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EFFECT OF BAKERY-TYPE FOOD MATRICES AND THERMAL PROCESSING ON ELISA QUANTIFICATION OF CASHEW ALLERGEN ANA O 3

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ABSTRACT

The aim of this study was to evaluate the combined effects of cashew kernel processing, baking conditions, and food matrix type on the detectability of cashew allergens and, consequently, on the reliability of commercial ELISA kits used for allergen quantification in processed foods. The experimental design compared two baking regimes applied to muffin matrices (170 °C for 23 min and 200 °C for 18 min) with a non-thermal matrix represented by model raw bars. Cashew kernels subjected to three processing treatments (native, roasted, and blanched) were incorporated into the matrices using the geometric dilution method at nominal concentrations of 5, 10, 20, and 50 mg/kg. The results demonstrated that moisture loss during baking substantially increased the concentration of matrix solids (5.83% evaporation at 170 °C and 8.58% at 200 °C), highlighting the need for mathematical correction of allergen concentrations. Samples baked at 170 °C exhibited matrix-induced enhancement of the analytical signal and, in several cases, saturation of the assay response (ULOQ), likely due to partial protein denaturation and increased exposure of Ana o 3 epitopes. In contrast, baking at 200 °C resulted in pronounced signal suppression, with recoveries decreasing to 14.5% at 50 mg/kg of roasted cashew, a pattern consistent with extensive protein aggregation and epitope masking potentially associated with Maillard reaction products, as reported in previous studies. In the non-thermal matrix, preservation of the native protein structure led predominantly to signal enhancement and rapid saturation of the immunoassay response. Overall, the findings demonstrate that both thermal processing conditions and matrix composition substantially influence ELISA performance, potentially leading to either overestimation or underestimation of cashew allergen levels in processed food.

Keywords: cashew nut, allergen Ana o 3, bakery products, ELISA, thermal processing

INTRODUCTION

Cashew *(Anacardium occidentale L.)* originated in Brazil and was subsequently disseminated to Africa and Asia by Portuguese explorers during the sixteenth century **(Oliveira *et al.*, 2019)**. Today, it is extensively cultivated in tropical regions worldwide and represents one of the most economically important tree nuts in international trade. Côte d'Ivoire, India, and Vietnam are currently among the largest producers of cashew nuts globally **(Hirpara *et al.*, 2025)**. The increasing global consumption of cashew-containing products has also heightened concerns regarding cashew allergy and the need for reliable allergen detection methods in processed foods **(Mendes *et al.*, 2019)**. Allergen characteristics, the impact of processing on allergenicity, and future perspectives in food have been described in several studies **(Zeleňáková *et al.*, 2019; Žiarovská *et al.*, 2021; Jiang *et al.*, 2026)**. Recent advances in analytical methods have markedly improved the detection of nut allergens; however, accurate identification remains challenging due to food processing effects, matrix complexity, and allergen cross-reactivity **(Žiarovská *et al.*, 2016, 2019).** In the European Union, the declaration of allergenic ingredients is mandatory under **Regulation (EU) No. 1169/2011**. In addition, the application of precautionary allergen labelling (PAL) is increasingly guided by risk-based approaches, particularly the Voluntary Incidental Trace Allergen Labelling (VITAL) framework, which supports the assessment and management of unintended allergen presence in food products **(Allergen Bureau, 2019).** The main allergens identified in cashew nuts are the storage proteins Ana o 1 (7S globulin, vicilin), Ana o 2 (11S globulin, legumin), and especially Ana o 3 (2S albumin). The latter, Ana o 3, is characterized by a highly rigid structure stabilized by disulfide bridges, which is why it is considered the primary marker for immunoassay detection **(Breiteneder *et al.*, 2025; Mattison *et al.*, 2016; Shen *et al.*, 2025)**. In industrial practice, the ELISA (Enzyme-Linked Immunosorbent Assay) method is the standard for routine monitoring of cross-contamination and verification of the accuracy of precautionary allergen labeling (PAL). Despite its high sensitivity and selectivity, the analytical response of sandwich systems is often influenced by so-called matrix effects. During culinary and industrial food processing, high temperatures and mechanical forces are applied, and interactions occur between the proteins of the contaminant and the components of the accompanying food matrix (carbohydrates, lipids) **(Zeleňáková *et al.*, 2016; Luparelli *et al.*, 2022)**. Thermal processes, such as baking or roasting, induce structural changes in macromolecules, irreversible denaturation, the formation of insoluble aggregates, and modifications through non-enzymatic browning (the Maillard reaction) **(Vissers *et al.*, 2011)**. These changes can lead either to the destruction and masking of antigenic determinants (epitopes), causing false-negative results, or to the unfolding of the tertiary structure and exposure of previously hidden epitopes, leading to an overestimation of the analytical signal **(Johnson *et al.*, 2018; Masthoff *et al.*, 2013; Parker *et al.*, 2015)**.
This study evaluates the impact of food processing technologies and matrix effects on the reliability of cashew allergen (*Anacardium occidentale*) quantification using a commercial sandwich ELISA kit targeting the major allergen Ana o 3.

Scientific Hypothesis

H1: The processing treatment of cashew kernels (native, roasted, blanched) is associated with substantial differences in the quantification of the Ana o 3 allergen by sandwich ELISA.
H2: The intensity of thermal processing applied to the food matrix (170 °C vs. 200 °C) is associated with substantial differences in the ELISA recovery of cashew allergens.
H3: The non-thermally processed matrix (raw bars) differs from the thermally processed muffin matrices in its ELISA-detectable cashew allergen response; because the two matrix types differ simultaneously in composition and thermal history, this comparison reflects the overall effect of matrix type rather than an isolated effect of heat treatment.

MATERIAL AND METHODS

Material and experimental design

For the experimental part of the study, bakery products representing different levels of food processing were prepared. The following products were used as model samples:

- Muffins (thermal matrix): Composed of buckwheat flour (45 g), cane sugar (15 g), vegetable oil (8.5 g), milk (20 g), egg (10 g), and baking powder (1.5 g).
- Raw bars (non-thermal matrix): Composed of buckwheat flakes (50 g), date paste (35 g), and maple syrup (15 g) without the application of heat.

Model muffin and raw bar samples were prepared according to formulations standardized to 100 g of batter and mixture, respectively. Cashew kernels were subjected to three distinct processing treatments:

- Native (N): raw and unheated kernels
- Roasted (R): industrial dry-air roasting kernels
- Blanched (B): kernels dehusked using a hydrothermal process

The processed kernels were subsequently ground and incorporated into the respective food matrices by spiking the analyte into a solid matrix using the geometric dilution method. Final nominal concentrations of cashew kernels in the batter/mixture were 5, 10, 20, and 50 mg/kg.

The model muffin batter was divided into silicone molds with a target weight of 100 g per sample. Baking was carried out in a convection oven under two baking conditions:

- Mode 1: Temperature 170 °C, exposure time 23 minutes (n = 15).
- Mode 2: Temperature 200 °C, exposure time 18 minutes (n = 15).

Each sample was weighed to the nearest 0.01 g immediately before being placed in the oven and after cooling to room temperature. Relative weight loss due to water evaporation was determined gravimetrically and used for subsequent mathematical correction.

Raw bars were prepared by homogenizing dates and maple syrup into a cohesive paste, followed by the incorporation of buckwheat flakes. The mixture was processed into a uniform, formable mass, rolled to the desired thickness, and cut into bars of defined dimensions. The bars were refrigerated for 1-2 h to ensure structural stability.

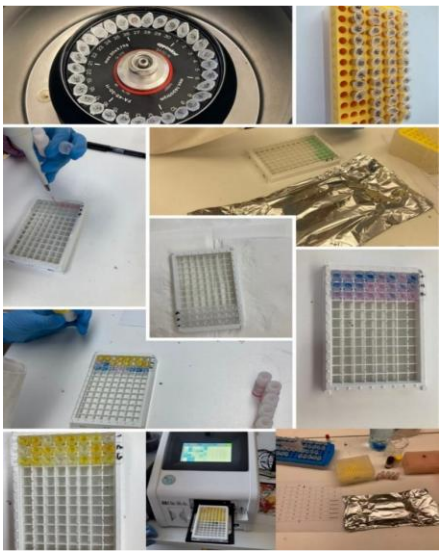
Methods

The allergen concentration in the analyzed samples was quantified using a commercial sandwich ELISA kit (RIDASCREEN® FAST Cashew, R-Biopharm AG, Germany). The analysis was performed according to the manufacturer's instructions.

Sample Preparation and Extraction

Prior to analysis, representative samples (50 g) were homogenized using a laboratory mill. Subsequently, 1 g of the homogenized sample was weighed into a reaction tube, mixed with 20 mL of preheated and diluted allergen extraction buffer, and extracted at 60 °C for 10 min with vigorous shaking. After extraction, the samples were centrifuged at 2500 × g for 10 min, and the resulting supernatants were used for subsequent immunoassay analysis.

ELISA analysis



Picture 1 Workflow of allergen determination by ELISA

The immunoassay was performed according to the manufacturer's instructions using a microplate coated with monoclonal antibodies specific to the Ana o 3 protein (Picture 1). Briefly, 100 µL of standards and sample extracts were added to the wells and incubated for 20 min at room temperature. Following a washing step, 100 µL of enzyme conjugate was added and the plate was incubated for an additional 20 min. After a second washing cycle, 100 µL of TMB substrate was added and the reaction was allowed to develop for 10 min in the dark. Colour development was stopped by the addition of stop solution, and the absorbance was measured at 450 nm. According to the manufacturer's specifications, the validated quantitative working range of the assay was 2.5–20 mg/kg (LLOQ = 2.5 mg/kg; ULOQ = 20 mg/kg); measurements outside this range were treated as censored observations (reported as <2.5 or >20 mg/kg) rather than as precise point estimates in subsequent calculations (R-BIOPHARM AG, 2023).

Data processing and statistical analysis

Correction for evaporation

Due to moisture loss during baking, the mass of non-volatile matrix solids (including the spiked cashew allergen) becomes concentrated within a smaller total sample mass. A mass-balance correction is therefore required to distinguish this trivial concentrating effect from genuine changes in ELISA-detectable allergen signal. The derivation assumes that the mass of spiked allergen material is conserved during baking, i.e. only water is lost.

Let C0 denote the nominal, as-formulated allergen concentration on a pre-baking mass basis and let Cmeasured denote the allergen concentration determined by ELISA using an extraction based on the post-baking sample mass. Because the allergen mass (C0 × m0) is conserved while mf < m0, water loss alone causes Cmeasured to exceed C0 by a factor equal to the pre-/post-baking mass ratio, independently of any change in immunoreactivity. This ratio is defined as the evaporation factor:

Fevaporation = m0 / mf

To express the ELISA-determined concentration on the same mass basis as the nominal, pre-baking fortification level – thereby removing the trivial mass-concentrating effect of evaporation before evaluating detectability – the measured concentration must be divided by the evaporation factor (equivalently, multiplied by mf/m0):

Ccorrected = Cmeasured / Fevaporation

Recovery (%) was then calculated as (Ccorrected / C0) × 100. Under this definition, any deviation of the recovery from 100% reflects genuine ELISA-detectability effects (e.g., matrix interactions, epitope accessibility) rather than the mass-concentrating artefact of water loss, which is removed by the correction.

Worked example: for a muffin sample weighing 100.0 g before baking (m0) and 91.4 g after baking (mf), Fevaporation = 100.0 / 91.4 = 1.094. A hypothetical ELISA reading of Cmeasured = 10.94 mg/kg on the baked extract would therefore give Ccorrected = 10.94 / 1.094 = 10.00 mg/kg. For a nominal fortification level of C0 = 10.00 mg/kg, this corresponds to a recovery of (10.00 / 10.00) × 100 = 100%, i.e. a scenario in which water loss alone accounts for the raw analytical signal and immunoreactivity is fully retained.

ELISA data analysis

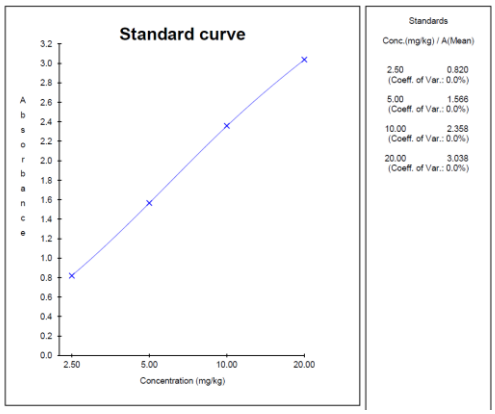


Figure 1 Standard curve for detection of cashew allergens

The optical density (OD) of the solutions was measured on a microplate reader at a wavelength of 450 nm. The calibration curve (Figure 1) was constructed based on standards supplied by the kit manufacturer, and the relationship between concentration and OD was modeled using four-parameter logistic regression (4PL). To ensure robust analytical representation, each homogenized food matrix sample was analyzed in four technical ELISA replicate wells (C1–C4) per microplate run. For measurements performed at 200 °C, the first of these four readings (C1) was consistently excluded from the calculated mean for each sample, in accordance with the laboratory's data-recording convention at the time of analysis; the remaining three readings (n = 3) were used to calculate mean recovery

and SD. For measurements at 170 °C, all four replicate readings were retained (n = 4). These technical ELISA replicates, rather than independent sample extractions, served as the experimental unit for the descriptive statistics reported in Table 2.

Statistical analysis

Descriptive statistics (mean and standard deviation, SD) were calculated for all experimental data. For the gravimetric weight-loss measurements (Table 1; n = 15 independent muffin samples per baking regime), differences in evaporation between the 170 °C and 200 °C baking regimes were evaluated using an independent-samples Student's t-test (or the non-parametric Mann-Whitney U test where the normality assumption was not met); statistical significance was set at $p<0.05$. For the ELISA-based allergen recovery data, four technical replicate readings were obtained per sample (n = 4 at 170 °C; n = 3 at 200 °C after exclusion of the first reading, C1, per the laboratory's data-recording convention – see "Sample Preparation and Extraction"), and mean apparent recovery (%) and SD were calculated from these replicates for each treatment combination (Table 2). Because analytical responses frequently fell outside the validated quantitative range (signal saturation >ULOQ at 170 °C; suppression <LLOQ at 200 °C), formal inferential hypothesis testing (e.g., factorial ANOVA) was not applicable across the full recovery dataset. Recovery data were therefore evaluated through descriptive comparative analysis, focusing on the magnitude and direction of matrix- and treatment-induced signal enhancement or suppression rather than on formal significance testing.

RESULTS AND DISCUSSION

Weight loss during baking and its impact on allergen quantification

Interpretive note on mechanisms. The present study directly measured ELISA-detectable allergen signal (optical density and derived recovery) under different combinations of cashew pretreatment, food matrix, and baking condition. Protein unfolding, aggregation, Maillard-type modification, and epitope masking or exposure were not directly assessed (e.g., by circular dichroism, SDS-PAGE, or peptide-level mass spectrometry) and are therefore discussed below as plausible explanations consistent with previous literature, rather than as mechanisms demonstrated by the present data. Accordingly, the results reported here show that ELISA detectability/recovery varies substantially among the investigated combinations of matrix and processing condition, while the underlying molecular causes remain hypothetical.

Monitoring changes in the weight of model muffin food matrices before and after thermal processing is essential for the accurate interpretation of immunoassay results. During baking, water evaporation leads to a reduction in the total weight of the product, resulting in a relative increase in the concentration of dry matrix components, including the added cashew allergen. Without correction for evaporation, allergen concentrations may be systematically overestimated, reflecting moisture loss rather than the actual amount of allergen present prior to processing **(Tuzimski *et al.*, 2020).**

To evaluate this effect, two baking regimes were compared: a moderate regime (170 °C for 23 min) and a high-temperature regime (200 °C for 18 min). For each treatment, 15 independent muffin samples were analyzed.

Table 1 Descriptive statistics for weight changes and moisture loss (n = 15)

Parameter	170 °C / 23 min	200 °C / 18 min
Average weight before baking (g)	97.61	97.80
Standard deviation before baking	0.94	0.99
Average weight after baking (g)	91.92	89.41
Average evaporation (%)	5.83	8.58
Standard deviation of evaporation	0.99	1.75

The data showed (Figure 2) that baking at 170 °C for 23 min resulted in an average weight loss of 5.83% (± 0.99%). Under the more intense baking regime (200 °C for 18 min), the average moisture loss increased to 8.58% (± 1.75%). The difference in evaporation between both temperature regimes was statistically significant ($p<0.05$). The maximum recorded weight loss at 200 °C was 10.74%, indicating a substantially higher degree of dehydration of the baked matrix.

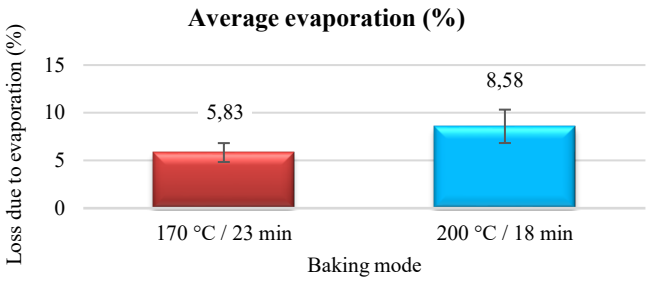
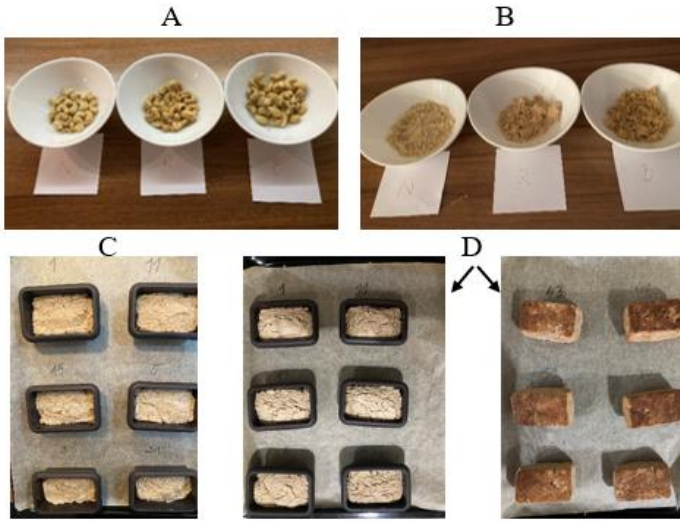


Figure 2 Comparison of moisture loss (%) between muffins baked at 170 °C and 200 °C

Differences in the structure and appearance of the baked matrices were also visually apparent (Picture 2). The higher temperature induced faster formation of a surface crust and a more porous interior, which accelerated the loss of internal moisture.



Picture 2 Visual comparison of muffin matrices before and after baking.
Note:
A – Native, roasted and blanched cashew kernels.
B – Ground cashew material used for muffin preparation.
C – Muffins batter before baking
D – Muffins after baking (170 °C and 200 °C)

Therefore, the apparent concentration of matrix components, including cashew proteins, increased by approximately 9.4% solely due to water evaporation (at 200 °C). To eliminate this source of bias, all ELISA-derived allergen concentrations were corrected using individual evaporation and extraction factors for each sample. This correction ensured that the reported results reflected the detectability of the originally added cashew material rather than changes caused by moisture loss during baking.

Effect of thermal processing on allergen detection in baked matrices (muffins)

Heat treatment plays a dominant role in modifying the conformation of plant storage proteins. The interaction between high temperature, baking time, and the components of the complex muffin matrix – particularly the presence of reducing sugars from cane sugar and lipids from vegetable oil – radically alters the accessibility of antigenic determinants (epitopes) to the specific antibodies used in commercial ELISA kits.

Table 2 Recovery (%) of cashew allergen detection by ELISA in muffin matrices after thermal processing.

Temperature regimen	Cashew treatment	5 mg/kg	10 mg/kg	20 mg/kg	50 mg/kg
Muffin 170 °C	Native (N)	> 200.0*	187.3 ± 1.8	> 100.0*	> 40.0*
	Roasted (R)	62.4 ± 1.1	74.6 ± 1.2	59.0 ± 0.6	> 40.0*
	Blanched (B)	65.0 ± 1.1	153.1 ± 1.6	89.5 ± 0.8	> 40.0*
Muffin 200 °C	Native (N)	112.4 ± 3.1	35.0 ± 0.5	40.5 ± 0.7	29.3 ± 0.8
	Roasted (R)	< 46.0***	< 25.0***	17.5 ± 0.6	14.5 ± 0.3
	Blanched (B)	56.0 ± 1.0	32.0 ± 0.8	37.0 ± 0.7	38.4 ± 0.6

Note: *Recovery values at or above the upper limit of quantification (ULOQ = 20 mg/kg) are reported as lower-bound estimates (>X%), since the true concentration could not be quantified beyond this point. *** Recovery values at or below the lower limit of quantification (LLOQ = 2.5 mg/kg) are reported as upper-bound estimates (<X%), since the true concentration could not be precisely quantified below this point.

Recoveries exceeding 100% indicate enhanced antibody recognition, likely caused by heat-induced unfolding of protein structures and improved exposure of immunoreactive epitopes. At lower fortification levels (5 and 10 mg/kg), native cashew samples showed substantial signal enhancement. However, with increasing allergen concentration, apparent recovery decreased markedly, suggesting matrix-dependent suppression and limited linearity of the analytical response. The effect of both temperature regimes on the analytical response and stability for individual technological modifications of cashew nut kernels (native, roasted, blanched) revealed substantial deviations from linearity.

Increasing baking temperature from 170 °C to 200 °C resulted in a dramatic reduction in ELISA recoveries across all cashew treatments, consistent with progressive epitope masking, protein aggregation and reduced extractability of allergenic proteins (Figure 3).

At concentrations where the analytical response exceeded the linear range of the method, recoveries were not reported as exact values but were classified as exceeding the upper limit of quantification (ULOQ); the corresponding bars are therefore omitted from Figure 3 (see Table 2 for the censored values).

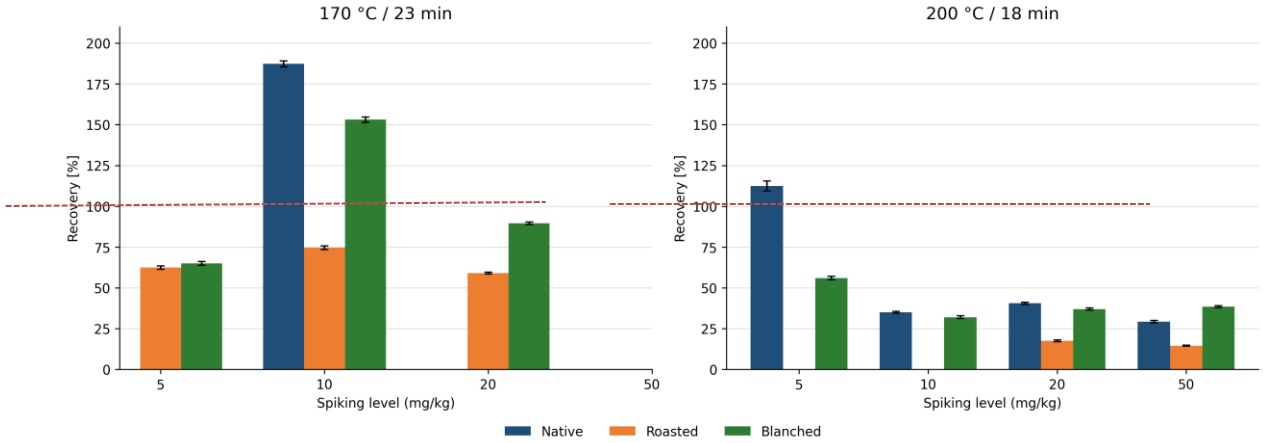


Figure 3 Recovery (%, mean ± SD) of cashew allergen detection by ELISA in muffin matrices, by cashew kernel treatment (native, roasted, blanched), spiking level and baking temperature. Bars are omitted for treatment–concentration combinations in which the analytical response fell outside the validated quantitative range (2.5–20 mg/kg; see Table 2 and its footnotes).

Samples baked at 170 °C exhibited substantial signal enhancement, particularly for native cashew kernels, where recoveries exceeded 200% at the lowest fortification level. This effect is likely associated with partial protein unfolding and increased exposure of antibody-recognized epitopes. In contrast, baking at 200 °C resulted in markedly reduced recoveries across all cashew treatments. The strongest suppression was observed for roasted cashew kernels, where recoveries fell below 25% at 10 mg/kg. These findings suggest that extensive thermal treatment promotes protein aggregation, Maillard-type reactions and epitope masking, thereby reducing antibody accessibility and ELISA detectability.

The substantial decrease in ELISA recovery observed at 200 °C is consistent with the findings of **Ravindran and Ramaswamy (2023)**, who reported that severe thermal processing markedly reduces allergen immunoreactivity due to structural modifications of proteins and reduced antibody recognition.

Similar observations were reported by **Santos *et al.* (2021)**, who demonstrated that thermal processing can substantially alter the immunoreactivity of food allergens and consequently affect their detection by commercial ELISA assays.

According to **He (2023)**, thermal processing can either enhance or reduce the immunoreactivity of food allergens, depending on the degree of protein denaturation, aggregation, and the formation of Maillard reaction products.

Collectively, these findings demonstrate that thermal processing not only affects the concentration of extractable proteins but also substantially changes the immunochemical behaviour of cashew allergens, potentially leading to either overestimation or underestimation of allergen content when using ELISA-based methods.

Matrix-Induced Signal Enhancement (170 °C)

At 170 °C, ELISA recoveries frequently exceeded the expected values, particularly at low fortification levels. In some cases, the analytical response exceeded the validated calibration range of the assay (>ULOQ), indicating pronounced matrix-induced signal enhancement **(Dupont, 2022)**. This effect can be attributed to partial thermal denaturation of cashew storage proteins, which may expose previously inaccessible epitopes and improve antibody recognition in the sandwich ELISA format **(Stanic-Vucinic and Velickovic, 2012; Polloni *et al.*, 2011)**. Consequently, mild thermal processing may lead to an overestimation of allergen content, although such analytical bias is generally considered less critical from a consumer safety perspective than allergen underestimation **(Allergen Bureau, 2019; Monaci and Visconti, 2009)**.

Suppression of Detection and Masking of Epitopes (200 °C)

In contrast, baking at 200 °C markedly reduced ELISA recoveries, indicating severely impaired allergen detectability. Based on previous literature, this reduction is likely associated with extensive protein denaturation, aggregation, and decreased extractability of thermally modified proteins **(Parker *et al.*, 2015)**. Furthermore, it is hypothesised that Maillard reaction products, formed between proteins and reducing sugars under intense heat, may covalently mask antibody-binding sites and further limit immunochemical recognition **(Vissers *et al.*, 2011)**.

As a result, ELISA-based methods may critically underestimate allergen concentrations in such highly processed bakery products, increasing the probability of false-negative results **(Goodman *et al.*, 2015)**.

Evaluation of the response in a non-thermally processed matrix (raw bars)

The analysis of model raw bars was included in the experimental design as a distinct, non-thermally processed model food matrix. Because raw bars differ fundamentally from muffins not only in the absence of heat treatment but also in their biochemical composition (e.g., presence of date paste and maple syrup, absence of egg and baking powder), this system does not serve as a direct thermal control; rather, it isolates the ELISA detectability of cashew allergens within a structurally distinct, unheated, high-sugar, lipid-interacting environment, reflecting the combined outcome of matrix composition, mechanical incorporation, and homogenisation (R-Biopharm AG 2023). In this non-baked matrix, the native protein structure is expected to be preserved in the absence of thermal degradation; consistent with this, the method's linearity limits were frequently reached. This pattern should nevertheless be interpreted as characteristic of the raw-bar system, rather than as evidence isolating the absence of heat as the sole causal factor.

Picture 3 Raw bars (visual appearance and matrix homogeneity)

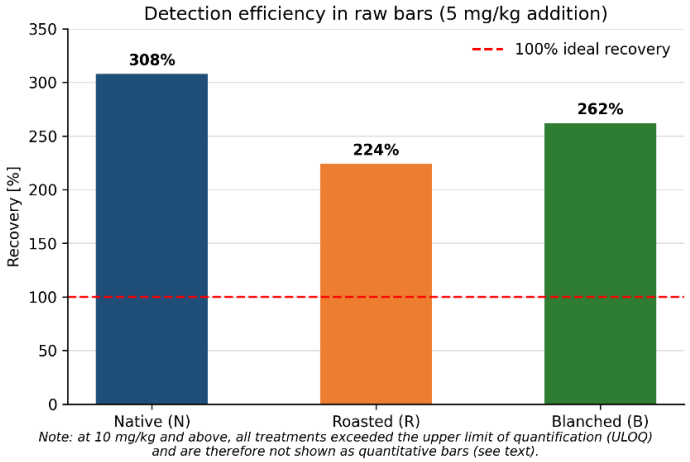


Figure 4 Recovery (%) of cashew allergen detection by ELISA in the non-thermal raw-bar matrix at the 5 mg/kg fortification level, by cashew kernel treatment (Native, Roasted, Blanched), relative to the 100% ideal-recovery reference line. At 10 mg/kg and above, all treatments exceeded the ULOQ and are therefore not shown as quantitative bars.

In the non-thermal raw bar system, a consistent trend of elevated analytical response was observed across all technological treatments of cashew kernels. Even at the baseline fortification level of 5 mg/kg, the samples showed an apparent signal overestimation above the theoretical 100% recovery (Figure 4). As the added concentration was increased (to 10 mg/kg and higher), the analytical response rapidly reached saturation, and the resulting optical density values exceeded the validated working range of the assay. Consequently, these results were classified as exceeding the upper limit of quantification (ULOQ), making it impossible to determine exact percentage recoveries within the linear range of the method. Samples of the finished raw bars exhibited a dense, highly homogeneous structure, which is characteristic of this type of product (Picture 3).

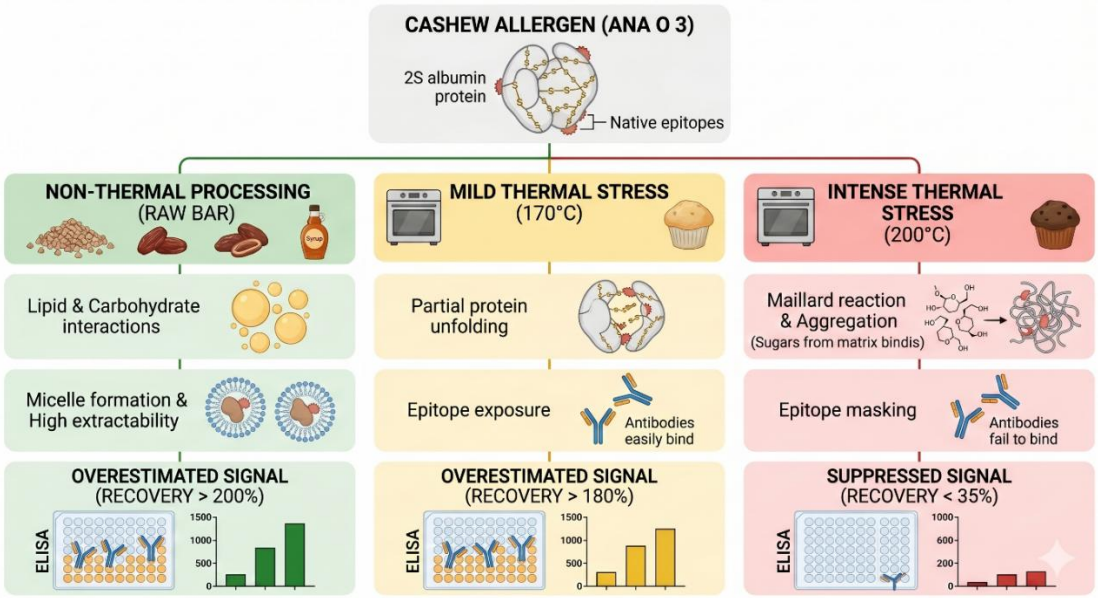
These findings indicate that cashew proteins in the raw bar matrix remained highly extractable and readily detectable by the ELISA method. In the absence of thermal processing, protein epitopes are expected to remain largely accessible to antibody recognition. In addition, the homogeneous structure of the raw bars may facilitate efficient extraction of proteins into the aqueous phase. Consequently, the observed signal enhancement most likely reflects high extraction efficiency and favorable matrix effects rather than an actual increase in allergen concentration.

Comparative evaluation of the impact of technological processing

By comparing all three model food systems investigated (raw bars, muffins baked at 170 °C, and muffins baked at 200 °C), several fundamental principles governing the influence of food processing on allergen detectability can be identified:

1. Effect of thermal processing intensity. Within the muffin matrix, where composition was held constant, the analytical response obtained by ELISA decreased as baking temperature increased from 170 °C to 200 °C: partial protein denaturation at 170 °C was associated with epitope exposure and, in some cases, apparent signal overestimation, whereas baking at 200 °C was associated with pronounced signal suppression, consistent with protein aggregation, reduced extractability, and epitope masking. ELISA responses for the same nominal allergen concentration differed by more than fifteen-fold between these two baking regimes. The non-thermally processed matrix (raw bars) also showed elevated signal and high epitope accessibility relative to the 200 °C muffins; however, because this matrix differs from the muffins in composition as well as in thermal history, this difference should be attributed to overall matrix type rather than to thermal intensity alone.
2. Effect of prior processing of cashew kernels. Cashew kernels that had undergone roasting before incorporation into the model products consistently exhibited the lowest recoveries and the greatest reduction in detectability across all matrix types. The combination of roasting followed by baking at 200 °C produced the most pronounced decrease in ELISA response, suggesting cumulative effects of repeated thermal exposure on protein structure. In contrast, native and blanched kernels generally maintained higher levels of immunoreactivity, likely due to differences in protein conformation and extractability.

A schematic overview of the mechanisms affecting the immunochemical detection of cashew allergens as a function of technological processing is presented in the Picture 4.



Picture 4 Schematic overview of the mechanisms influencing the immunochemical detection of cashew allergens under different thermal processing conditions.

CONCLUSION

The recoveries reported in this study describe the ELISA-detectable analytical signal relative to the nominal fortification level, and not a directly measured change in the total mass of allergenic protein or in its biological allergenic potency. A recovery departing from 100% indicates that the immunoassay under-detects or over-detects the added cashew material under the tested conditions; it does not, by itself, establish that the true allergen content or clinical allergenicity of the food changed to the same extent. This distinction is particularly relevant for the low-recovery scenarios discussed below, where the practical concern is analytical under-detection rather than an actual reduction in the allergen present in the product.

The present study demonstrated that the performance of commercial ELISA kits for cashew allergen detection is strongly influenced by both food matrix characteristics and technological processing conditions. In addition, weight loss caused by water evaporation during baking resulted in an apparent increase in analyte concentration, highlighting the need for appropriate correction factors when interpreting analytical results.

Experimental evaluation revealed two contrasting analytical phenomena associated with different processing conditions:

- **Signal enhancement (matrix enhancement)** was observed in non-thermally processed raw bar matrices and, to a lesser extent, in muffins baked at 170 °C, with ELISA responses frequently exceeding the upper limit of quantification (ULOQ). This pattern is consistent with high extractability and increased epitope accessibility, plausibly linked to partial protein

denaturation at 170 °C, although these mechanisms were not directly measured in this study.

- **Signal suppression and epitope masking** were observed following baking at 200 °C. This suppression is consistent with extensive protein denaturation, aggregation, and Maillard-type reactions reducing allergen extractability and antibody recognition, as reported for related systems in the literature, although the present study did not directly measure these structural changes. The lowest recovery value (14.5%) was recorded for roasted cashews subjected to combined thermal processing, indicating a considerable reduction in allergen detectability and a potential risk of allergen underestimation.

These findings demonstrate that analytical results obtained using ELISA cannot be interpreted independently of the processing history of the food matrix. Thermal treatment, as well as prior processing of cashew kernels, substantially affected allergen detection and may lead to either overestimation or underestimation of allergen content.

From a practical perspective, allergen monitoring strategies should consider matrix-specific effects and processing-dependent changes in protein structure. For highly processed food products, complementary analytical approaches such as LC-MS/MS may provide additional reliability and improve the detection of thermally modified allergenic proteins. Furthermore, optimization of extraction procedures may help reduce matrix-related masking effects and improve analytical performance.

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