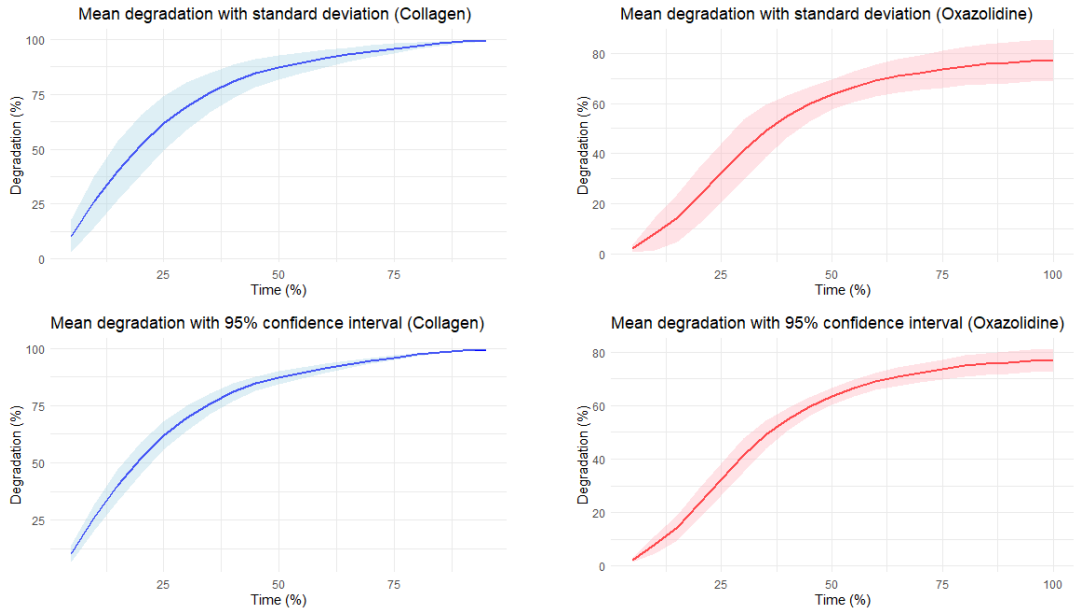

Figure 2. Mean degradation with standard deviation and mean degradation with 95% confidence interval. collagen in blue and oxazolidine in red.

To evaluate sample variability, coefficients of variation were calculated (Tables 1 and 2). Variability was considered low when coefficients were below 12%, a threshold considered favorable given the high intrinsic variability typically observed in biological assays.

For both sample types, coefficients dropped below this threshold from approximately 35–40% of normalized time onward, suggesting that the differences in degradation values across trials were insignificant for both collagen and oxazolidine.

Collagen	
Time (%)	CV (%)
5	70.7
10	44.3
15	33.2
20	26.2
25	19.8
30	15.5
35	12.0
40	9.29
45	7.59
50	6.34
55	5.33
60	4.37
65	3.42
70	2.87
75	2.32
80	1.59
85	1.08
90	0.71
95	0.31

a

Oxazolidine	
Time (%)	CV (%)
5	62.7
10	80.1
15	65.7
20	47.6
25	36.7
30	29.5
35	21.2
40	15.0
45	11.3
50	9.38
55	9.17
60	9.25
65	9.44
70	9.73
75	10.0
80	10.4
85	10.5
90	10.6
95	10.7
100	10.8

b

Table 1. Coefficient of variation of collagen (a) and oxazolidine (b) biodegradation curves in the 15 trials performed.

Pearson correlation coefficients for collagen comparisons between trials were all above 0.89, while for oxazolidine, the correlation was 0.86. These high correlations confirm that biodegradation kinetics for collagen were highly similar across the 15 trials, with a similar trend observed for oxazolidine-tanned leather (Figure 3).

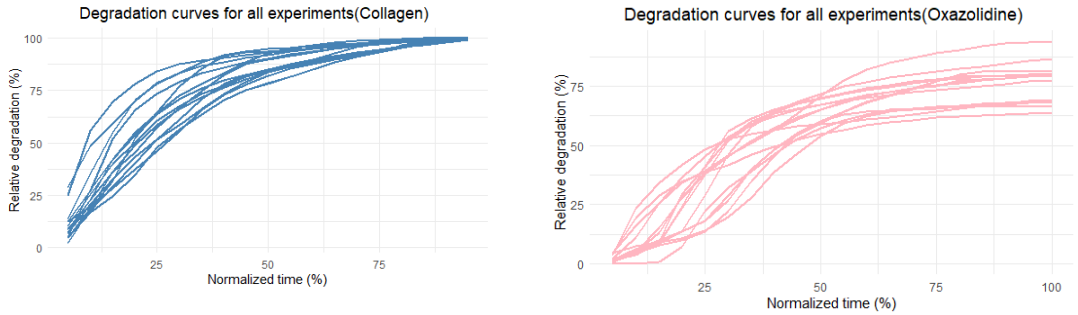

Figure 3. Pearson correlation coefficient from 15 trials for collagen (blue) and oxazolidine (red).

As previously noted, Lin's concordance correlation coefficient assesses both shape and magnitude agreement between biodegradation curves. For both collagen and oxazolidine, most values were close to 1, indicating high agreement (Figure 4).

For collagen, this outcome was expected since all values were normalized to 100%. For oxazolidine, although not all trials reached the same final degradation value due to normalization relative to collagen, Lin's coefficient remained high, confirming that differences were minimal.

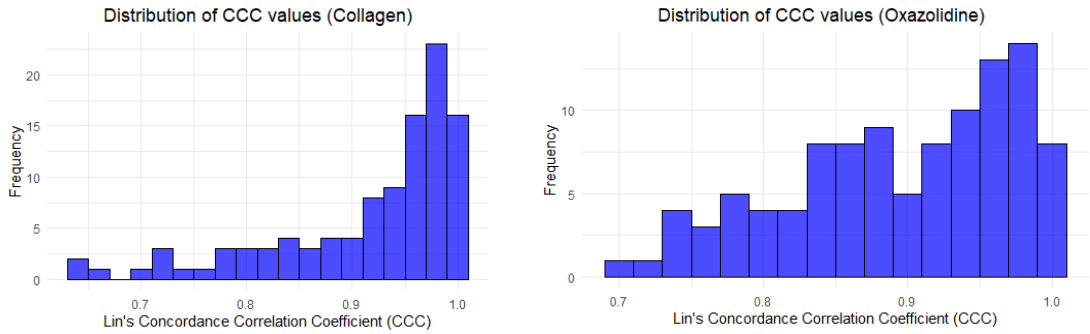

Figure 4. Similarity and magnitude coefficient according to Lin between the biodegradation curves of collagen (blue) and oxazolidine (red).

The Kolmogorov test was applied only to collagen data, as oxazolidine samples reached different final degradation values, introducing slight variations in mean curve values that precluded assuming a common distribution.

For collagen, the Kolmogorov test determined whether two curves originated from a common distribution, implying a shared degradation pattern with minor individual differences, thus providing strong support for the reproducibility of collagen degradation results.

For this hypothesis to be valid, p-values must exceed 0.05. This criterion was met for all experiment pairs, confirming that all collagen degradation curves followed a common distribution (Figure 5).

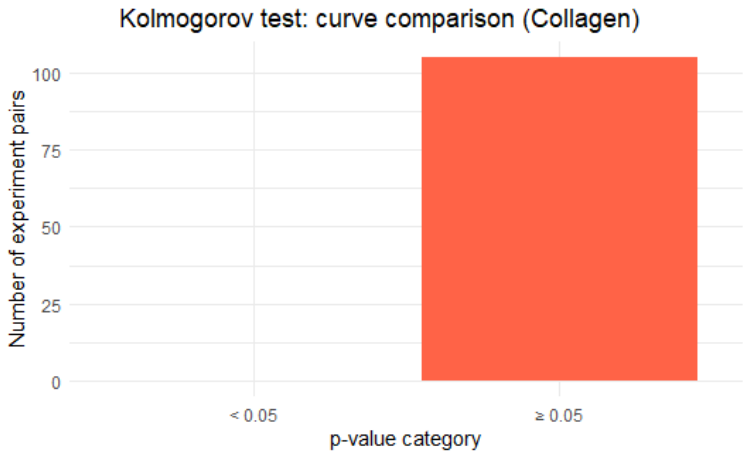

Figure 5. Common distribution test for collagen using the Kolmogorov test

To assess whether the small observed differences between experiments were statistically significant, Bland–Altman plots were generated for each pair of collagen (Table 2, a) and oxazolidine (Table 2, b) experiments.

These plots show the average difference between two trials over time, define significance thresholds, and display individual time-point differences. A percentage of outliers around 5% was expected to confirm non-significant differences.

For collagen, both mean and median values were below 5%, while for oxazolidine, values were approximately 5%, indicating that differences between experiment pairs were not statistically significant.

Collagen						
Statistic	Minimum	1st Quartile	**Median**	**Mean**	3rd Quartile	Maximum
Points outside (%)	0.000	0.000	**0.000**	**2.807**	5.263	10.526

Oxazolidine						
Statistic	Minimum	1st Quartile	**Median**	**Mean**	3rd Quartile	Maximum
Points outside (%)	0.000	5.000	**5.000**	**5.238**	10.000	15.000

Table 2. Evaluation of statistical differences between pairs of collagen and oxazolidine experiments using Bland–Altman.

Finally, Quality Control Charts (QCC) were used to verify whether variability observed during the initial phases was statistically significant (Figure 6). These charts display the initial slope values of each experiment, the average initial slope, and significance limits.

For both collagen and oxazolidine, no data points exceeded the defined limits. Therefore, even though most variability occurred at the beginning of the trials, these differences were not statistically significant.

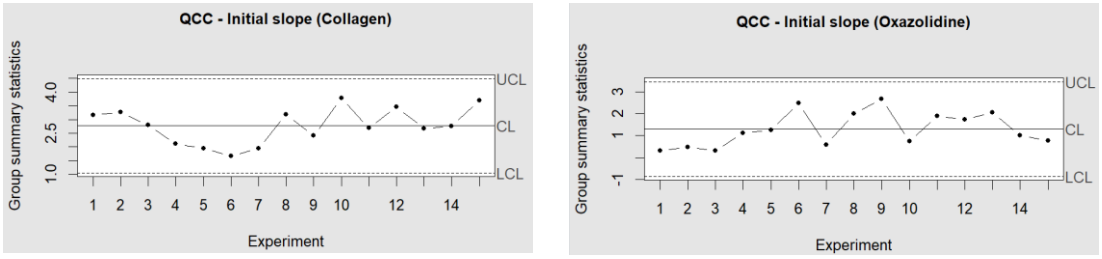

Figure 6. Analysis of initial slope significance in biodegradation assays of collagen (a) and oxazolidine (b) using Quality Control Charts (QCC)

4. Conclusion

This study evaluated the reproducibility of leather biodegradation tests in liquid media following ISO 20136:2020, using oxazolidine-tanned leather as the test material and pure collagen as the reference control, in accordance with the standard's protocol.

To present the data uniformly, results were expressed as relative biodegradation percentages, as defined in by ISO 20136:2020.

Based on statistical analyses of 15 trials over a five-year period (2019–2025), the following conclusions can be drawn:

Statistical analysis of collagen, as evidenced in the results section, supports the hypothesis of reproducibility.

High correlations between trials, indicated by coefficients Pearson, CCC, and CV coefficients, confirmed this finding. Differences observed between trials fell within statistically acceptable boundaries, as confirmed by QCC and Bland–Altman plots.

The Kolmogorov test further validated that all collagen degradation curves originated from a common distribution.

Conversely, since the collagen served as the normalization reference, oxazolidine-tanned leather exhibited variability in biodegradation values across trials—an inherent feature of biological assays. Therefore, it cannot be concluded that all oxazolidine degradation curves derive from a single distribution.

Nonetheless, statistical analyses indicate that the curves share similar shapes and dispersions, albeit with slightly different mean values. Consequently, reproducibility remains a valid hypothesis for these experiments.
Overall, thorough analysis of the 15 trials for both collagen and oxazolidine confirmed data consistency despite differences in year, inoculum, and environmental conditions (which affected only the inoculum, as laboratory test conditions were controlled).
The observed degradation patterns were highly similar across trials, indicating that results were not due to chance or circumstantial factors but rather reflected stable and repeatable behavior.
Therefore, it can be concluded that the biodegradation tests are reproducible, lending strength and reliability to conclusions drawn from them.

5. Acknowledgement

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