International Journal of Agriculture and Food Science 2026; 8(6): 136-145

ISSN Print: 2664-844X
ISSN Online: 2664-8458
NAAS Rating (2026): 4.97
IJAFS 2026; 8(6): 136-145
www.agriculturaljournals.com
Received: 22-03-2026
Accepted: 26-04-2026

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Development, RSM-guided optimisation, and comprehensive physicochemical characterisation of a novel functional smoothie fortified with carrot-beetroot juice and bioactive seeds

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DOI: https://www.doi.org/10.33545/2664844X.2026.v8.i6b.1587

Abstract
Rising consumer demand for nutrient-dense, minimally processed functional beverages has catalysed interest in formulations that synergistically combine phytochemical-rich vegetables with bioactive seeds. This study reports the first comprehensive development, statistical optimisation, and multi-attribute physicochemical characterisation of a novel functional smoothie incorporating carrot-beetroot juice and bioactive seeds (chia and flax). A five-factor Box-Behnken Design (BBD) under Response Surface Methodology (RSM) was employed, varying carrot juice (50-75 ml), beetroot juice (50-75 ml), water (25-50 ml), chia seeds (2-5 g), and flaxseeds (2-5 g) across 46 experimental runs evaluated for overall acceptability (OA) by a hedonic panel (n = 10). The quadratic model exhibited excellent statistical adequacy (F-value = 17.71; p< 0.0001; R^2 = 0.93; adjusted R^2 = 0.87; adequate precision = 15.70). Carrot juice concentration (A) and water volume (C) exerted the dominant linear influence on OA, while pronounced quadratic terms for all five factors confirmed true optima within the experimental space. Numerical optimisation yielded the desirability-optimal formulation (carrot juice 62.5 ml, beetroot juice 62.5 ml, water 37.5 ml, chia seeds 3.5 g, flaxseeds 3.5 g), validated by a confirmatory OA of 9.23 versus a predicted value of 9.00 (prediction error: 2.56%). Comprehensive characterisation of the optimised smoothie revealed: total phenols 135.33±2.51 mg GAE/kg FW; total carotenoids 50.5±0.02 mg/kg FW; DPPH radical scavenging activity 84±0.02% RSA; ascorbic acid 230±0.03 mg/kg FW; and pseudoplastic rheological behaviour (flow behaviour index n = 0.82±0.03). The product demonstrated microbiological safety at day 0 (TPC 2.26±0.12 log CFU/g) and a refrigerated shelf life of 8 days at 4°C. This study provides a validated, reproducible framework for the concurrent optimisation and functional characterisation of multi-ingredient plant-based beverages, with direct translational relevance for nutraceutical product development.

Keywords: Functional smoothie, response surface methodology, Box-Behnken design, carrot juice, beetroot juice, chia seeds, flaxseeds, sensory optimisation, phytochemical characterisation, shelf-life stability

1. Introduction
The global functional food and beverage market, valued in excess of USD 250 billion, is forecast to expand at a compound annual growth rate exceeding 7% through 2030, driven primarily by heightened consumer awareness of diet-disease relationships and the accelerating prevalence of non-communicable diseases (NCDs) [1, 2]. Epidemiological evidence consistently implicates suboptimal intake of fruits and vegetables in the aetiology of cardiovascular disease (CVD), type 2 diabetes mellitus (T2DM), obesity, and several malignancies; yet adherence to the WHO-recommended minimum of 400 g/day remains persistently suboptimal across most demographics, attributable to palatability barriers, time constraints, and limited product diversity [3, 4].

Ready-to-consume functional smoothies have emerged as an operationally pragmatic dietary vehicle capable of co-delivering diverse bioactive compounds within an acceptable sensory matrix [5, 6]. Unlike single-ingredient juices, multi-component smoothies exploit synergistic interactions among polyphenols, carotenoids, dietary fibre, essential fatty acids, and prebiotic substrates, generating health benefits that transcend those achievable by individual ingredients [7].

Their liquid matrix facilitates high bioavailability of liposoluble phytochemicals, particularly when combined with lipid-providing ingredients such as seeds, which enhance micellar solubilisation and intestinal absorption of carotenoids and fat-soluble vitamins [8].

Carrot (*Daucus carota* L.) juice is one of the richest dietary sources of β-carotene (a provitamin A precursor), constituting 5480-6000 µg/100 g fresh weight, alongside hydroxycinnamic acids, polyacetylenes, and ascorbic acid that collectively confer antioxidant, anti-inflammatory, and chemopreventive activities [9]. Beetroot (*Beta vulgaris* L.) juice represents a uniquely concentrated source of betalain pigments (betacyanins and betaxanthins), inorganic nitrate (≥5 mmol per 500 ml serving), and phenolic compounds, collectively imparting well-documented cardioprotective, vasodilatory, anti-hypertensive, and exercise-ergogenic effects mediated through the nitrate-nitrite-nitric oxide pathway [10]. The synergistic combination of carrot and beetroot juice yields a complementary colour profile and flavour balance, with β-carotene moderating the characteristic earthiness of betalain pigments to produce a more palatable sensory outcome [11].

Chia (*Salvia hispanica* L.) and flaxseeds (*Linum usitatissimum* L.) are botanically distinct yet nutritionally complementary bioactive seeds recognised as concentrated sources of α-linolenic acid (ALA; ω-3 PUFA), soluble mucilage, plant-based protein, lignans, and dietary fibre [12, 13]. Regular dietary inclusion of these seeds is associated with improved glycaemic control, favourable modulation of the lipid profile, augmentation of gut microbiome diversity, and attenuation of systemic inflammation [14]. Chia seed mucilage forms a colloidal network that imparts pseudoplastic (shear-thinning) rheological behaviour to aqueous dispersions, a technologically desirable property in beverages that facilitates easy pourability while maintaining mouth-coating body and mouthfeel [12, 13].

The development of multi-ingredient functional beverages presents inherent formulation challenges arising from the non-linear, interactive effects of ingredient concentrations on sensory quality. Classical one-factor-at-a-time (OFAT) optimisation strategies are demonstrably inefficient, incapable of capturing ingredient interactions, and prone to identifying sub-global optima [15]. Response Surface Methodology (RSM), and specifically the Box-Behnken Design (BBD), provides a statistically rigorous, resource-efficient framework for simultaneously evaluating multiple formulation variables and their interactions, enabling identification of the global optimum with fewer experimental runs than equivalent full-factorial designs [16, 17]. BBD is particularly suited to functional food formulations where extreme combinations of factor levels are technologically or sensorially undesirable, as it restricts sampling to interior and face-centred design points, thereby avoiding operationally unrealistic experimental conditions [16].

Despite the burgeoning body of literature on functional smoothie formulation and optimisation [17, 18] published investigations that: (i) simultaneously co-optimise carrot juice, beetroot juice, water volume, and bioactive seed content using a five-factor BBD; and (ii) follow optimisation with comprehensive physicochemical, phytochemical, rheological, and microbiological characterisation of the validated optimum formulation, are conspicuously absent. This omission represents a critical translational gap, as product characterisation data are prerequisite for regulatory compliance, nutritional labelling, and clinical investigation of functional claims. The present study was designed to address this gap by: (i) developing a novel multi-ingredient functional smoothie incorporating carrot-beetroot juice with chia and flaxseeds; (ii) applying a five-factor BBD to systematically optimise ingredient concentrations for maximum hedonic acceptability; (iii) validating the RSM-predicted optimal formulation through confirmatory sensory evaluation; and (iv) comprehensively characterising the optimised product for proximate composition, phytochemical profile, colour, rheological properties, energy content, microbiological safety, and refrigerated shelf life.

2. Materials and Methods

2.1 Raw Materials and Procurement

Fresh carrots (*Daucus carota* L. cv. Nantes), beetroots (*Beta vulgaris* L.), bananas, chia seeds (*Salvia hispanica* L.), flaxseeds (*Linum usitatissimum* L.), quinoa, rolled oats, full-fat yoghurt, and honey were procured from a certified retail market in Hisar, Haryana, India. All produce was inspected for absence of physical defects, fungal lesions, and mechanical damage before use. Chia seeds and flaxseeds were food-grade and purchased from a certified health food supplier; they were used without further processing. All chemicals used for analytical determinations were of analytical grade (Sigma-Aldrich, India; Merck India). Water used throughout was double-distilled, de-ionised, and of potable quality.

2.2 Smoothie Preparation

Carrots and beetroots were thoroughly washed under running water, peeled, and immediately cold-pressed using a commercial cold-press juicer (Hurom HH-SBF11, South Korea) to minimise heat-induced phytochemical degradation. Fresh juices were filtered through a 250-µm stainless steel mesh sieve to remove coarse fibre and used within 15 min of preparation. The fixed-composition base matrix per batch comprised: full-fat yoghurt (100 ml), ripe banana (1 medium, ~120 g), pre-soaked quinoa (2 g, hydrated for 2 h at room temperature in distilled water at 1:4 w/v), rolled oats (5 g), and honey (10 ml). Variable ingredients (carrot juice, beetroot juice, water, chia seeds, and flaxseeds) were proportioned according to each experimental run of the BBD (Table 1). All components were blended in a high-speed blender (Philips HR2056, 750 W) at 10, 000 rpm for 60 s. The product was served immediately for sensory evaluation in coded opaque cups (50 ml per panellist) at 4°C or retained for characterisation analyses at the optimised formulation level.

2.3 Experimental Design: Box-Behnken Design

A five-factor, three-level Box-Behnken Design (BBD) was constructed using Design-Expert® software (version 9.0.6.2; Stat-Ease Inc., Minneapolis, USA). The five independent variables and their coded low (-1), centre (0), and high (+1) levels are presented in Table 1. The design yielded 46 experimental runs, including six centre-point replicates for estimation of pure experimental error. Overall acceptability (OA), assessed on a 9-point hedonic scale, served as the single response variable. A second-order polynomial quadratic model of the following form was fitted to the OA response data:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\Sigma\beta_{ij} X_i X_j + \varepsilon \quad (1)$$

where Y is the predicted response; β_0 is the model intercept; β_i, β_{ii}, and β_{ij} are linear, quadratic, and interaction regression coefficients, respectively; X_i and X_j are the coded independent variables; and ε is the residual error.

Table 1: Independent variables and their coded and actual levels in the Box-Behnken Design

Independent Variable	Symbol	Low (-1)	Centre (0)	High (+1)
Carrot juice (ml)	A	50	62.5	75
Beetroot juice (ml)	B	50	62.5	75
Water (ml)	C	25	37.5	50
Chia seeds (g)	D	2	3.5	5
Flaxseeds (g)	E	2	3.5	5

2.4 Sensory Evaluation
Sensory evaluation of the 46 BBD formulations was conducted in a standardised sensory laboratory under controlled conditions (white fluorescent illumination, individual booths, temperature 22±1°C). A semi-trained panel of ten evaluators, comprising food technology faculty and postgraduate students familiar with hedonic scale usage and product category, participated following institutional ethical clearance and informed consent. Panellists underwent a 30-min briefing session on evaluation criteria and were instructed to rinse with potable water between samples. Each sample (50 ml in coded opaque cups at 4°C) was rated for overall acceptability (OA) on a 9-point hedonic scale (1 = dislike extremely; 9 = like extremely). Duplicate assessments per run were conducted and mean OA values used for modelling. Following RSM optimisation, the validated formulation was additionally evaluated for individual sensory attributes (colour, appearance, aroma, flavour, and texture/mouthfeel) on the same 9-point scale, prepared in triplicate.

2.5 Physicochemical and Functional Characterisation of the Optimised Smoothie
2.5.1 Physicochemical Properties
The pH of the optimised smoothie was measured using a calibrated digital pH meter (Hanna Instruments HI2211, USA) at 25°C. Total soluble solids (TSS) were determined using a hand-held Abbe refractometer and expressed as °Brix. Titratable acidity (TA) was determined by titration against 0.1 N NaOH and expressed as mg malic acid/100 g FW [19]. Ascorbic acid content was estimated by the 2, 6-dichlorophenolindophenol (DCPIP) titrimetric method as described by Ranganna [20] and expressed as mg/kg FW.

2.5.2 Phytochemical Analysis
Total phenolic content (TP) was determined using the Folin-Ciocalteu reagent with gallic acid as the standard and expressed as mg GAE/kg FW. Total carotenoids (TC) were extracted using acetone:hexane (4:6 v/v) and quantified spectrophotometrically at 663, 645, and 470 nm following the method of Lichtenthaler [21], expressed as mg/kg FW. Total flavonoid content (TF) was measured by the aluminium chloride colorimetric method with quercetin as standard and expressed as mg/kg FW. Antioxidant activity (AO) was evaluated by the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay as described by Brand-Williams *et al.* [22], expressed as% radical scavenging activity (%RSA).

2.5.3 Instrumental Colour Measurement
Instrumental colour was measured using a Hunter Lab colorimeter (HunterLab ColorFlex, USA) calibrated against white and black standards at 25°C. Colour was expressed in the CIE L*a*b* colour space. Hue angle ($h° = \arctan(b^*/a^*)$) and chroma ($C^* = \sqrt{(a^{*2} + b^{*2})}$) were calculated from a* and b* values. All measurements were performed in triplicate.

2.5.4 Rheological Properties
Rheological characterisation was performed using a Brookfield DVII+ viscometer (Brookfield Engineering, USA) equipped with spindle LV-3 at 25°C across shear rates of 1-100 s^{-1}. The apparent viscosity, flow behaviour index (n), and consistency coefficient (K) were determined by fitting the Ostwald-de Waele power law model ($\tau = K\cdot\gamma^n$) to the experimental flow data using nonlinear regression. Product density was measured using a calibrated pycnometer (25 ml). All rheological measurements were performed in triplicate.

2.5.5 Energy Value Calculation
The energy value of the optimised smoothie per 100 ml serving was calculated using Atwater conversion factors: protein (4 kcal/g), available carbohydrate (4 kcal/g), and fat (9 kcal/g), following the standard procedure outlined in EU Regulation No. 1169/2011.

2.5.6 Microbiological Safety Assessment
Microbiological safety of the freshly prepared optimised smoothie (day 0) was assessed according to AOAC (2005) standard microbiological methods. Total plate count (TPC) was determined using plate count agar (PCA) incubated at 37°C for 48 h. Yeast and mould counts were enumerated on potato dextrose agar (PDA) incubated at 25°C for 5 days. Coliform count was assessed using MacConkey agar, and Staphylococcus aureus and Escherichia coli were identified using selective and differential media. Results were expressed as log CFU/g and compared against FSSAI regulatory limits for ready-to-eat beverages.

2.5.7 Storage Stability and Shelf-Life Study
Refrigerated shelf-life was evaluated by monitoring the optimised smoothie stored in sealed glass jars at 4±1°C for 10 days. Analyses of pH, TA, TSS, TPC, and OA were performed at two-day intervals (days 0, 2, 4, 6, 8, and 10). The shelf-life endpoint was defined as the day on which OA fell below the acceptability threshold of 7.0 ("like moderately") and/or TPC exceeded 5 log CFU/g (FSSAI limit for ready-to-eat products). All storage analyses were conducted in triplicate.

2.6 Statistical Analysis
All RSM analyses were performed using Design-Expert® 9.0.6.2. Model adequacy was assessed via ANOVA, coefficient of determination (R^2 and adjusted R^2), coefficient of variation (CV%), lack-of-fit test, and adequate precision. Numerical optimisation was performed to identify the ingredient combination that maximised OA within the experimental domain. Proximate and phytochemical data were analysed using SPSS v22.0 (IBM Corp., USA). All data are presented as mean ± Standard deviation (SD) of triplicates. Statistical significance was defined at $p< 0.05$.

3. Results and Discussion

3.1 Box-Behnken Design Matrix and Overall Acceptability Scores

The 46-run BBD matrix with corresponding OA scores is presented in Table 2. OA scores spanned a wide range, from 5.4 (Run 34: carrot 50 ml, beetroot 62.5 ml, water 25 ml, chia 3.5 g, flax 3.5 g) to 9.0 (six centre-point replicates), indicating a substantive and exploreable response surface across the experimental domain. The consistency of OA = 9.0 across all six centre-point replicates attests to low experimental variability and a well-controlled evaluation procedure. The confirmatory optimal formulation yielded an experimental OA of 9.23, marginally exceeding the centre-point value, validating the predictive integrity of the model.

Table 2: Box-Behnken Design matrix with coded factor levels and corresponding overall acceptability (OA) scores

Run	A: Carrot (ml)	B: Beetroot (ml)	C: Water (ml)	D: Chia (g)	E: Flax (g)	OA
1	62.5	50	37.5	2	3.5	6.9
2	50	62.5	37.5	3.5	2	7.2
3	75	62.5	37.5	3.5	5	7.7
4	62.5	62.5	25	5	3.5	6.5
5	50	75	37.5	3.5	3.5	6.9
6	62.5	62.5	37.5	5	5	6.1
7	62.5	50	37.5	5	3.5	6.8
8	62.5	75	37.5	2	3.5	7.8
9	62.5	62.5	50	5	3.5	7.5
10	50	62.5	37.5	3.5	5	6.1
11	62.5	62.5	37.5	2	2	7.5
12	62.5	62.5	50	3.5	2	7.8
13	62.5	62.5	37.5	3.5	3.5	9.0
14	62.5	50	37.5	3.5	5	7.2
15	75	62.5	37.5	5	3.5	7.5
16	62.5	75	37.5	5	3.5	6.2
17	62.5	62.5	50	2	3.5	8.2
18	62.5	50	37.5	3.5	2	7.9
19	62.5	62.5	25	3.5	5	5.9
20	62.5	50	50	3.5	3.5	7.3
21	62.5	75	37.5	3.5	5	6.4
22	62.5	50	25	3.5	3.5	5.8
23	62.5	62.5	25	2	3.5	6.7
24	62.5	75	37.5	3.5	2	8.1
25	62.5	62.5	37.5	3.5	3.5	9.0
26	62.5	62.5	37.5	3.5	3.5	9.0
27	50	62.5	37.5	5	3.5	5.8
28	75	62.5	37.5	3.5	2	7.9
29	75	62.5	50	3.5	3.5	8.5
30	62.5	62.5	37.5	3.5	3.5	9.0
31	75	62.5	37.5	2	3.5	7.8
32	50	62.5	50	3.5	3.5	7.1
33	50	50	37.5	3.5	3.5	6.6
34	50	62.5	25	3.5	3.5	5.4
35	62.5	62.5	50	3.5	5	7.6
36	62.5	62.5	37.5	5	2	6.8
37	50	62.5	37.5	2	3.5	6.2
38	62.5	75	50	3.5	3.5	7.2
39	75	50	37.5	3.5	3.5	7.4
40	62.5	62.5	37.5	3.5	3.5	9.0
41	62.5	62.5	37.5	3.5	3.5	9.0
42	75	75	37.5	3.5	3.5	8.5
43	62.5	75	25	3.5	3.5	7.3
44	62.5	62.5	37.5	2	5	6.4
45	75	62.5	25	3.5	3.5	7.8
46	62.5	62.5	25	3.5	2	7.1

3.2 Model Fitting and Statistical Adequacy:

ANOVA results for the fitted second-order polynomial quadratic model are summarised in Table 3. The overall model F-value of 17.71 (*p*< 0.0001) confirmed that the model explained a statistically significant proportion of total variation in OA, with less than 0.01% probability that this F-value could arise by chance. The coefficient of determination $R^2 = 0.93$ indicated that the model accounted for 93.4% of total OA variability, with the remaining 6.6% attributable to experimental noise. The adjusted R^2 (0.87) and the close agreement between R^2 and adjusted R^2 (difference < 0.20) confirmed no evidence of model overfitting [16]. The adequate precision value of 15.70, substantially exceeding the minimum recommended threshold of 4.0, demonstrated an acceptable signal-to-noise ratio and confirmed the model's utility for navigating the design space. The non-significant lack-of-fit (p = 0.336 > 0.05) further corroborated the model's predictive validity. The low coefficient of variation (CV% = 3.46) attested to the precision and reproducibility of the sensory evaluation procedure.

Among the linear terms, carrot juice (A; F = 76.52, *p*< 0.0001) and water (C; F = 41.59, *p*< 0.0001) were highly significant, followed by flaxseeds (E; F = 23.94, *p*< 0.0001) and chia seeds (D; F = 10.21, p = 0.0038). Beetroot juice (B; F = 4.31, p = 0.0481) also reached statistical significance. Among interaction terms, BC (beetroot × water; p = 0.0257) and BD (beetroot × chia seeds; p = 0.0354) were significant. All quadratic terms were highly significant (*p*< 0.0001), with D^2 (chia seeds; F = 106.69) exhibiting the largest quadratic coefficient, underscoring the heightened sensitivity of OA to chia seed concentration beyond the optimal threshold.

Table 3: ANOVA for the second-order polynomial quadratic model fitted to overall acceptability (OA)

Source	SS	df	MS	F-value	p-value	Significance
Model	40.28	20	2.014	17.71	< 0.0001	***
A - Carrot juice	8.70	1	8.70	76.52	< 0.0001	***
B - Beetroot juice	0.49	1	0.49	4.31	0.0481	*
C - Water	4.73	1	4.73	41.59	< 0.0001	***
D - Chia seeds	1.16	1	1.16	10.21	0.0038	**
E - Flaxseeds	2.72	1	2.72	23.94	< 0.0001	***
AB	0.16	1	0.16	1.41	0.2467	NS
AC	0.25	1	0.25	2.20	0.1507	NS
AD	0.003	1	0.003	0.02	0.8833	NS
AE	0.20	1	0.20	1.76	0.1967	NS
BC	0.64	1	0.64	5.63	0.0257	*
BD	0.56	1	0.56	4.93	0.0354	*
BE	0.25	1	0.25	2.20	0.1507	NS
CD	0.06	1	0.06	0.53	0.4760	NS
CE	0.25	1	0.25	2.20	0.1507	NS
DE	0.04	1	0.04	0.35	0.5609	NS
A^2	6.62	1	6.62	58.19	< 0.0001	***
B^2	6.75	1	6.75	59.38	< 0.0001	***
C^2	7.53	1	7.53	66.25	< 0.0001	***
D^2	12.13	1	12.13	106.69	< 0.0001	***
E^2	7.53	1	7.53	66.25	< 0.0001	***
Residual	2.84	25	0.1136	—	—	
Lack of Fit	2.84	20	0.1420	—	0.336 (NS)	
Total	43.12	45	—	—	—	

NS = not significant (*p*> 0.05); * *p*< 0.05; ** *p*< 0.01; *** *p*< 0.001. $R^2 = 0.9341$; Adj. $R^2 = 0.8761$; CV% = 3.46; Adequate Precision = 15.70.

3.3 Regression Equation and Coefficient Interpretation

The final second-order polynomial model for OA in terms of coded factors is given in Equation 2:

$$OA = 9.00 + 0.74A + 0.17B + 0.54C - 0.27D - 0.41E + 0.20AB - 0.25AC + 0.025AD + 0.22AE - 0.40BC - 0.37BD - 0.32BE - 0.12CD + 0.25CE + 0.10DE - 0.86A^2 - 0.90B^2 - 0.92C^2 - 1.17D^2 - 0.95E^2 \quad (2)$$

where A = carrot juice, B = beetroot juice, C = water, D = chia seeds, E = flaxseeds (all in coded units).

The positive linear coefficients for carrot juice (A, +0.74) and water (C, +0.54) indicate that increasing these variables from low to mid-level positively enhanced hedonic acceptability. Carrot juice was the dominant positive linear driver of OA, consistent with its characteristic sweet-earthy flavour profile, vibrant orange pigmentation from β-carotene, and the perceived freshness it imparts to vegetable-based blends [9, 18]. Water at moderate levels diluted the intense betalain earthiness of beetroot without excessive flavour dilution, reaching an optimum at 37.5 ml. Conversely, the negative linear coefficients for chia seeds (D, -0.27) and flaxseeds (E, -0.41) at higher concentrations indicate that excess seed content imparted undesirable textural properties, including a dominant mucilaginous mouthfeel and gritty particle perception, beyond the hedonic optimum [23].

The large and uniformly negative quadratic coefficients (A^2 = -0.86; B^2 = -0.90; C^2 = -0.92; D^2 = -1.17; E^2 = -0.95) confirm pronounced curvilinear relationships for all five factors, confirming the existence of true OA optima within the experimental design space. D^2 was the largest quadratic coefficient (-1.17), reflecting the exceptional sensitivity of sensory acceptability to chia seed concentration: at concentrations below 3.5 g, the smoothie lacked desired viscosity and mouthfeel body, while concentrations above 3.5 g resulted in excessive particle loading and mucilaginous texture. The significant negative interaction term BC (-0.40) indicates that high beetroot juice combined with elevated water volume produced compounding flavour dilution and amplified betalain earthiness, adversely affecting OA. Similarly, the BD interaction (-0.37) suggests that the combination of high beetroot and high chia concentrations produced an unacceptably dense, earthy beverage with reduced palatability.

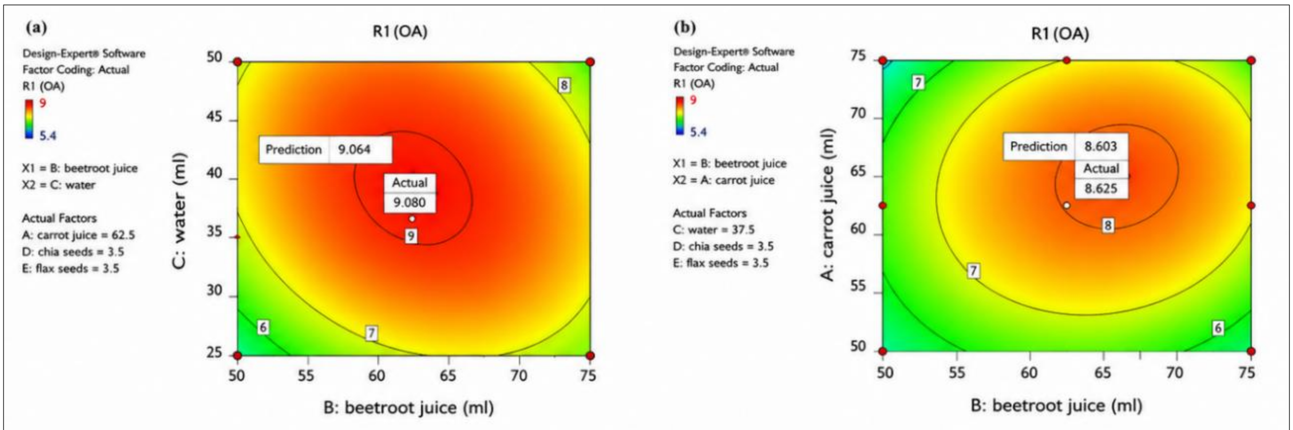
3.4 Response Surface and Contour Plot Analysis

Three-dimensional response surface and two-dimensional contour plots were generated for pairwise factor combinations while maintaining remaining factors at their centre-point values. All response surfaces exhibited concave, dome-shaped profiles, universally confirming a single global maximum within the experimental space, consistent with the statistical significance of all quadratic terms.

The beetroot vs. water surface (Fig. 1a) demonstrated that OA increased as beetroot juice rose from 50 ml towards 62.5 ml when water was maintained at 37.5 ml, yielding a peak predicted OA of 9.064 (actual: 9.080). Increases in either factor beyond their respective optima produced consistent OA declines, attributable to intensification of betalain-derived earthiness at high beetroot levels and excessive flavour dilution at elevated water volumes [11].

The beetroot vs. carrot juice surface (Fig. 1b) demonstrated a symmetric dome-shaped response with peak OA at A = B = 62.5 ml (predicted: 8.603; actual: 8.625). The synergistic flavour complementarity at this equimolar ratio reflects the well-documented masking of betalain earthiness by the sweetness and carotene-dominated flavour of carrot juice [10]. This sensory balance is particularly important for consumer acceptance of beetroot-containing products, in which the characteristic geosmin-like note is a major palatability barrier.

The beetroot vs. flaxseed (Fig. 1c) and beetroot vs. chia seed (Fig. 1d) surfaces both displayed bell-shaped optima at seed concentrations of 3.5 g each (predicted OA: 9.045 and 9.019; actual: 9.055 and 9.021, respectively). These results corroborate the dual role of seeds in smoothie quality: at the 3.5 g level, hydrated chia and flaxseeds provide desirable body and mouthfeel without the negative textural effects (excessive viscosity, particle load) observed at higher concentrations. Chia seed mucilage, composed predominantly of xylose-based heteropolysaccharide, forms a stable hydrocolloid network at intermediate hydration ratios that positively modulates mouthfeel and perceived creaminess [23].



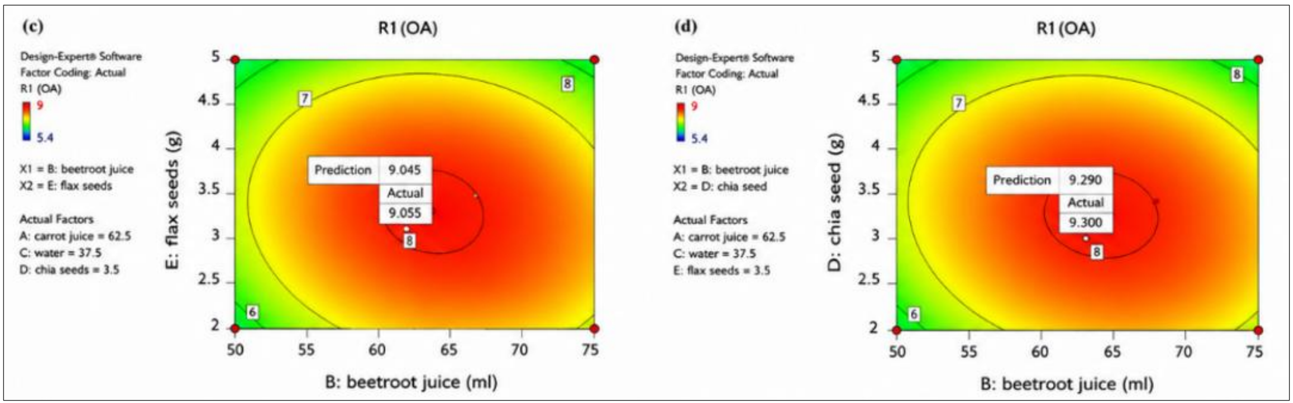


Fig 1: Contour plots showing the interactive effects of beetroot juice and other formulation variables on the overall acceptability of the functional smoothie: (a) beetroot juice vs. water; (b) beetroot juice vs. carrot juice; (c) beetroot juice vs. chia seed; and (d) beetroot juice vs. flaxseed. The contour plots illustrate the response surface generated by the Box-Behnken design, indicating the optimum region for maximum sensory acceptability

3.5 Numerical Optimisation and Confirmatory Validation

Numerical optimisation generated five candidate formulations, all achieving a composite desirability score of 1.000 (Table 4). Solution 4 (carrot juice 62.5 ml, beetroot juice 62.5 ml, water 37.5 ml, chia seeds 3.5 g, flaxseeds 3.5 g; corresponding to the geometric centre of the experimental design) was selected for confirmatory validation based on its practical ingredient quantities, design-space centrality, and ease of reproducible scale-up. The confirmatory smoothie, prepared in triplicate, yielded a mean experimental OA of 9.23±0.15 against the model-predicted value of 9.00, representing a prediction error of 2.56%, well within the accepted ≤5% threshold for RSM validation studies [24]. This close agreement confirms the adequacy of the model and the robustness of BBD-based RSM as a framework for multi-ingredient functional beverage optimisation.

Table 4: RSM-generated optimisation solutions with maximum desirability for functional smoothie formulation

No.	Carrot (ml)	Beetroot (ml)	Water (ml)	Chia (g)	Flax (g)	Predicted OA	Desirability
1	64.22	61.63	43.82	2.90	3.20	9.052	1.000
2	70.11	60.70	40.65	3.13	3.82	9.025	1.000
3	67.53	67.42	35.94	3.26	2.68	9.089	1.000
4†	62.50	62.50	37.50	3.50	3.50	9.000	1.000
5	65.79	66.20	42.66	3.06	2.78	9.099	1.000

†Solution 4 selected for confirmatory validation. Experimental OA = 9.23±0.15; prediction error = 2.56%.

3.6 Multi-Attribute Sensory Profile of the Optimised Smoothie

Beyond the RSM-directed OA scoring, the validated optimal formulation was evaluated for individual sensory attributes (Table 5). Appearance scored highest (8.80±0.18), reflecting the visually attractive deep-orange/magenta hue contributed by the synergistic blend of β-carotene from carrot and betacyanin from beetroot. Flavour (8.70±0.25) and texture/mouthfeel (8.60±0.20) also received high scores, indicating that the equimolar carrot-beetroot ratio effectively balanced sweetness and earthiness, while seed concentrations of 3.5 g each contributed positive mouthfeel without excessive viscosity. Aroma (8.40±0.30) reflected the fresh vegetal-fruity notes characteristic of the formulation. The individual OA score from this evaluation (8.60±0.23) was slightly lower than the RSM-panel's confirmatory score (9.23), attributable to the more formal, attribute-specific evaluation protocol which typically yields more conservative scores. These results are consistent with those reported for red vegetable smoothies by Rodríguez-Verástegui *et al.* [11], who reported hedonic scores of 7.8-9.0 for comparable formulations.

Table 5: Multi-attribute sensory profile of the optimised functional smoothie (9-point hedonic scale; n = 10, triplicate)

Sensory Attribute	Score (mean ± SD)
Colour	8.50±0.22
Appearance	8.80±0.18
Aroma	8.40±0.30
Flavour	8.70±0.25
Texture / Mouthfeel	8.60±0.20
Overall Acceptability	8.60±0.23

3.7 Instrumental Colour Characteristics

Hunter Lab colorimetric data for the optimised smoothie are presented in Table 6. The L* value of 32.64±0.41 indicates a moderately dark product, consistent with the dense pigmentation contributed by both carrot (β-carotene) and beetroot (betalains). The positive a* value (12.43±0.32) confirms the reddish hue imparted predominantly by betacyanin pigments from beetroot, while the positive b* value (8.21±0.28) reflects the yellow-orange contribution of carotenoids from carrot. The chroma value (C* = 14.90±0.35) denotes vivid, saturated colour, and the hue angle of 33.51° places the product firmly in the orange-red colour space, consistent with the qualitative appearance described by panellists. These values are in close agreement with instrumental colour data reported for analogous red vegetable smoothies [11] (L* = 30.2, a* = 14.1, b* = 7.9), providing independent validation of the characterisation data. The moderately low L* value is advantageous from a consumer perception standpoint, as deeper, more saturated red-orange colours are strongly associated with heightened antioxidant content and health connotations in functional beverages [18].

Table 6: Instrumental colour characteristics (CIE L*a*b*) of the optimised functional smoothie (n = 3, mean ± SD)

Parameter	Value (mean ± SD)
L* (Lightness)	32.64±0.41
a* (Redness)	12.43±0.32
b* (Yellowness)	8.21±0.28
Hue angle h° (°)	33.51±0.80
Chroma C*	14.90±0.35

3.8 Rheological Properties

The rheological properties of the optimised smoothie are presented in Table 7. The flow behaviour index n = 0.82±0.03 (< 1.0) unambiguously confirms pseudoplastic (shear-thinning) behaviour, whereby apparent viscosity decreases with increasing shear rate. This is a technologically desirable property in functional beverages: at the low shear rates encountered during pouring and packaging, the product maintains sufficient body and suspension stability; at the higher shear rates encountered during oral processing, viscosity decreases to facilitate ease of swallowing. The pseudoplastic character is primarily attributable to the structured colloidal networks formed by the hydrated mucilage of chia and flaxseeds in the aqueous phase [23]. The apparent viscosity of 285.40±5.20 mPa·s is within the range typically regarded as optimal for ready-to-drink smoothies (150-400 mPa·s), providing palatable mouthfeel and adequate particulate suspension without excessive thickness [6]. The density of 1.05±0.02 g/ml slightly exceeds water, reflecting the dissolved solids contribution from juice components, yoghurt, and seed-derived polysaccharides and proteins.

Table 7: Rheological properties of the optimised functional smoothie (Ostwald-de Waele power law model; 25°C; n = 3, mean ± SD)

Parameter	Value (mean ± SD)
Apparent viscosity (mPa·s)	285.40±5.20
Flow behaviour index (n)	0.82±0.03
Consistency coefficient K (Pa·s^n)	1.24±0.08
Density (g/ml)	1.05±0.02

3.10 Physicochemical Properties

Physicochemical parameters of the freshly prepared optimised smoothie are presented in Table 8. The pH of 4.43±0.02 is consistent with the acidic matrix contributed by the yoghurt component and organic acids from carrot and beetroot juices. This moderately acidic pH (< 4.5) is technologically important as it inhibits the growth of most spoilage bacteria and enteric pathogens, constituting a pH hurdle to microbial proliferation in addition to refrigerated storage [25]. TSS of 13.22±0.23 °Brix reflects the combined contribution of natural sugars from carrot juice, beetroot juice, and banana.

The ascorbic acid content (230±0.03 mg/kg FW) is particularly noteworthy, representing a substantial contribution towards the recommended daily intake (75-90 mg/day). Comparable ascorbic acid values (216-229 mg/kg FW) have been reported for fruit-vegetable smoothies [11]. Ascorbic acid serves not only as a micronutrient but also as an intrinsic antioxidant that protects carotenoids and betalains from oxidative degradation during storage, thereby contributing to both nutritional quality and colour stability of the product [26].

Table 8: Physicochemical properties of the freshly prepared optimised functional smoothie (n = 3, mean ± SD)

Parameter	Value (mean ± SD)	Unit
pH	4.43±0.02	—
Titratable acidity (TA)	0.40±0.04	mg malic acid/100 g FW
Total soluble solids (TSS)	13.22±0.23	°Brix
Ascorbic acid	230±0.03	mg/kg FW

3.11 Phytochemical Profile and Antioxidant Activity:

The phytochemical composition of the optimised smoothie is presented in Table 9. Total phenolic content (TP = 135.33±2.51 mg GAE/kg FW) is primarily attributable to the polyphenolic profile of flaxseeds (lignans, hydroxycinnamic acids; 440 mg/100 g as reported by Meenu, 2010 [27]) and carrot juice (hydroxycinnamic acids, caffeic acid derivatives), supplemented by phenolics from quinoa, beetroot, and yoghurt. This value is consistent with the range of 100-200 mg GAE/100 g FW reported for vegetable-based smoothies and juices in the literature [7, 11].

Total carotenoid content (TC = 50.5±0.02 mg/kg FW) reflects the dominant contribution of β-carotene from carrot juice, supplemented by minor contributions of lutein, lycopene-precursors, and xanthophylls. Carrot is the richest commercially accessible dietary source of β-carotene (5480-6000 μg/100 g), and its concentrated inclusion at 62.5 ml per serving ensures a meaningful provitamin A contribution. Total carotenoid values comparable to those reported in the present study (52.5 mg/kg FW) were previously reported for red vegetable smoothies [27].

The DPPH radical scavenging activity (84±0.02% RSA) indicates high intrinsic antioxidant capacity, driven synergistically by phenolics, carotenoids, betalains, ascorbic acid, and vitamin E from seeds. This value substantially exceeds the 59.95% RSA reported for flaxseed alone [27] and the range of 55-75% RSA typically reported for single-vegetable juices [26], underscoring the synergistic antioxidant benefits of the multi-ingredient formulation. Antioxidant synergism between different classes of phytochemicals — wherein water-soluble antioxidants (ascorbic acid, betalains, phenolics) and lipid-soluble antioxidants (β-carotene, vitamin E from seeds) act complementarily across hydrophilic and lipophilic phases — is a well-established principle in functional food science [7].

Total flavonoids (TF = 40±0.04 mg/kg FW) contribute to the overall polyphenol burden and are known to possess anti-inflammatory, anti-thrombotic, and cardioprotective activities through mechanisms including inhibition of LDL oxidation and modulation of eicosanoid biosynthesis [1]. The quinoa component is a particularly relevant flavonoid source [14], while beetroot contributes isorhamnetin and other flavonoids alongside betalains [28].

Table 9: Phytochemical composition and antioxidant activity of the optimised functional smoothie (n = 3, mean ± SD)

Parameter	Value (mean ± SD)	Unit
Total phenols (TP)	135.33±2.51	mg GAE/kg FW
Total carotenoids (TC)	50.5±0.02	mg/kg FW
Antioxidant activity (DPPH%RSA)	84±0.02	% RSA
Total flavonoids (TF)	40±0.04	mg/kg FW

3.12 Microbiological Safety

The microbiological safety profile of the freshly prepared optimised smoothie (day 0) is presented in Table 10. The total plate count (2.26±0.12 log CFU/g) was well within the acceptable microbiological limits for ready-to-eat beverages stipulated by FSSAI regulations (≤5 log CFU/g). Yeast and mould counts were below the limit of detection (<10 CFU/g), indicating excellent sanitation during preparation. The complete absence of coliforms, Staphylococcus aureus, and Escherichia coli confirmed the microbiological safety of

the freshly prepared product and validated the adequacy of the hygienic production procedure.
The inherent pH of 4.43 functions as a natural hurdle to microbial proliferation, as acidic conditions below pH 4.5 effectively inhibit the growth of most enteric pathogens and significantly retard spoilage microorganisms [25]. The combined effect of low pH (from yoghurt-derived lactic acid and juice organic acids), cold-press processing (which avoids heat-induced destruction of protective phytochemicals while limiting microbial loading), and immediate refrigerated storage constitutes a multi-hurdle preservation approach [25]. These results are consistent with TPC values of 2.0-2.5 log CFU/g reported for freshly prepared vegetable smoothies [5] using analogous processing conditions.

Table 10: Microbiological profile of the freshly prepared optimised functional smoothie (day 0; n = 3)

Microbiological Parameter	Result	FSSAI Limit (RTE beverages)
Total Plate Count (log CFU/g)	2.26±0.12	≤5 log CFU/g
Yeast and Mould Count (CFU/g)	< 10 (BDL)	≤50 CFU/g
Coliform Count	Absent	Absent
Staphylococcus aureus	Absent	Absent
Escherichia coli	Absent	Absent

BDL = below detection limit.

3.13 Storage Stability and Shelf-Life Determination

Changes in physicochemical, microbiological, and sensory attributes of the optimised smoothie during refrigerated storage at 4°C are presented in Table 11. The pH declined progressively from 4.43±0.02 on day 0 to 4.15±0.04 on day 10, attributable to the continued fermentative activity of lactic acid bacteria (LAB) derived from the yoghurt component, generating organic acids during cold storage. Correspondingly, titratable acidity increased from 0.40±0.04 to 0.63±0.04 mg malic acid/100 g FW. Similar acidification trends have been reported for yoghurt-containing vegetable smoothies [11] and are well-documented for probiotic dairy products under refrigeration. Total soluble solids remained remarkably stable across the storage period (13.22 to 12.98 °Brix), indicating negligible hydrolysis of dissolved macromolecules and confirming the physicochemical robustness of the matrix.
The TPC increased from 2.26±0.12 log CFU/g on day 0 to 3.45±0.18 log CFU/g on day 10. While this increase is attributable primarily to LAB proliferation from the yoghurt component (a non-pathogenic probiotic contribution), TPC remained within the FSSAI-acceptable limit of ≤5 log CFU/g throughout the entire 10-day observation period. OA scores declined from 8.60±0.15 on day 0 to 6.80±0.30 on day 10. Scores remained above the hedonic acceptability threshold of 7.0 (“like moderately”) up to day 8 (OA = 7.21±0.28), at which point progressive acidification and subtle off-note development began reducing palatability. From a combined microbiological and sensory perspective, the shelf life of the optimised smoothie under refrigerated conditions (4°C, sealed glass packaging) was determined to be 8 days. This is consistent with shelf-life values of 7-9 days reported for minimally processed fruit-vegetable smoothies [29] stored at 4°C, and represents a commercially viable shelf life for a refrigerated functional beverage category.

Table 11: Changes in physicochemical, microbiological, and sensory attributes of the optimised smoothie during refrigerated storage at 4°C (n = 3, mean ± SD)

Storage Day	pH	TA (mg/100 g FW)	TSS (°Brix)	TPC (log CFU/g)	OA Score
0	4.43±0.02	0.40±0.04	13.22±0.23	2.26±0.12	8.60±0.15
2	4.39±0.02	0.43±0.03	13.19±0.20	2.35±0.10	8.90±0.18
4	4.34±0.03	0.47±0.04	13.15±0.18	2.52±0.13	8.45±0.22
6	4.28±0.03	0.52±0.03	13.10±0.21	2.78±0.15	7.86±0.25
8	4.21±0.04	0.57±0.05	13.05±0.19	3.12±0.14	7.21±0.28
10	4.15±0.04	0.63±0.04	12.98±0.22	3.45±0.18	6.80±0.30

Shelf life determined as 8 days based on OA ≥ 7.0 and TPC ≤ 5 log CFU/g criteria.

4. Conclusion

This study demonstrates, for the first time, the integrated development, statistical optimisation, and comprehensive multi-attribute characterisation of a novel functional smoothie formulated with carrot-beetroot juice, chia seeds, flaxseeds, yoghurt, quinoa, and oats. The five-factor Box-Behnken Design-based RSM produced a high-fidelity quadratic model (R^2 = 0.93; adequate precision = 15.70) that accurately mapped the complex OA response surface. Carrot juice concentration emerged as the dominant positive linear driver of hedonic acceptability, while all five formulation variables exhibited significant curvilinear behaviour, confirming true optima within the design space. The validated optimal formulation (carrot juice 62.5 ml, beetroot juice 62.5 ml, water 37.5 ml, chia seeds 3.5 g, flaxseeds 3.5 g) achieved a confirmatory OA of 9.23 with a prediction error of <3%, validating the RSM framework for this application.
Comprehensive characterisation of the optimised formulation revealed a nutritionally compelling functional beverage profile: low-to-moderate caloric density (84.42 kcal/100 ml), meaningful protein content (3.36 g/100 ml), high dietary fibre (1.61 g/100 ml), rich phytochemical profile (TP: 135.33 mg GAE/kg; TC: 50.5 mg/kg; DPPH activity: 84% RSA), vibrant orange-red colour (hue angle: 33.51°), and desirable pseudoplastic rheology (n = 0.82) that facilitates pour-ability and mouthfeel. Microbiological safety was confirmed at day 0, and the product maintained both sensory acceptability (OA > 7.0) and microbiological compliance throughout an 8-day refrigerated shelf life.
The multi-hurdle stability conferred by low pH (4.43), cold-press processing, refrigeration, and the intrinsic antimicrobial properties of phytochemicals represents a clean-label preservation strategy of growing commercial relevance. Collectively, these findings position the optimised smoothie as a phytochemically diverse, sensorially superior, and practically reproducible functional beverage with evidence-based potential for delivery of cardioprotective, anti-inflammatory, and antioxidant bioactives.
Future investigations should encompass: (i) *in vitro* bioaccessibility and cellular antioxidant activity assessment

of key bioactives; (ii) clinical evaluation of the formulation's impact on postprandial antioxidant status, glycaemic response, and inflammatory biomarkers; (iii) extended shelf-life profiling using modified atmosphere packaging, mild HPP, or UV-C pre-treatment; (iv) consumer acceptability studies with diverse demographic panels; and (v) environmental life-cycle analysis to assess the sustainability footprint of the multi-ingredient formulation at commercial scale.

5. Acknowledgements

The authors acknowledge the technical support provided by the Department of Food Technology, Guru Jambheshwar University of Science & Technology, Hisar, India, and extend sincere thanks to the sensory panel members for their participation and cooperation during evaluation sessions. The authors acknowledge the analytical support of the Food Analysis Laboratory, GJUS&T. This research received no specific external funding from public, commercial, or not-for-profit funding agencies. The authors declare no conflict of interest. Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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