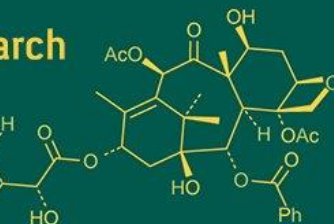
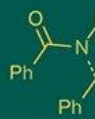


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Shelf-life study of protein-rich vegan cocoa beverage during refrigerated storage condition

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Abstract

Shelf-life evaluation is essential for establishing the storage stability and marketability of newly developed food products. The present study evaluated the physicochemical, microbiological and sensory stability of an optimized Protein-rich Vegan Cocoa Beverage (PVCB), formulated from amaranth milk, roasted horse gram flour, peanut protein concentrate (PPC-80), cocoa powder and sugar, during refrigerated storage (4-7 °C) over 30 days, with sampling at 3-day intervals. Titratable acidity increased marginally from 0.02 to 0.03% citric acid (non-significant, $p < 0.05$). Viscosity declined gradually from 124.20 ± 1.20 to 119.00 ± 0.50 cP (4.19% reduction), while serum separation increased steadily and significantly from 0.00 to $1.78 \pm 0.03\%$ (CV = 3.91%), representing the most statistically robust indicator of physical destabilization. Colour coordinates L^* , a^* and b^* declined by 10.48%, 11.18% and 15.97%, respectively, consistent with non-enzymatic browning and pigment settling. The aerobic plate count increased only marginally, from 1.30 ± 0.01 to 1.69 ± 0.14 log CFU/mL, remaining well within FSSAI safety limits, while yeast, mould and coliform counts remained not detected throughout storage. The overall acceptability (OA) score declined progressively from 8.50 ("liked very much") on day 0 to 5.30 ("dislike slightly") on day 30, falling below the consumer rejection threshold (6.0) after day 27. On the basis of sensory acceptability, the shelf life of the developed PVCB under refrigerated storage was established as 27 days, while microbiological safety was maintained throughout the entire 30-day study period.

Keywords: Vegan cocoa beverage, refrigerated storage, shelf life, serum separation, sensory acceptability, microbial safety, storage stability

1. Introduction

Shelf-life evaluation is a critical step in the development of any food product intended for commercial production, determining the period during which the product remains safe, nutritionally acceptable and organoleptically desirable under specified storage conditions (Gupta *et al.*, 2023) [7]. During storage, physical, chemical, microbiological and sensory changes may occur, resulting in deterioration of product quality, with the rate of change depending on product composition, processing conditions, packaging and storage environment. Plant-based beverages, being inherently unstable colloidal dispersions of proteins, starches and fine particulates in an aqueous serum phase, are especially prone to instability phenomena such as creaming, flocculation and serum separation during extended storage (Karlsson *et al.*, 2019) [8].

A protein-rich vegan cocoa beverage (PVCB) was previously developed and optimized by combining amaranth milk with roasted horse gram flour, peanut protein concentrate (PPC-80), cocoa powder and sugar using Response Surface Methodology, yielding a product with 30.36% total solids, 10.84% crude protein and desirable sensory and functional attributes immediately after processing. However, the storage stability of this novel beverage system under refrigerated conditions had not been established. Comparable plant-based ready-to-drink beverages have been reported to undergo progressive sensory and physicochemical decline during refrigerated storage (Divyashree, 2025; Alemayehu *et al.*, 2025) [4, 2]. The present study was therefore undertaken to evaluate the physicochemical, microbiological and sensory changes occurring in the developed PVCB during refrigerated storage, and to determine its shelf life on the basis of sensory acceptability and microbiological safety.

2. Materials and Methods

2.1 Preparation and packaging of PVCB

The optimized PVCB (5.25 g roasted horse gram flour, 12.00 g PPC-80, 4.0 g cocoa powder and 12.61 g sugar per 100 mL amaranth milk) was prepared following the standardized process: amaranth milk (extracted from grains soaked 8 h and wet-milled in a colloidal mill) was diluted with water (1:5 v/v), preheated to 40 °C, blended with the pre-mixed dry ingredients, and thermally processed at 75–80 °C for 7 min. The beverage was hot-filled into 500 mL PET bottles, sealed, and stored under refrigerated conditions (4–7 °C) for the duration of the storage study.

2.2 Storage study design

Samples were analysed at 3-day intervals over a 30-day refrigerated storage period, until the product reached the limit of acceptable quality based on sensory evaluation and microbiological safety. At each sampling interval, the beverage was evaluated for titratable acidity, viscosity, colour values (L^* , a^* , b^*) and serum separation, alongside microbiological quality (aerobic plate count, yeast and mould count, coliform count) and sensory overall acceptability.

2.3 Analytical, microbial and sensory methods

Titratable acidity was determined following the method of Gupta *et al.* (2010) [6] by titration against 0.1 N NaOH using phenolphthalein indicator and expressed as percent citric acid. Viscosity was measured using a Brookfield viscometer (DV-II + Pro, spindle No. 62, 50 rpm, 25 °C). Colour values

(L^* , a^* , b^*) were recorded using a Lovibond Tintometer (Model: RT850i) based on the CIE Lab* colour space. Serum separation was determined following Adedokun *et al.* (2017) [1] by monitoring the volume of serum separated from a 100 mL sample held at room temperature, expressed as a percentage. Microbiological quality was assessed by standard plate count methods (AOAC, 2019) [3] for aerobic plate count (nutrient agar, 37±0.5 °C, 24 h), yeast and mould count (potato dextrose agar, 25±0.5 °C, 48–72 h) and coliform count (MacConkey agar, 37±0.5 °C, 48 h), expressed as log CFU/mL. Sensory overall acceptability was evaluated using a 9-point hedonic scale.

2.4 Statistical analysis

All analyses were performed in triplicate and results expressed as Mean ± SD. Data generated during the storage study were subjected to one-way analysis of variance (ANOVA) under a Completely Randomized Design (CRD) to determine the effect of storage period on quality characteristics. Where significant differences were observed, treatment means were separated using Duncan's Multiple Range Test (DMRT) at the 5 percent level of significance ($p < 0.05$), consistent with the statistical framework applied by Alemayehu *et al.* (2025) [2] for beverage storage studies.

3. Results and Discussion

The complete set of quality characteristics monitored during the 30-day refrigerated storage period is presented in Table 1.

Table 1: The complete set of quality characteristics monitored during the 30-day refrigerated storage period is presented

Storage day	TA (% citric acid)	Viscosity (cP, 25 °C)	L^*	a^*	b^*	Serum separation (%)	OA score	APC (log CFU/mL)
0	0.02±0.00	124.20±1.20 ^a	35.21±0.18 ^a	8.32±0.08 ^a	14.34±0.13 ^a	0.00±0.00 ^k	8.50±0.14 ^a	1.30±0.01
3	0.02±0.00	123.27±1.33 ^{ab}	35.31±0.21 ^a	7.91±0.06 ^{bc}	13.58±0.18 ^b	0.15±0.03 ^j	8.28±0.18 ^{ab}	1.34±0.03
6	0.03±0.00	123.33±2.10 ^{ab}	33.69±0.16 ^b	7.77±0.12 ^{de}	13.28±0.26 ^c	0.32±0.04 ⁱ	8.00±0.18 ^{bc}	1.40±0.10
9	0.02±0.00	122.30±1.95 ^{abc}	32.53±0.35 ^c	7.78±0.11 ^{ede}	12.48±0.06 ^{de}	0.48±0.03 ^h	7.72±0.19 ^c	1.45±0.05
12	0.03±0.00	121.27±1.51 ^{bcd}	32.45±0.04 ^c	7.98±0.10 ^b	13.43±0.05 ^{bc}	0.64±0.04 ^g	7.38±0.17 ^d	1.49±0.04
15	0.03±0.00	121.20±2.11 ^{bcd}	32.38±0.19 ^c	7.84±0.05 ^{cd}	12.65±0.16 ^d	0.85±0.03 ^f	7.06±0.14 ^e	1.54±0.13
18	0.03±0.00	121.03±0.75 ^{bcd}	32.34±0.14 ^{cd}	7.75±0.06 ^{de}	12.55±0.07 ^{de}	1.02±0.06 ^e	6.67±0.18 ^f	1.59±0.10
21	0.03±0.00	120.37±0.91 ^{cd}	32.08±0.12 ^{de}	7.69±0.08 ^{ef}	12.35±0.06 ^{ef}	1.22±0.02 ^d	6.50±0.11 ^{fg}	1.62±0.03
24	0.03±0.00	119.87±1.95 ^d	31.83±0.13 ^{ef}	7.56±0.07 ^f	12.23±0.06 ^g	1.41±0.04 ^c	6.25±0.14 ^{gh}	1.65±0.07
27	0.03±0.00	119.00±2.03 ^d	31.59±0.15 ^g	7.41±0.04 ^g	12.07±0.06 ^g	1.61±0.01 ^b	6.10±0.22 ^h	1.67±0.05
30	0.03±0.00	119.00±0.50 ^d	31.52±0.06 ^g	7.39±0.03 ^g	12.05±0.03 ^g	1.78±0.03 ^a	5.30±0.14 ⁱ	1.69±0.14
SEm(±)	0.003	0.92	0.10	0.05	0.07	0.02	0.10	-
CD (P)	NS	2.69	0.30	0.14	0.21	0.06	0.28	-
CV (%)	17.30	1.31	0.54	1.06	0.96	3.91	2.34	-

Each observation is a Mean ± SD of three replicate experiments ($n = 3$). Means within a column bearing different superscript letters differ significantly ($P = 0.05$, DMRT). TA = titratable acidity; APC = aerobic plate count; OA = overall acceptability.

3.1 Effect of refrigerated storage on titratable acidity

Titratable acidity of the PVCB, recorded as 0.02% citric acid in the fresh sample, rose marginally to 0.03% citric acid over 30 days of refrigerated storage, a change that was statistically non-significant ($p < 0.05$; CD = NS). The gradual rise is commonly attributed to residual microbial activity leading to slow acid accumulation, an effect markedly slowed under refrigeration and with limited sugar/acid content restricting microbial proliferation, consistent with observations in plant-based and fruit-based beverages reported by Alemayehu *et al.* (2025) [2]. The high coefficient of variation (17.30%) reflects the very small absolute magnitude of change relative to measurement precision rather than a substantive quality shift.

3.2 Effect of refrigerated storage on viscosity

Viscosity decreased from 124.20±1.20 cP (day 0) to 119.00±0.50 cP (day 30), a reduction of 4.19% over the storage period. The decline was gradual and near-linear, with a slightly steeper drop between days 6 and 12, without any abrupt fluctuation, consistent with a slow, continuous structural change rather than a sudden destabilization event. The significance groupings (a, ab, abc, bcd, cd) indicate the decline emerged progressively: day 0 was not significantly different from day 3, but was significantly different from day 24 onward. The low coefficient of variation (1.31%) confirms this was a precise, reproducible trend. PVCB, being a colloidal dispersion of proteins, starches and fine particulates in an aqueous serum phase, undergoes

progressive particle aggregation and settling during storage, depleting the continuous phase of suspended solids and lowering bulk viscosity, consistent with reports on plant-based milk alternatives by Karlsson *et al.* (2019) [8].

3.3 Effect of refrigerated storage on colour values

All three colour coordinates exhibited an overall declining trend over 30 days, with reductions of 10.48%, 11.18% and 15.97% for L*, a* and b*, respectively. The L* value declined smoothly and monotonically from 35.21±0.18 to 31.52±0.06, following a marginal, non-significant increase between day 0 and day 3. The a* and b* values, by contrast, declined from day 0 to day 9, followed by a distinct rebound at day 12 (a*: 7.78-7.98; b*: 12.48-13.43) before resuming their downward trend through day 30; the low coefficients of variation (1.06% and 0.96%, respectively) confirm this transient reversal is a genuine, reproducible feature rather than experimental variability, possibly reflecting a partial redistribution of pigmented cocoa particulates within the serum phase at that sampling interval. The overall darkening and dulling across all coordinates are attributable to slow non-enzymatic browning between residual reducing sugars and free amino groups of proteins, gradual oxidation of cocoa polyphenols, and progressive settling of pigmented cocoa particulates reducing the dispersion's light-scattering capacity, consistent with colour-stability changes reported in hazelnut milk by Tsai *et al.* (2018) [9].

3.4 Effect of refrigerated storage on serum separation

Serum separation increased steadily from 0.00% at production to 1.78±0.03% by day 30, following an almost linear trajectory with no plateau or lag phase, indicating progressive and continuous physical instability. Each of the eleven storage-day means received a distinct superscript letter (k to a), indicating serum separation differed significantly from every preceding and succeeding sampling day (CD = 0.06), which together with the low coefficient of variation (3.91%) establishes serum separation as the most statistically robust and reproducible indicator of physical destabilization recorded in this study. This trend closely mirrors sediment formation reported in UHT-treated milk during long-term cold storage, arising from a decline in serum viscosity and a corresponding increase in particle settling velocity in accordance with Stokes' law (Karlsson *et al.*, 2019) [8], and directly parallels the concurrent viscosity decline observed in the present beverage, likely contributing substantially to the decline in sensory acceptability with increasing storage duration.

3.5 Effect of refrigerated storage on microbial quality

The aerobic plate count (APC) increased gradually from 1.30±0.01 log CFU/mL at production to 1.69±0.14 log CFU/mL by day 30, an increase of only about 0.40 log cycles over the entire storage period. Yeast and mould count and coliform count remained not detected (ND) throughout all 30 days of storage without exception. The slow and limited rise in APC indicates that standardized thermal processing, hygienic handling and aseptic bottling achieved a low initial microbial load, while continuous refrigeration (4-7 °C) effectively suppressed the multiplication of mesophilic spoilage organisms. APC values remained well within FSSAI microbiological safety limits throughout the storage period, and the complete absence of yeast, mould and coliform organisms confirms the microbiological safety

and hygienic quality of the product for the full 30-day study duration.

3.6 Effect of refrigerated storage on overall acceptability and shelf-life determination

The overall acceptability (OA) score declined progressively from 8.50±0.14 ("liked very much") on day 0 to 5.30±0.14 ("dislike slightly") on day 30, an overall reduction of approximately 37.65%. The decline followed a gradual, consistent downward trajectory, with a slightly steeper rate of decrease between days 18 and 27, but without abrupt or discontinuous deterioration at any single point. Statistical analysis revealed a clear progressive pattern of significance: day 0 (a) and day 3 (ab) were not significantly different from each other, but the OA score declined significantly from day 6 onward, with each subsequent group of storage days forming progressively lower and largely distinct homogeneous subsets through day 30. The low coefficient of variation (2.34%) confirms high reproducibility of this sensory trend.

The OA score fell below the commonly accepted consumer rejection threshold of 6.0 after day 27 (score 6.10, "neither like nor dislike") and reached 5.30 by day 30. Based on sensory evaluation, the shelf life of the developed PVCB under refrigerated storage conditions (4-7 °C) was therefore established as 27 days. Since the concurrent decline in viscosity and rise in serum separation directly affect visual appeal and mouthfeel, these physical changes are likely to have been major contributors to the observed decline in sensory scores. Similar findings were reported by Divyashree M. (2025) [4], who observed a gradual decline in OA score from 8.21±0.06 to 6.66±0.07 in a millet milk-based ready-to-drink beverage during 35 days of refrigerated storage.

4. Conclusion

The developed protein-rich vegan cocoa beverage exhibited a characteristic and reproducible pattern of physicochemical change during 30 days of refrigerated storage (4-7 °C): a marginal, non-significant rise in titratable acidity; a gradual, near-linear decline in viscosity (4.19%); a progressive, highly significant increase in serum separation, identified as the most statistically robust indicator of physical instability; and a consistent darkening across all colour coordinates attributable to non-enzymatic browning and pigment settling. Microbiological safety was maintained throughout the entire storage period, with aerobic plate counts remaining well within FSSAI limits and yeast, mould and coliform organisms undetected at every sampling point. However, the overall acceptability score declined below the consumer rejection threshold after day 27, establishing the sensory-based shelf life of the beverage at 27 days under refrigerated conditions. These findings indicate that physical destabilization particularly serum separation and the associated decline in viscosity and colour rather than microbiological spoilage was the primary factor limiting the shelf life of the developed beverage, providing a clear target for future stabilization strategies such as hydrocolloid incorporation or homogenization to extend product shelf life.

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