Enhancement of Iron Bioavailability in Cold Plasma-Treated and Iron-Fortified *Cicer arietinum L.* Through Organic Acid Soaking

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Abstract

Iron deficiency anaemia (IDA) remains one of the most prevalent micronutrient deficiencies globally, disproportionately affecting women of reproductive age and children in low- and middle-income countries. *Cicer arietinum* L. (chickpea) is a promising carrier for iron fortification; however, bioavailability is constrained by antinutritional factors such as polyphenols and phytic acid. This study evaluated a two-stage strategy combining cold atmospheric plasma (CAP) pre-treatment (9.5 kV and 11.5 kV, 30 kHz, 5 min) with organic acid soaking (citric acid 1 g/250 mL; ascorbic acid 0.1% w/v, 10 h) to enhance iron fortification of chickpea seeds using Na[Fe(EDTA)] or $FeSO_4 \cdot 7H_2O$ (200–4200 µg g^{-1}). Organic acid soaking marginally reduced phenolic content (2.4–5.6%), antioxidant activity (1.0–4.8%), and lightness (Δ1.15–1.60), while significantly reducing lipoxygenase activity (13–30%) and preserving iron retention. Importantly, in vitro iron bioavailability improved substantially: post-soaking values for Na[Fe(EDTA)] reached 11.42–17.94% (9.5 kV) and 10.82–15.88% (11.5 kV), representing 36–58% and 25–44% improvements over pre-soaking values, respectively. For $FeSO_4 \cdot 7H_2O$, improvements ranged from 32–46%. These findings demonstrate that CAP-assisted iron fortification combined with organic acid soaking offers an effective, scalable strategy for developing iron-enriched pulse-based foods to address iron deficiency.

Keywords - *Cold atmospheric plasma; Iron fortification; Chickpea.; Iron bioavailability; Antinutritional factors*

1. Introduction

Iron Deficiency Anaemia (IDA) continues to be one of the most common micronutrient deficiencies worldwide. In the Global Burden of Disease 2021, anaemia was identified as one of the most important causes of years lived with disability, with the predominant cause being iron deficiency, affecting an estimated 1.2 billion people, largely women of reproductive age and children in South Asian and sub-Saharan African countries [1] [2]. The inability to absorb the iron in the diet is the most important cause in these populations; in these populations, the plant-based diet is predominant and rich in antinutrient compounds that form insoluble complexes in the gut [3] [4].

Chickpea is one of the most important grain legumes of the world and is widely grown in South Asia, the Middle East and sub-Saharan Africa. It is a nutritious food, providing protein (18–23 %), dietary fibre, folate and non-haem iron (~40–100 mg per kg DW) [5]. Yet, the bioavailability of Fe from chickpea is greatly affected by the chelating properties of phytic acid, tannins and polyphenols and the ability to solubilize Fe in the lumen of the gastrointestinal tract [6] [5]. Fortification of chickpeas at the food level may be an effective, low-cost, and culturally acceptable method to reduce IDA in populations that consume chickpeas as a staple food, but the bioavailability of the iron added to the food needs to be optimized. Cold Atmospheric Plasma Jet is an emerging non-thermal technology that utilises the formation of reactive oxygen and nitrogen species (RONS), UV photons and charged particles, which interact with the surface of seeds without significant thermal damage [7] [8]. In legume seeds, CAP has been shown to enhance the surface hydrophilicity, reduce condensed tannins and phytic acid, inactivate oxidative enzymes like lipoxygenase, and alter cell wall structure that could enable iron uptake during fortification [9] [10] [11]. The surface modification, especially the etching of the seed coat and the introduction of oxygenated functional groups, provides a plausible basis to explain the improved iron absorption from fortified seeds in a mechanical manner. Organic acids are known to increase the absorption of non-haem iron. Ascorbic acid will reduce Fe3+ to Fe2+ and chelate iron in a soluble form that is not readily precipitated at the pH of the upper gastrointestinal tract, and citric acid forms soluble iron-citrate complexes and helps prevent phytate-induced inhibition in the upper gastrointestinal tract [12] [13]. A rationally designed dual-stage intervention, combining a

CAP pre-treated structurally modified seed matrix with a chelating acidic soaking medium, is therefore the combined application of two complementary mechanisms of iron bioavailability enhancement. The present study extends this line of inquiry by quantifying iron concentration and in vitro bioavailability in seeds treated at two fixed CAP regimes (9.5 kV and 11.5 kV, 30 kHz, 5 min) based on preliminary optimisation trials with the same argon plasma jet reactor, wherein voltages below 9 kV produced insufficient RONS to modify seed coat permeability and voltages above 12 kV caused structural seed damage, consistent with the effective operating window of 7–15 kV reported in the CAP literature [14] [11] confirmed that plasma intensity within this range progressively enhances seed coat permeability and water absorption kinetics in chickpea, while [10] demonstrated that moderate CAP intensities effectively reduced phytic acid and tannins in legume seeds without compromising nutritional quality. Later the CAP treated seeds were fortified with Na[Fe(EDTA)] or $FeSO_4 \cdot 7H_2O$ at concentrations of 200–4200 µg/g before and after organic acid soaking, while also characterizing the effects of soaking on total phenolic content, antioxidant activity, surface colour (L*), and lipoxygenase activity, and comparing the performance of both iron sources across all treatment regimes.

2. Materials and Methods

2.1 Plant Material and Plasma Treatment

Chickpea (*Cicer arietinum* L.) variety PUSA 3062 seeds, previously subjected to cold atmospheric plasma (CAP) treatment and iron fortification, were used as the baseline material for this study. [14] [11]. A Cold Atmospheric Plasma Jet reactor with argon as the plasma gas was operated under two optimised voltage conditions: 9.5 kV (CPT 1) and 11.5 kV (CPT 2) at a fixed frequency of 30 kHz for a treatment duration of 5 minutes. Following plasma exposure, seeds were independently fortified with two chemically distinct iron sources — ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) (IS 1), an inorganic iron salt, and sodium iron ethylenediaminetetraacetate (Na[Fe(EDTA)]) (IS 2), an organic iron chelate — each applied separately at iron concentrations ranging from 200 to 4200 µg/g. Both iron sources were evaluated in parallel across all CAP treatment conditions to allow a direct comparison of their fortification efficiency, iron retention, and influence on physico-chemical parameters. The plasma-treated and iron-fortified seeds prepared with each of the two iron sources were subsequently used as the control material to assess the independent and comparative effects of organic acid soaking on iron concentration and related quality attributes.[15]

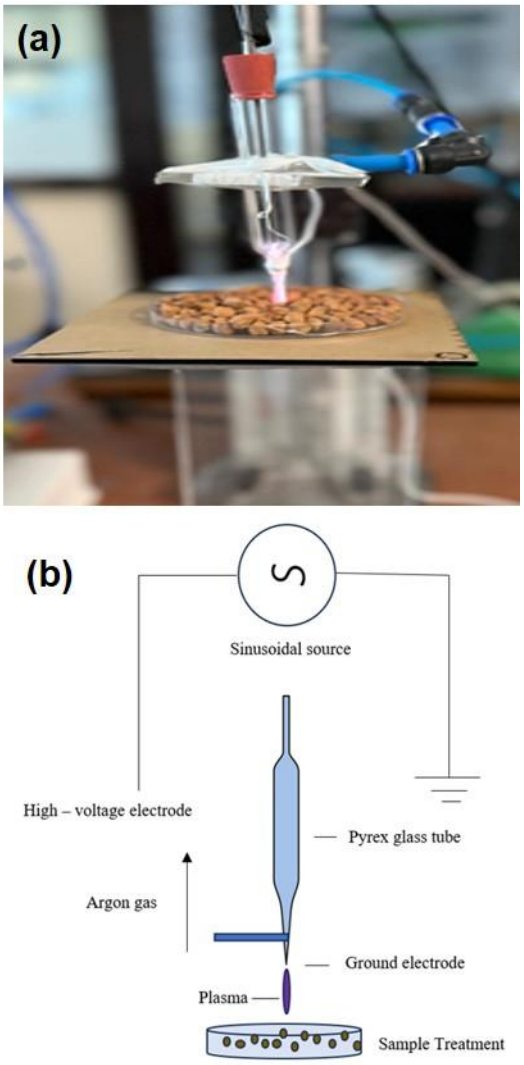

Fig 1. (A) Diagrammatic representation of Cold Atmospheric Plasma Setup, (B) Cold Atmospheric Plasma with sample treatment.

2.2 Preparation of Citric–Ascorbic Acid Soaking Solution

The soaking solution was prepared fresh for each experiment to minimize the oxidation of ascorbic acid. Food-grade citric acid (1 g) and ascorbic acid were dissolved in 250 mL of distilled water under continuous magnetic stirring until complete dissolution, resulting in a citric acid concentration of 4 g/L and an ascorbic acid concentration of 0.1% (w/v). These concentrations were selected based on established literature, which indicates that 0.1% (w/v) ascorbic acid is the minimum effective concentration required for the reduction of Fe^{3+} to Fe^{2+} and the formation of soluble iron–ascorbate complexes, thereby enhancing iron bioavailability [12]. Similarly, a citric acid concentration of 4 g/L falls within the optimal range of 2–6 g/L reported to competitively inhibit phytate–iron binding in legume-based food systems [4], citric acid acted as an iron absorption enhancer, while ascorbic acid functioned both as an iron absorption

promoter and a reducing agent, converting Fe^{3+} to the more bioavailable Fe^{2+} form in the soaking medium.

2.3 Post-Fortification Soaking and Sample Processing

The seeds of chickpeas were soaked in plasma-treated and iron-fortified freshly prepared citric–ascorbic acid soaking solution in a seed-to-soaking solution ratio such that the seed surface was completely submerged and had uniform exposure to the solution. Soaking was carried out for 10 hours at ambient temperature (25 ± 2°C) with static conditions. After soaking, the seeds were drained, excess surface solution removed by gently blotting with sterile filter paper and then dried in a hot air oven, 50–55°C, until they reached a constant weight. Dried seeds were finely ground with a laboratory mixer-grinder to make a fine flour. All flour samples were stored in airtight containers at 4°C until subsequent analyses were performed. Samples collected immediately after iron fortification but before soaking were designated as pre-soaking samples, while those processed through the complete soaking protocol were designated as post-soaking samples [16].

2.5 Analytical Methods

2.5.1 Iron Concentration

Total iron was quantified by atomic absorption spectrophotometry (AAS) following Hot plate acid digestion (HNO_3–$HClO_4$, 3:1, 180 °C, 20 min). Digests were diluted to 25 mL with 1% HNO_3 and analysed against certified iron standards. Results are expressed as mg iron/100 g dry weight (mg/100 g). All analyses were performed in triplicate (n = 3) [17]

Iron concentration was calculated as:

$$Iron\ (mg/100\ g) = \frac{A \times F \times V}{W} \quad (1)$$

Where:
A = absorbance reading from AAS
F = calibration factor derived from the standard curve
V = final volume of digest (mL)
W = sample weight (g)

2.5.2 In Vitro Iron Bioavailability

In vitro iron bioavailability was estimated using a simulated gastrointestinal pepsin-pancreatin dialysis model [18]. Dialysable iron in the dialysate fraction was quantified by AAS and expressed as the percentage of total iron transferred across the dialysis membrane (% dialysable iron).

$$Iron\ Bioavailability\ (\%) = \frac{100 \times Y}{Z} \quad (2)$$

Where:
Y = iron content in dialysate (mg/100 g)
Z = total iron content (mg/100 g)

2.5.3 Surface Colour (L)*

Colour parameters were measured by using a CR-6 calibrated colourimeter, and presented in the following colour scale: Colour space CIE Lab*. The powdered samples were put in a uniform layer in a petri dish; Standardized lighting was used for the measurements. The instrument was calibrated using a white reference plate (L* = 97.17, a* = 0.11, b* = 1.06). L* represents lightness (0 = black, 100 = white); a* is the red–green chromaticity measurement; b* is the yellow–blue chromaticity measurement. Chromaticity [19].

2.5.4 Total Phenolic Content (TPC)

TPC was measured by the Folin-Ciocalteu method [20] [21]. Methanolic extracts (80%, 0.5 g/10 mL, 2 h shaking) were centrifuged (3000 × g, 10 min). Aliquots (0.1 mL) were reacted with Folin-Ciocalteu reagent and 7.5% Na_2CO_3 (2 h, room temperature). Absorbance was read at 760 nm and results expressed as mg gallic acid equivalents per g DW (mg GAE/g DW).

TPC was calculated using the following equation:

$$TPC\ (mg\ GAE/g) = \frac{C \times V}{M} \quad (3)$$

Where:
C = concentration of phenolic compounds in the extract (mg/mL), derived from the gallic acid standard curve
V = total volume of extract (mL)
M = mass of sample used for extraction (g, dry weight basis)

2.5.5 Antioxidant Activity (AOX)

AOX was determined by the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay. Methanolic seed extracts were reacted with 0.1 mM DPPH solution for 30 min in the dark at room temperature. Absorbance was measured at 517 nm and results expressed as % radical scavenging activity relative to a Trolox standard curve [22].

$$\text{Inhibition } (\%) = \frac{A_0 - A_S}{A_0} \times 100 \quad (4)$$

Where:
A_0 = control absorbance
As = sample absorbance
EC50 values were calculated from dose–response curves and expressed as mg extract required to reduce DPPH radical concentration by 50%.

2.5.6 Lipoxygenase (LOX) Activity

LOX activity was determined spectrophotometrically using linoleic acid as substrate (Axelrod et al., 1981). Crude enzyme extract was prepared by homogenising

seeds in 50 mM sodium phosphate buffer (pH 6.5) at 4 °C. Absorbance increase at 234 nm (conjugated diene formation) was monitored for 2 min, and LOX activity was expressed as U min^{-1} g^{-1} fresh weight.

$$LOX\ Activity\ (U/min/g) = \frac{\Delta A_{234}/\Delta t \times V_0 \times V_2}{0.01 \times V_1 \times M} \quad (5)$$

Where ΔA_{234} represents the change in absorbance measured at 234 nm during the reaction, Δt denotes the total reaction time (min), and 0.01 corresponds to the absorbance change defined as one unit of LOX activity per minute. In addition, V_0 is the total reaction volume (mL), V_1 is the volume of enzyme extract used in the assay (mL), V_2 is the total volume of crude enzyme extract prepared (mL), and M is the sample weight (g). The final LOX activity is expressed as $U \cdot min^{-1} \cdot g^{-1}$ [23]

3. Results

3.1 Iron Concentration

The baseline iron concentration of the control and CAP-treated unfortified seeds is presented in Table 1. The control seeds contained 7.40 mg/100 g of iron. The CAP treatment at 9.5 kV (CPT 1) and 11.5 kV (CPT 2) reduced this marginally to 6.90 and 6.83 mg/100 g, respectively. Post-soaking, CAP-only samples did not show measurable iron concentrations (no fortification baseline to report), whereas control seeds did not undergo soaking in the fortification protocol. Tables 2 and 3 present iron concentration data for all fortified treatments before and after organic acid soaking. For Na[Fe(EDTA)]-fortified seeds at 9.5 kV, iron concentrations increased from 8.57 ± 0.08 mg/100 g DW (200 µg/g) to 20.26 ± 0.18 mg/100 g (4200 µg/g) pre-soaking, with post-soaking values marginally lower (8.23–19.34 mg/100 g). $FeSO_4 \cdot 7H_2O$ achieved substantially higher iron concentrations at equivalent concentrations, reaching 55.96 ± 0.18 mg/100 g at 4200 µg/g and 9.5 kV pre-soaking, declining slightly to 55.01 ± 0.22 mg/100g post-soaking. At 11.5 kV, Na[Fe(EDTA)] concentrations were lower (7.43–18.92 mg/100g pre-soaking), while $FeSO_4 \cdot 7H_2O$ remained high (11.88–51.05 mg/100 g). Post-soaking iron losses across all treatments ranged from approximately 0.4 to 4.6% ($p < 0.05$).

Table 1. Iron concentration (mg/100 g) and in vitro iron bioavailability (%) of control and CAP-treated unfortified chickpea seeds before and after organic acid soaking. Values are mean ± SD (n = 3).

Treatment	Iron concentration pre-soaking (mg/100 g)	Iron concentration Post Soaking (mg/100 g)	Bio Pre Soaking (%)	Bio Post Soaking (%)
Control	7.40 ± 0.021	7.08 ± 0.11	3.10 ± 0.12	5.85 ± 0.18
CPT 1	6.90 ± 0.13	6.68 ± 0.13	4.40 ± 0.15	7.92 ± 0.24
CPT 2	6.83 ± 0.19	6.65 ± 0.08	4.10 ± 0.14	7.35 ± 0.22

Table 2. Iron concentration (mg/100 g) of (IS-1) and (IS-2) fortified chickpea seeds at CPT 1 before and after organic acid soaking. Values are mean ± SD (n = 3).

Conc. (µg/g)	IS 1 (CPT 1) Pre	IS 1 (CPT 1) Post	Conc. (µg/g)	IS 2 (CPT 1) Pre	IS 2 (CPT 1) Post
200	8.57±0.08	8.23±0.10	**200**	14.13±0.09	13.62±0.08
700	9.11±0.11	8.88±0.09	**700**	16.93±0.11	16.21±0.10
1200	10.55±0.10	9.89±0.13	**1200**	18.31±0.10	18.03±0.12
1700	12.37±0.14	12.02±0.11	**1700**	23.82±0.14	23.32±0.11
2200	13.81±0.12	13.05±0.15	**2200**	31.96±0.13	31.31±0.15
2700	15.16±0.17	14.86±0.14	**2700**	37.72±0.17	36.98±0.14
3200	17.01±0.15	16.91±0.19	**3200**	43.36±0.16	42.55±0.18
3700	18.01±0.20	17.74±0.16	**3700**	49.09±0.20	48.04±0.17
4200	20.26±0.18	19.34±0.22	**4200**	55.96±0.18	55.01±0.22

Table 3. Iron concentration (mg/100 g) of (IS-1) and (IS-2) - fortified chickpea seeds at (CPT 2) before and after organic acid soaking. Values are mean ± SD (n = 3).

Conc. (µg/g)	IS 1 (CPT 2) Pre	IS 1 (CPT 2) Post	Conc. (µg/g)	IS 2 (CPT 2) Pre	IS2 (CPT 2) Post
200	7.43±0.07	6.98±0.09	**200**	11.88±0.08	11.81±0.09
700	7.98±0.10	7.01±0.08	**700**	13.33±0.10	13.12±0.08
1200	8.89±0.09	8.49±0.11	**1200**	15.43±0.09	15.07±0.11
1700	9.97±0.13	9.04±0.10	**1700**	16.94±0.12	15.86±0.10

2200	11.45±0.12	11.39±0.14	**2200**	21.26±0.13	21.08±0.14
2700	13.44±0.16	12.98±0.13	**2700**	24.32±0.15	23.19±0.13
3200	15.68±0.15	15.08±0.18	**3200**	35.34±0.14	34.87±0.17
3700	16.92±0.19	16.09±0.16	**3700**	41.89±0.19	41.12±0.16
4200	18.92±0.17	18.61±0.21	**4200**	51.05±0.18	50.98±0.21

3.2 Iron Bioavailability

The in vitro iron bioavailability data for all treatments are presented in Table 4. The unfortified control showed pre-soaking bioavailability of 3.10 ± 0.12%, rising significantly to 5.85 ± 0.18% post-soaking. CAP treatment at 9.5 kV increased pre-soaking bioavailability to 4.40 ± 0.15%, with a post-soaking value of 7.92 ± 0.24%; the corresponding values for 11.5 kV were 4.10 ± 0.14% and 7.35 ± 0.22%, respectively. For Na[Fe(EDTA)]-fortified seeds at 9.5 kV, bioavailability increased progressively with fortification concentration: from 7.23 ± 0.23% (pre-soaking, 200 µg/g) to 10.98 ± 0.42% (pre-soaking, 4200 µg/g), with post-soaking values reaching 11.42 ± 0.27% to 17.94 ± 0.51%. At 11.5 kV, post-soaking values for Na[Fe(EDTA)] ranged from 10.82 ± 0.26% (200 µg/g) to 15.88 ± 0.49% (4200 µg/g). $FeSO_4 \cdot 7H_2O$ achieved lower bioavailability: post-soaking values at 9.5 kV ranged from 9.12 ± 0.24% (200 µg/g) to 14.61 ± 0.41% (4200 µg/g); at 11.5 kV, from 9.18 ± 0.25% to 13.92 ± 0.40%. All soaking-induced bioavailability increases were statistically significant ($p < 0.05$).

Table 4. In vitro iron bioavailability (%) of (IS-1 and (IS-2) -fortified chickpea seeds at (CPT 1) and (CPT 2) before and after organic acid soaking. Values are mean ± SD (n = 3).

Conc. (µg/g)	**IS 1 (CPT 1) Pre (%)**	**IS 1 (CPT 1) Post (%)**	**Conc. (µg/g)**	**IS 2 (CPT 1) Pre (%)**	**IS 2 (CPT 1) Post (%)**
200	7.23±0.23	11.42±0.27	**200**	5.56±0.19	9.12±0.24
700	8.26±0.26	12.96±0.30	**700**	6.82±0.23	10.38±0.28
1200	9.00±0.29	14.08±0.33	**1200**	7.71±0.27	11.47±0.31
1700	9.45±0.31	15.22±0.36	**1700**	8.27±0.25	12.26±0.35
2200	9.83±0.33	16.01±0.39	**2200**	8.80±0.30	12.94±0.33
2700	10.16±0.35	16.47±0.42	**2700**	9.17±0.28	13.41±0.39
3200	10.46±0.38	17.08±0.45	**3200**	9.45±0.34	13.88±0.37
3700	10.73±0.40	17.61±0.48	**3700**	9.70±0.31	14.22±0.43
4200	10.98±0.42	17.94±0.51	**4200**	10.36±0.38	14.61±0.41
Conc. (µg/g)	**IS 1 (CPT 2) Pre (%)**	**IS 1 (CPT 2) Post (%)**	**Conc. (µg/g)**	**IS 2 (CPT 2) Pre (%)**	**IS 2 (CPT 2) Post (%)**
200	7.67±0.22	10.82±0.26	**200**	6.27±0.20	9.18±0.25
700	8.72±0.25	12.14±0.31	**700**	7.58±0.24	10.41±0.29
1200	9.51±0.29	13.21±0.34	**1200**	8.40±0.27	11.32±0.32
1700	10.00±0.27	14.06±0.32	**1700**	9.07±0.25	12.04±0.35
2200	10.59±0.34	14.78±0.38	**2200**	9.66±0.31	12.68±0.33
2700	10.95±0.31	15.12±0.42	**2700**	10.08±0.29	13.12±0.39
3200	11.05±0.39	15.41±0.40	**3200**	10.46±0.36	13.54±0.37
3700	10.93±0.36	15.58±0.47	**3700**	10.79±0.34	13.81±0.43
4200	11.03±0.41	15.88±0.49	**4200**	11.03±0.41	13.92±0.40

3.3 Total Phenolic Content (TPC)

TPC values for all selected treatments are presented in Table 5. The unfortified control showed a pre-soaking TPC of 1.1608 ± 0.021 mg GAE/g DW, declining slightly to 1.1326 ± 0.019 mg GAE/g post-soaking (2.43% reduction). CAP treatment at 9.5 kV increased pre-soaking TPC to 1.6809 ± 0.034 mg GAE/g, which decreased to 1.6214 ± 0.032 mg GAE/g post-soaking (3.54%). Na[Fe(EDTA)]-fortified seeds showed higher TPC than $FeSO_4 \cdot 7H_2O$-fortified seeds at equivalent CAP voltage and iron concentration. TPC declined progressively with increasing iron concentration across all treatments. Soaking-induced TPC reductions ranged from 2.75% ($FeSO_4$, 9.5 kV, 200 µg/g) to 5.64% (both salt types at 4200 µg/g, 11.5 kV). All reductions were statistically significant ($p < 0.05$).

Table 5. Total phenolic content (TPC, mg GAE/g) of selected treatments before and after organic acid soaking, with percentage reduction. Values are mean ± SD (n = 3).

Treatment	Pre-Soaking TPC (mg GAE/g)	Post-Soaking TPC (mg GAE/g)	% Reduction
Control	1.1608±0.021	1.1326±0.019	2.43
CPT 1 9.5 kV / 30 kHz / 5 min	1.6809±0.034	1.6214±0.032	3.54
IS 1 CPT 1 200 µg/g	1.6693±0.033	1.6218±0.032	2.85
IS 1 CPT 1 4200 µg/g	1.0809±0.040	1.0328±0.038	4.45
IS 1 CPT 2 200 µg/g	2.0303±0.041	1.9716±0.040	2.89
IS 1 CPT 2 4200 µg/g	1.8483±0.053	1.7441±0.051	5.64
IS 2 CPT 1 200 µg/g	0.7137±0.014	0.6941±0.013	2.75
IS 2 CPT 1 4200 µg/g	0.6798±0.019	0.6557±0.018	3.55
IS 2 CPT 2 200 µg/g	0.8992±0.018	0.8726±0.017	2.96
IS 2 CPT2 4200 µg/g	0.7374±0.028	0.6958±0.027	5.64

3.4 Antioxidant Activity (AOX)

AOX data for all treatments are shown in Table 6. The control showed pre-soaking AOX of 16.86 ± 0.32%, reducing to 16.69 ± 0.29% post-soaking (1.01% reduction). CAP treatment increased pre-soaking AOX substantially: 21.14 ± 0.41% (9.5 kV) and 25.14 ± 0.48% (11.5 kV), with post-soaking values of 20.82 ± 0.39% and 24.64 ± 0.46%, respectively. AOX increased markedly with iron fortification concentration for both iron sources and both CAP regimes. Na[Fe(EDTA)]-fortified seeds at 11.5 kV and 4200 µg/g showed the highest pre-soaking AOX (48.28 ± 0.96%), declining to 45.96 ± 0.91% post-soaking (4.80% reduction). $FeSO_4 \cdot 7H_2O$-fortified seeds showed lower AOX than Na[Fe(EDTA)] at equivalent conditions (36.77 ± 0.73% at 11.5 kV, 4200 µg/g pre-soaking; 35.18 ± 0.70% post-soaking). All soaking-induced AOX reductions were statistically significant ($p < 0.05$) but remained small relative to the fortification-induced gains.

Table 6. Antioxidant activity (% DPPH radical scavenging) of selected treatments before and after organic acid soaking. Values are mean ± SD (n = 3).

Treatment	Pre-Soaking AOX (%)	Post-Soaking AOX (%)	% Change
Control	16.86±0.32	16.69±0.29	−1.01
CAP 9.5 kV / 30 kHz / 5 min	21.14±0.41	20.82±0.39	−1.51
CPT 2 11.5 kV / 30 kHz / 5 min	25.14±0.48	24.64±0.46	−1.99
IS 1 CPT 1 200 µg/g	8.93±0.21	8.67±0.20	−2.91
IS 1 CPT 1 4200 µg/g	39.69±0.79	38.12±0.76	−3.95
IS 1 CPT 2 200 µg/g	13.92±0.28	13.48±0.27	−3.16
IS 1 CPT 2 4200 µg/g	48.28±0.96	45.96±0.91	−4.80
IS 2 CPT 1 200 µg/g	6.01±0.18	5.86±0.17	−2.50
IS 2 CPT 1 4200 µg/g	31.96±0.63	30.71±0.61	−3.91
IS 2 CPT 2 200 µg/g	12.03±0.25	11.71±0.24	−2.66
IS 2 CPT 2 4200 µg/g	36.77±0.73	35.18±0.70	−4.33

3.5 Surface Colour (L*)

The total colour change and Lightness (L*) values of all the sample are..... presented in Table 7. The control exhibited the highest L* (64.50 ± 0.42), declining progressively with increasing CAP voltage: 62.60 ± 0.38 (9.5 kV) and 59.10 ± 0.35 (11.5 kV). Iron fortification caused further, concentration-dependent reductions in L*: for Na[Fe(EDTA)] at 9.5 kV, L* ranged from 59.84 ± 0.32 (200 µg/g) to 52.85 ± 0.46 (4200 µg/g) pre-soaking; $FeSO_4 \cdot 7H_2O$ caused greater darkening at equivalent concentrations (e.g., 50.82 ± 0.48 at 9.5 kV, 4200 µg/g pre-soaking). The 11.5 kV treatment produced

lower L* values overall. Organic acid soaking reduced L* further by 1.15–1.60 units across all treatments; the maximum δL* of −1.60 was recorded for $FeSO_4 \cdot 7H_2O$ at 11.5 kV and 4200 µg/g. All soaking-induced L* changes were statistically significant ($p < 0.05$) but were small in absolute terms.

Table 7. Surface lightness (L*) of chickpea seeds before and after organic acid soaking across all treatment groups. Values are mean ± SD (n = 3). L* = post-soaking minus pre-soaking L*

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Treatment	Pre-Soaking L* (Mean±SD)	Post-Soaking L* (Mean±SD)	ΔL*
Control	64.50±0.42	63.28±0.39	−1.22
CPT 1 9.5 kV / 30 kHz / 5 min	62.60±0.38	61.24±0.36	−1.36
CPT 2 11.5 kV / 30 kHz / 5 min	59.10±0.35	57.72±0.34	−1.38
IS 1 CPT 1 200 µg/g	59.84±0.32	58.69±0.30	−1.15
IS 1 CPT 1 4200 µg/g	52.85±0.46	51.43±0.45	−1.42
IS 1 CPT 2 200 µg/g	58.18±0.28	56.98±0.29	−1.20
IS 1 CPT 2 4200 µg/g	50.45±0.47	49.05±0.46	−1.40
IS 2 CPT 1 200 µg/g	58.55±0.27	57.27±0.27	−1.28
IS 2 CPT 1 4200 µg/g	50.82±0.48	49.31±0.48	−1.51
IS 2 CPT 2 200 µg/g	56.45±0.26	55.06±0.26	−1.39
IS 2 CPT 2 4200 µg/g	48.32±0.50	46.72±0.50	−1.60

3.6 Lipoxygenase (LOX) Activity

LOX activity data for all treatments are given in Table 8. Control pre-soaking LOX was 82.4 ± 2.5 U min^{-1} g^{-1}, decreasing to 71.5 ± 2.1 U min^{-1} g^{-1} post-soaking (13.23% inhibition). CAP treatment reduced LOX substantially: 61.8 ± 1.9 (9.5 kV, pre-soaking) and 46.7 ± 1.4 (11.5 kV, pre-soaking) U min^{-1} g^{-1}. Iron fortification further reduced LOX in a concentration-dependent manner; Na[Fe(EDTA)] at 11.5 kV and 4200 µg/g showed the lowest pre-soaking LOX (26.7 ± 0.8 U min^{-1} g^{-1}), decreasing to 18.8 ± 0.6 U min^{-1} g^{-1} post-soaking (29.59% inhibition). $FeSO_4 \cdot 7H_2O$ at equivalent conditions produced comparable LOX inhibition (32.5 ± 1.0 to 23.4 ± 0.7 U min^{-1} g^{-1}). All soaking-induced LOX reductions were statistically significant ($p < 0.05$).

Table 8. Lipoxygenase (LOX) activity (U min^{-1} g^{-1}) of chickpea seeds before and after organic acid soaking. Values are mean ± SD (n = 3). % Inhibition computed relative to pre-soaking LOX of the same treatment.

Treatment	Pre-Soaking LOX (U min^{-1} g^{-1})	Post-Soaking LOX (U min^{-1} g^{-1})	% Inhibition
Control	82.4±2.5	71.5±2.1	13.23
CPT 1 9.5 kV / 30 kHz / 5 min	61.8±1.9	49.6±1.6	19.74
CPT 2 11.5 kV / 30 kHz / 5 min	46.7±1.4	34.2±1.1	26.77
IS 1 CPT 1 200 µg/g	57.5±1.7	45.8±1.4	20.35
IS 1 CPT 1 4200 µg/g	34.5±1.0	25.8±0.8	25.22
IS 1 CPT 2 200 µg/g	43.5±1.3	31.8±1.0	26.90
IS 1 CPT 2 4200 µg/g	26.7±0.8	18.8±0.6	29.59
IS 2 CPT 1 200 µg/g	61.8±1.9	49.6±1.6	19.74
IS 2 CPT 1 4200 µg/g	36.5±1.1	27.3±0.8	25.21
IS 2 CPT 2 200 µg/g	46.7±1.4	34.2±1.0	26.77
IS 2 CPT 2 4200 µg/g	32.5±1.0	23.4±0.7	28.00

4. Discussion

4.1 Role of CAP in Modifying Seed Surface and Iron Uptake

The modest reduction in endogenous iron concentration observed following CAP treatment (7.40 to 6.83–6.90 mg/100 g) is consistent with surface ablation effects previously documented in plasma-treated cereals and legumes. RONS generated during CAP discharge interact with the seed coat, oxidising and partially solubilising surface-bound mineral–polyphenol complexes [7]. More critically, the CAP-induced surface modifications—increased roughness, etching of the cuticle, and introduction of hydrophilic oxygenated functional groups—are known to facilitate greater aqueous solution penetration, which is mechanistically important for subsequent iron fortification by immersion [24] [25]. [11]. Specifically demonstrated that CAP plasma treatment of chickpea cultivars significantly reduced the Peleg rate constant K_1, indicative of enhanced water absorption kinetics—a directly analogous mechanism enabling deeper iron solution penetration during fortification. The higher iron concentrations achieved at 9.5 kV compared to 11.5 kV for Na[Fe(EDTA)] may reflect an optimum plasma energy for seed coat permeabilization without excessive structural disruption that might limit iron retention, consistent with findings by [26]. On legume flour. CAP-induced RONS also degraded phytic acid and tannin complexes on the seed surface, as evidenced by the significantly higher pre-soaking bioavailability in CAP-treated seeds (4.10 - 4.40%) compared to control (3.10%). This is mechanistically consistent with the findings of [10]. In guar bean, who reported 20–40% reductions in phytic acid following atmospheric cold plasma pretreatment, and with the broader observation that RONS directly oxidise the myo-inositol phosphate backbone of phytic acid, reducing its chelating capacity for iron. The 11.5 kV regime, while producing greater surface modification (lower L*, higher bioavailability enhancement from baseline), resulted in lower absolute iron uptake for Na[Fe(EDTA)], suggesting that excessive plasma energy may reduce surface pore size or alter seed coat permeability in ways that impede larger-molecule EDTA chelate penetration while still benefiting from smaller ferrous salts ($FeSO_4 \cdot 7H_2O$).

4.2 Iron Concentration and Comparative Performance of Iron Sources

Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) achieved 2.5–2.8-fold higher absolute iron concentrations than sodium iron ethylenediaminetetraacetate (Na[Fe(EDTA)]) at equivalent fortification levels across both CAP regimes. This differential is explained by the markedly higher solubility and smaller molecular size of the ferrous sulphate complex compared to the bulkier iron-EDTA chelate. The ferrous cation (Fe^{2+}) from $FeSO_4$ diffuses more readily through the seed coat aqueous channels generated by CAP treatment, enabling greater iron loading per unit mass. This is consistent with the findings of [6], who reported that $FeSO_4 \cdot 7H_2O$ fortification of chickpea flour at 2000 µg/g yielded substantially higher iron content than Na[Fe(EDTA)] at equivalent concentrations. Similarly, [27] that iron content in fortified lentils was critically dependent on iron source solubility and particle contact time.

The concentration-dependent increase in iron loading for both sources is well established in fortification literature and reflects a simple diffusion gradient mechanism: higher external iron concentration increases the driving force for iron diffusion into seed tissue. The marginal post-soaking iron losses (0.4–4.6%) are expected consequences of soaking in an acidic solution, which solubilizes surface-associated iron. These losses are small and clinically negligible relative to the total iron gain from fortification, and are consistent with the leaching losses reported by [27]. For fortified lentils stored under various conditions.

4.3 Iron Bioavailability

The most practically significant outcome of this study is the substantial post-soaking improvement in iron bioavailability across all fortified treatments. Organic Soaking refers to the soaking of food using organic acids. The combination of citric acid and ascorbic acid as a medium works with several complementary mechanisms. mechanisms. The ascorbic acid is the strongest nutritional promoter of non-haem iron, complexing with the Fe^{2+}. It converts Fe^{3+} to the more soluble and membrane-permeable Fe^{2+}, forming a complex with the Fe^{2+}. Labile, water-soluble, precipitate-resistant iron ascorbate chelate pH [12] [4]. This is a type of reduction chemistry especially relevant. In situ reduction of Fe^{3+} to Fe^{2+} by ascorbic acid in the soaking buffer makes the Fe^{2+} observable in the X-ray data. The Fe^{3+} in the soaking buffer is reduced to Fe^{2+} in situ, which is visible in the X-ray data because of the ascorbic. This is complemented by the ability to form soluble iron-citrate complexes and competitive inhibition of the uptake of iron. Phytate binding to iron in the luminal environment, which would decrease the phytate inhibition [13] [12].

The bioavailability of Na[Fe(EDTA)] was higher than that of $FeSO_4 \cdot 7H_2O$ following the soaking process in both cases. The well-established protective function of the, which is responsible for the matching of conditions, is the reason for these mechanistic explanations. EDTA ligand. In gastrointestinal environment, EDTA chelates iron from the common non has no space for haem in the pool and prevents the interaction of the haem with inhibitory phytate and polyphenol molecules, which present iron to intestinal transport proteins in a more bioavailable form—a phenomenon that has been adequately documented in cereal- and legume-based diets [28] [4]. This protection of iron from inhibitory effects is provided by EDTA. The main reason for Na[Fe(EDTA)] always being superior to simple iron salts is complexation. In food matrices with high phytate content, this is the mechanism that is consistent with our results understanding. The superior bioavailability of Na[Fe(EDTA)] despite lower absolute iron content makes it the fortification compound of choice

in this context, agreeing with meta-analytic evidence from multiple legume fortification programmes [28].

The significantly greater post-soaking bioavailability at 9.5 kV compared to 11.5 kV for Na[Fe(EDTA)] (17.94% versus 15.88% at 4200 µg/g) is noteworthy and warrants mechanistic consideration. At higher plasma voltage, more extensive oxidative modification of the seed surface may promote the formation of iron-protein or iron-polyphenol complexes within the seed matrix that reduce iron dialysability during the in vitro digestion assay. This is consistent with the observation that 11.5 kV seeds had higher pre-soaking TPC (2.0–2.03 mg GAE/g for Na[Fe(EDTA)]) compared to 9.5 kV seeds (1.67 mg GAE/g at 200 µg/g), suggesting that higher plasma intensity paradoxically mobilised more surface phenolics available to interact with iron. Post-soaking total phenolic content reductions were larger under the 11.5 kV CAP regime (up to 5.64%), consistent with greater phenolic leaching, which would be expected to release inhibitor-bound iron and potentially improve bioavailability; however, the competing formation of iron-phenolate complexes within the seed matrix before soaking may have concurrently reduced the dialysable iron pool.

4.4 TPC: CAP-Mediated Enhancement and Soaking-Induced Leaching

The elevation of TPC following CAP treatment (1.68 mg GAE/g at 9.5 kV versus 1.16 mg GAE/g for control) is a well-documented and counterintuitive phenomenon in plasma food science. Plasma-generated RONS, particularly hydroxyl radicals and singlet oxygen, cause oxidative fragmentation of condensed tannins and high-molecular-weight polyphenols into lower-molecular-weight phenolic monomers and oligomers that are more efficiently extracted by methanol and more reactive with Folin-Ciocalteu reagent [29] [30]. This oxidative depolymerisation of condensed tannins has implications for iron bioavailability: lower-molecular-weight monomeric phenolics, particularly catechol-type compounds, can in fact enhance iron absorption at sub-inhibitory concentrations, while high-molecular-weight condensed tannins are the primary inhibitors (Piskin et al., 2022). The 11.5 kV treatment produced even higher TPC (2.03 mg GAE/g at 200 µg/g Na[Fe(EDTA)]), reflecting a greater degree of tannin depolymerisation at higher plasma energy. [31] similarly reported that RSM-optimized CAP conditions maximized TPC extraction from plant materials by promoting depolymerisation rather than destruction of phenolic compounds.

The concentration-dependent decline in TPC with increasing iron loading across all treatments is explained by iron-polyphenol complex formation. Fe^{3+} in particular forms intensely coloured, insoluble complexes with catechol-type polyphenols at the seed surface (the well-documented iron tannate reaction), thereby sequestering phenolics in complexes unavailable to the Folin-Ciocalteu reagent [32]. Organic acid soaking reduced TPC further (2.75–5.64%), consistent with the solubilization and leaching of free and loosely bound phenolics into the acidic soaking medium—a phenomenon extensively documented in legume soaking studies [5]. This reduction in free phenolics is nutritionally beneficial, as it directly reduces the inhibitory polyphenol burden on iron absorption.

4.5 Antioxidant Activity and LOX Inactivation

The dose-dependent increase in Antioxidant Activity with iron fortification concentration is a mechanistically interesting observation. Iron is a redox-active element and can directly scavenge DPPH radicals through Fenton-type chemistry at the concentrations used in this study; additionally, the ascorbic acid retained in Na[Fe(EDTA)]-fortified seeds post-soaking likely contributes significantly to the elevated AOX values observed for Na[Fe(EDTA)] compared to $FeSO_4 \cdot 7H_2O$ treatments. The small but significant post-soaking AOX reductions (1.0–4.8%) most likely reflect leaching of water-soluble antioxidant compounds—ascorbate itself and low-molecular-weight phenolics—into the soaking solution. These losses are consistent with findings from general legume soaking literature [5] and do not represent a meaningful nutritional disadvantage given the substantial iron bioavailability gain.

The progressive inactivation of LOX by CAP treatment and iron fortification represents a dual benefit of the processing protocol. CAP-generated RONS inactivate LOX through oxidative modification of the enzyme's iron-containing active site, histidine residues, and overall tertiary structure—a mechanism extensively characterized by [29] and [33], who reported 49.98% and 100% LOX inactivation in wheat germ and flour at 25 kV for 25 min and 6 min, respectively. In the present study, CAP treatment at 11.5 kV reduced LOX from 82.4 to 46.7 U min^{-1} g^{-1} pre-soaking (43.3% reduction), consistent with the voltage-dependent inactivation reported by Li et al. (2023) for legume LOX (reductions of 28–37% at 5 W, 10 min DBD). Iron fortification further inhibited LOX in a concentration-dependent manner, likely through competitive binding of exogenous iron ions at the enzyme's native Fe^{2+} active site, disrupting catalytic function. Soaking-induced LOX reductions (13–30%) reflect both enzyme leaching and acid-induced denaturation of the LOX protein structure. Collectively, these findings suggest that the combined treatment substantially reduces chickpea's susceptibility to oxidative rancidity and beany off-flavour development during storage—an important quality benefit beyond nutritional improvements [34].

4.6 Surface Colour and Consumer Acceptability

The darker the seed becomes (the lower the L* value), the higher the applied CAP voltage and iron. As the applied CAP voltage and iron increase, the seed gets darker (L* decreases). The concentration has significant consumer acceptability considerations. Plasma-induced oxidative reactions form surface browning by producing Maillard precursors and polyphenols. As observed in CAP-treated legumes [26], it is the oxidation that happens after the

treatment. Iron-polyphenol complex Form is the initial step in the process (especially Fe^{3+} forming blue-black ferric tannate), a well-known technical problem in iron-fortified foods—the darkening of the product. The darkening of the product is one of the problems recognized in food technology and is considered to be the primary reason for the darkening of the iron-fortified food. [32] Mentioned that food fortification is a great challenge. The high colour-fastness of the results showed that the sodium salt of EDTA (Na[Fe(EDTA)]) was superior to $FeSO_4 \cdot 7H_2O$ in terms of iron content. The results indicated that the sodium salt of EDTA (Na[Fe(EDTA)]) was superior to $FeSO_4 \cdot 7H_2O$ for iron content at equivalent iron levels. The fact that the chelated iron in Na[Fe(EDTA)] is sterically protected against polyphenol may account for this. As a result, the complexation by the EDTA ligand is consistent with the one suggested by [32] For tea fortification. The reductions in color L* caused by the soaking were always low (δL* −1.15). The use of the organic acid medium does not add an unacceptable additional to −1.60. colour change. Future sensory evaluation studies should verify that these observed L* values are within consumer acceptable levels for chickpea products.

5. Conclusions

This study demonstrates that iron fortification of chickpea (*Cicer arietinum* L. variety PUSA 3062) through a two-stage processing strategy — (Stage 1) cold atmospheric plasma (CAP) treatment with iron fortification using Na[Fe(EDTA)] or $FeSO_4 \cdot 7H_2O$, and (Stage 2) organic acid soaking in citric and ascorbic acid (10 h) — is scientifically valid and practically feasible. Na[Fe(EDTA)] consistently yielded superior iron bioavailability across all treatment combinations, with the highest dialysable bioavailability of 17.94 ± 0.51% at 9.5 kV and 4200 µg/g, representing a 36–58% improvement over pre-soaking values. Although $FeSO_4 \cdot 7H_2O$ achieved higher absolute iron concentrations (up to 55.96 mg/100 g), this did not translate into proportionally higher bioavailability, confirming that chelation chemistry rather than absolute iron load governs dialysable iron. CAP served primarily as a surface pre-conditioning step to enhance seed permeability, while organic acid soaking significantly inhibited lipoxygenase activity (13–30%) and produced nutritionally acceptable reductions in phenolic content (2.4–5.6%), antioxidant activity (1.0–4.8%), and lightness ($\Delta L^* \leq 1.60$). From a public health standpoint, this strategy is particularly relevant for Indian and other developing-country populations where chickpea is a dietary staple and iron deficiency anaemia remains a major concern, as ascorbic acid and citric acid act as effective iron adjuvants — the former converting Fe^{3+} to the more absorbable Fe^{2+} form, and the latter forming soluble iron complexes that inhibit phytate binding — without requiring specialised equipment. The process is also industrially scalable, as the CAP parameters identified (9.5 kV, 30 kHz, 5 min) fall within existing pilot-scale reactor operating ranges and can be integrated as an in-line step in pulse processing lines, with Na[Fe(EDTA)]'s WHO-approved status removing regulatory barriers for fortification programmes. The Na[Fe(EDTA)]–CAP (9.5 kV)–organic acid soaking regime is recommended for pilot-plant validation; future work should assess iron bioavailability retention under cooking and storage conditions and confirm findings through in vivo human trials.

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Declaration of Completing Interest

The authors have no competing interests.

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