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***Research article***

# Formulating preservatives to enhance stability of crude extracts of food allergens used for food allergy management and immunotherapy

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**Abstract:** Due to rapid degradation of food allergenic extracts, devising some optimal conditions is mandatory to boost shelf life of extracts for appropriate diagnosis of allergy and immunotherapy. In the current study, food extracts of *Bos domesticus* (cow) milk, *Gallus domesticus* (chicken) egg, *Trititcum aestivum* (wheat), *Gallus domesticus* (chicken) meat and *Arachis hypogaea* (peanut) were prepared and their protein estimation was evaluated by bicinchoninic acid (BCA) method. Stability of food extracts was evaluated under two groups of preservatives; storage with coca's solution (mixture of sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$)) and stabilizing buffer (sucrose and ethylene diamine tetra-acetic acid (EDTA)). Effect of cocktail of protease inhibitors ((phenyl methyl sulfonyl fluoride (PMSF) and dithiothreitol (DTT)) and glycerol was checked on food extracts under both groups of preservatives. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS PAGE) was performed for food protein extracts after 1, 3 and 6 months under both conditions. Size of obtained protein bands were compared with allergen database (http//: www.allergen.org) in order to check their potential allergenicity. Extracts stored with coca's solution remained more potent as compared to stabilizing buffer for up to 3 months except *Gallus domesticus* egg, whose proteins remained more potent in stabilizing buffer as compared to coca's solution. Most of the proteins deteriorated at 6 months of storage. Glycerol had shown better results under both conditions. A formulation containing a combination of coca's solution along with cocktail of protease inhibitors and glycerol improved the shelf life of food extracts for up to 3 months, showing better potential for stabilization of allergenic food extracts and their use for immunotherapy.

**Keywords:** allergy; food extracts; allergen stability; coca's solution; stabilizing buffer; immunotherapy

## 1. Introduction

Food allergy has recently been recognized as a growing public health burden and named as the "second wave" of the allergy epidemic, following asthma [1]. Recent studies revealed that food allergies are very common in some countries and are increasing in prevalence as compared to the previous year's [2]. Different factors such as geographical location, food patterns and environmental factors result in a strong impact on the basic common types of food allergies in each specific region. In most of the countries, cow's milk, egg, peanut, tree nut, fish, shellfish, wheat and soya contribute a major role in allergic inflammatory reactions. Food based allergic reactions and their types cause allergic reactions differently in different age groups. For example, milk allergy are more common among younger children, while allergic reactions occurring by peanut, tree nuts, fish and shellfish are more prevalent among adults [3,4].

Currently, the possible way to treat food allergy is allergen-specific immunotherapy (AIT) which involves several injections at increasing doses of the allergenic extracts to achieve immunological tolerance over time [5]. It is a skill that technically needs efficient training and proper handling. Extracts used for allergy diagnosis and therapy are a heterogeneous mixture of proteins [6]. They are prepared by collection of raw materials followed by steps of complex series. Allergen extract(s) are assumed to contain all the distinct minor and major allergens and be biologically potent [7]. The extracts show maximum potency when they are freshly made but deteriorate on storage. The loss in potency affects the results of allergy diagnosis and immunotherapy [8]. Inaccuracy or inappropriate approach leads to fatal allergic reactions in patients that are subjected to immunotherapy techniques. One of the most important aspects about immunotherapy is stability of allergen extracts and it can be affected by increasing relevant temperature, contamination with irrelevant aerosols, environmental particles and degradation of extract protein by proteolytic enzymes [9]. Therefore the efficacy of immunotherapy would be affected by protein degradation. In order to reduce the risk of extract potency loss and its stability, health care personnel and manufacturers take several specific measures [6].

Some salts like sodium chloride (NaCl) have the important property to stabilize proteins through their nonspecific electrostatic interactions depending on the ionic strength of the medium. Therefore a mixture of salt can be used to enhance the stability of allergic extract, known as coca's solution (the combination of sodium chloride and sodium bicarbonate (NaCl and $NaHCO_3$) [10]. There exists a controversy regarding the origin of the effect produced by salts, but the correlation between the effect of salt on protein structure has been elaborated by the Hofmeister series [11].

Some additional stabilizers have also been reported that have the ability to maintain the integrity of proteins and structure of allergens in solution and restrict them from binding to glass vials in which they are contained [12]. There are some additives such as sucrose and trehalose that have the ability to protect protein either in lyophilized form or stored under dry conditions [12]. Although, mechanism of additives for the stability of proteins is still controversial, but a hypothetical fact shows that dehydration of proteins follows recovery of proteins upon subsequent rehydration [13]. Another important protein stabilizer is ethylenediamine tetraacetic acid (EDTA). It is serine, cysteine, calpain and metalloproteases that is commonly used as metal chelator [14,15]. The loss of stability in

allergenic extract might also occur at lower or room temperature i.e. (22 °C) because of the possibility that proteases can be activated at these relevant temperatures and has ability to degrade the allergenic proteins present in extracts. In order to avoid this, protease inhibitors are added that inhibit the function of proteases to do proteolysis. Important protease inhibitors are phenyl methyl sulfonyl fluoride (PMSF) [14,15] and dithiothreitol (DTT) that provide protection to the extracts against the long term exposure to room temperature, as it has significant impact on proteolytic enzymes [6]. PMSF is an irreversible inhibitor of serine proteases, including trypsin and chymotrypsin. It also inhibits cysteine proteases and mammalian acetylcholinesterase [14].

Another major stabilizer that is used to enhance stability of proteins includes glycerol. The basic function of glycerol is to provide restriction to proteases to do protein degradation and stabilizes protein in extract. It also acts as bacteriostatic at concentration of ≥20%. Meanwhile there is one drawback of glycerol that it causes irritation to skin when present in higher concentration. By decreasing the concentration of glycerol, the potency of glycerol to stabilize allergic extracts also reduces [9].

The aim of the present study is to enhance the shelf life of crude food allergen extract with the help of coca's solution and stabilizing buffer under two conditions; with and without cocktail of protease inhibitors, with and without glycerol. These formulations may have promising role in stability and preservations of other proteins as well.

## 2. Material and methods

The acrylamide/bis-acrylamide (crosslinking degree of 2.7%), glycine, sodium dodecyl sulphate (SDS), ammonium persulphate (APS) and tris-Cl were purchased from Bio-Rad Company. Coomassie brilliant blue R-250 and N,N,N',N'-tetra-methyl-ethylenediamine (TEMED) were purchased from Sigma company. Coca's solution was prepared by adding 2.5 g of sodium chloride and 4.5 g of sodium bicarbonate in 100 mL of distilled water [16]. DTT (Lot 2W002753) was purchased from AppliChem GmbH, Germany. PMSF (Lot 42771) was purchased from neoFroxx GmbH, Germany. 0.5 M EDTA (pH 8) was prepared in the lab.

### *2.1. Collection, preparation and protein estimation of food samples*

For extract preparations of food allergens, *Bos domesticus* (cow) raw milk was taken from the Sahiwal-breed cow from Khokhar Pind near Lahore, Punjab, Pakistan. *Gallus domesticus* (chicken) meat and eggs were taken from the Tollinton market, Lahore, Punjab, Pakistan. *Trititcum aestivum* (wheat) seeds were taken from the market of Narowal, Punjab, Pakistan. *Arachis hypogaea* (peanut) samples were taken from the market of Chiniot, Punjab, Pakistan. Food extracts of *Bos domesticus* (Cow) milk, *Gallus domesticus* (chicken) egg, *Trititcum aestivum* (wheat) and *Arachis hypogaea* (peanut) were prepared as described by Zahra et al. [17] with some modifications. *Gallus domesticus* (chicken) meat extract was prepared as described by Omana et al. [18]. Bicinchoninic acid (BCA), a calorimetric method, was used for detection and estimation of allergenic proteins in the food samples [19].

*2.2. Preparation of coca's solution, stabilizing buffer, protease inhibitors and glycerol*

For the extraction method with coca's solution 1 g dry weight of sample was added in 10 mL of coca's solution. The mixture was stirred for 24 hours at 4 °C in a magnetic stirrer and centrifuged at 12000 × g for 10 min at 4 °C to obtain the supernatant [16]. Stabilizing buffer was used by preparing 200 mM EDTA and 50 mM sucrose with constant shaking for 72 hours. A cocktail of protease inhibitors was prepared by using 0.1 mM dithiothreitol (DTT) and 0.5 mM phenyl-methyl-sulfonyl fluoride (PMSF) with 3 hours of consistent shaking. Glycerol (10%) was prepared by adding 10 mL of glycerol in 90 mL of autoclaved distilled water.

*2.3. Storage of extracts with coca's solution, stabilizing buffer, protease inhibitors and glycerol*

Two sets of crude extracts were prepared. One set of food extracts was stored with coca's solution (as mentioned above) under two conditions; with and without protease inhibitors cocktail (100 µL), with and without glycerol (10%). The other set was stored with 50 µL of stabilizing buffer. A 100 µL cocktail of protease Inhibitors (PMSF and DTT each) and 10% glycerol solution was used to add in half of the samples. The other half of food extracts were proceeded without protease inhibitor cocktail and glycerol. The extracts were filtered through 0.22 µm filters and stored for the study of protein stability assays after 1, 3 and 6 months at 4 °C.

*2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*

Samples of food extract proteins were diluted in SDS-PAGE gel loading buffer prepared with β-mercaptoethanol added as a reducing agent. Samples were heated at ~95 °C for 5 min before loading in 10% resolving gel [20]. A 3 µL sample of pre-stained Precision Plus molecular weight marker proteins (Thermo fisher) was loaded on each gel to allow the estimation of apparent molecular weight (MW) of the proteins. Electrophoresis was conducted at 125 vdc for 100 min and stopped before the dye-front reached the gel edge. After separation, gels for staining were fixed in 7% (v/v) acetic acid, 40% (v/v) methanol for 1 hr, then stained overnight in colloidal brilliant blue R250 (Bio-Rad) [20]. Gels were de-stained by immersing in 10% (v/v) acetic acid, 25% (v/v) methanol de-staining solution to reduce the background staining for 1 min followed by multiple changes of 25% (v/v) methanol until the background was clear. Images were captured using a camera. Image intensities were adjusted manually for optimum reduction of background colour and band maximum band intensity [20]. This procedure was repeated for all the food extracts after 1, 3 and 6 months.

*2.5. Identification and determination of molecular weights of protein bands by comparison with allergen database*

An allergen database (www.allergen.org) contains approved and officially recognized allergens. Allergic proteins were determined by entering the scientific name of food such as cow milk (*Bos domesticus*), chicken (*Gallus domesticus*) meat and egg, wheat (*Triticum aestivum*) and peanut (*Arachis hypogaea*). Allergenic proteins of different molecular weights were identified by comparing them with already reported allergens in the database as described by Zahra et al. [17].

## 3. Results—analysis of food allergenic extracts stored with coca's solution and stabilizing buffer

### 3.1. *Bos domesticus (cow) milk*

Standard extract were resolved into 6 bands on SDS-PAGE and 4 allergenic bands as depicted by www.allergen.org. After one month of storage, extracts stored with coca's solution along with protease inhibitors cocktail and glycerol were closer to standard extract in terms of protein profile (Figure 1). Crude extracts stored with coca's solution were comparable to standard at month 3 as compared to the extracts stored with stabilizing buffer. After 6 months of storage, degradation was evident in all the extracts except coca's solution with protease inhibitors (Figure 2).

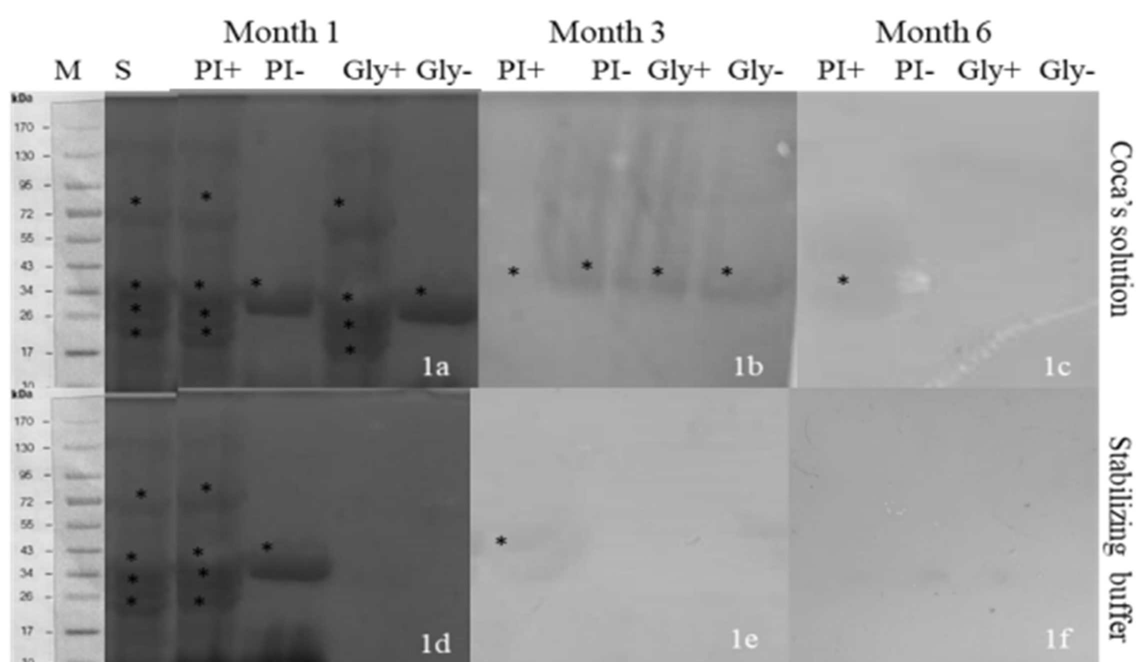


**Figure 1.** SDS-PAGE analysis for the stability assay of *Bos domesticus* (cow) milk allergenic extracts using coca's solution (a–c) and stabilizing buffer (d–f) under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months. M: prestained protein marker; S: standard extract. *sign shows allergenic band.

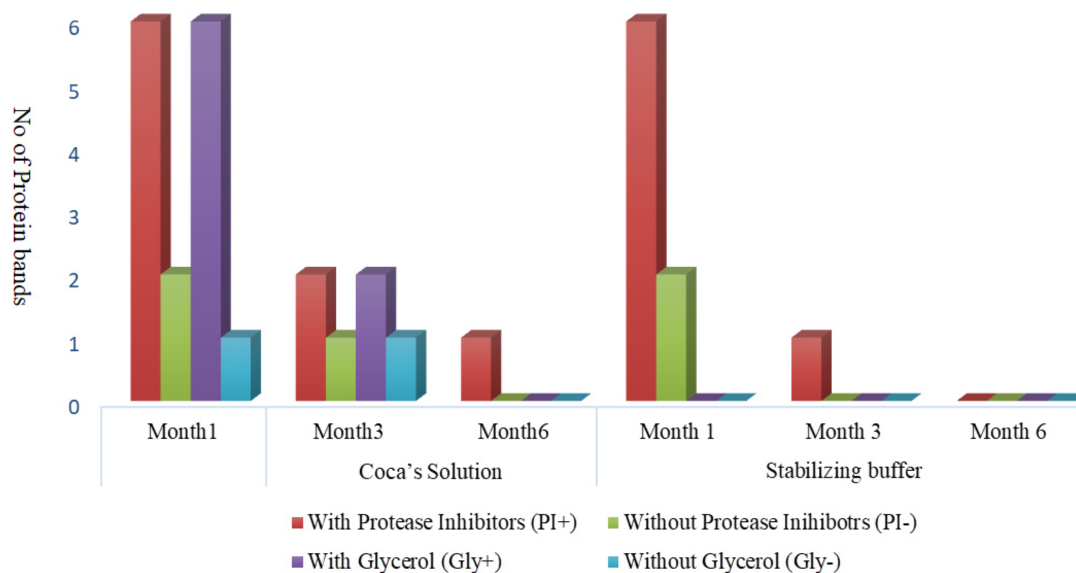


**Figure 2.** Graphical representation of SDS-PAGE for the stability assay of *Bos domesticus* (cow) milk allergenic extracts using coca's solution and stabilizing buffer under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months.

### *3.2. Gallus domesticus (chicken) egg*

After 1 month of storage, the protein bands of *Gallus domesticus* (chicken egg) extract in coca's solution showed significant number of bands in comparison to standard extract. Resolution of the bands was clear even after 3 months of storage (Figure 3). But, the allergenic proteins in coca's solution were seen a bit degraded after 6 months of storage. In case of stabilizing buffer, prominent protein band appeared after one-month storage that was significantly comparable to standard extract. After 3 months' storage, the bands appeared slightly degraded but more deterioration of proteins were seen in extracts after 6 months of storage. Extracts stored with stabilizing buffer and glycerol had shown better results as compared to extracts with coca's solution (Figure 4).

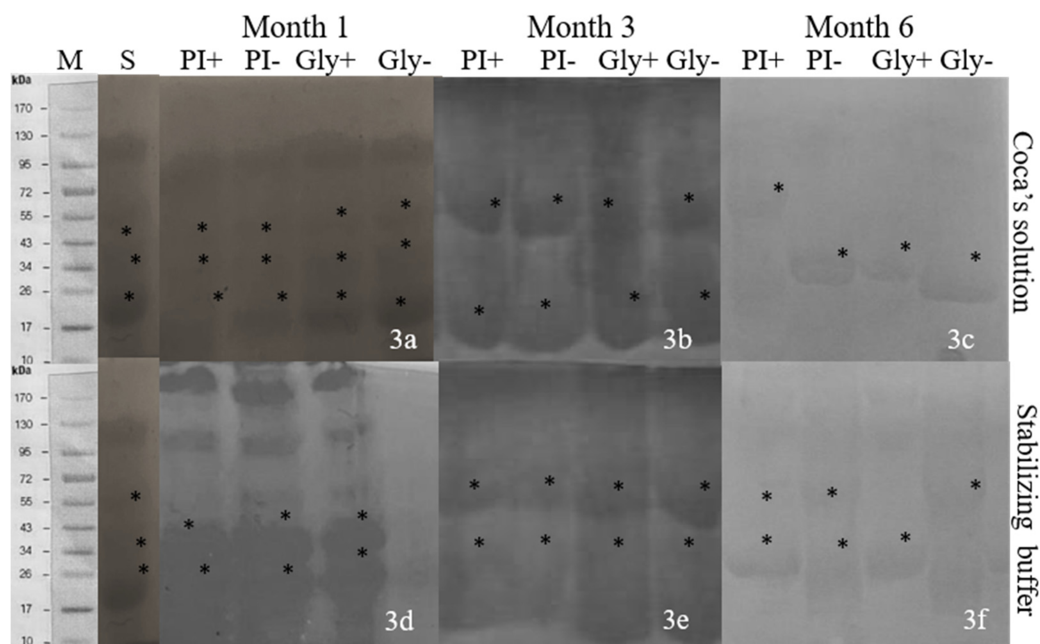


**Figure 3.** SDS-PAGE analysis for the stability assay of *Gallus domesticus* (chicken) egg allergenic extracts using coca's solution (a–c) and stabilizing buffer (d–f) under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months. M: prestained protein marker; S: standard extract. *sign shows allergenic bands.

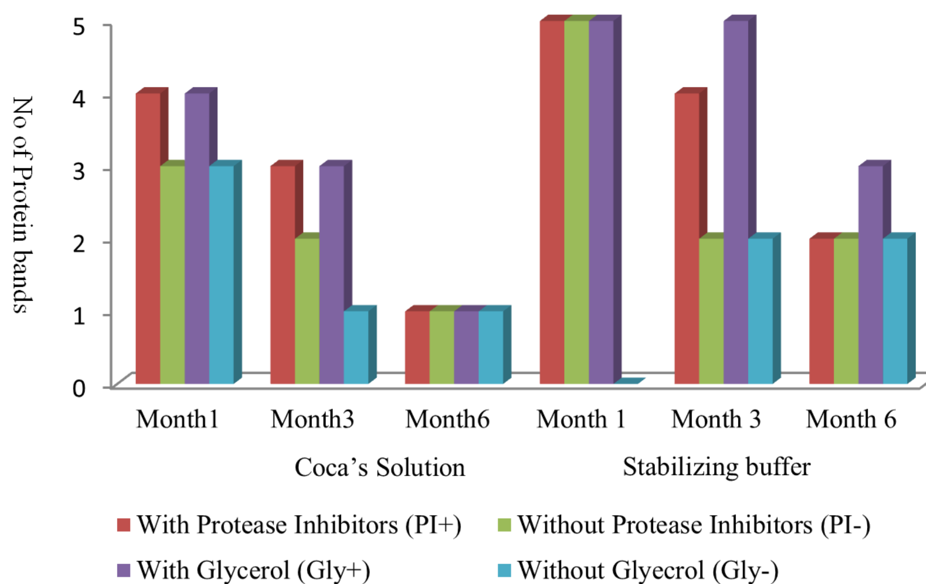


**Figure 4.** Graphical representation of SDS-PAGE analysis for the stability assay of *Gallus domesticus* (chicken) egg allergenic extracts using coca's solution and stabilizing buffer under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months.

### 3.3. *Triticum aestivum (wheat)*

Stability analysis of *Triticum aestivum* (wheat) was observed during 1, 3 and 6 months' storage (Figure 5). After 1 month of storage with coca's solution and stabilizing buffer, the protein bands of wheat extract were almost the same as in standard extract (Figure 6a). Number of protein bands gradually declined after 3 and 6 months in case of stabilizing buffer (Figure 6e,f) whereas results were better in case of coca's solutions even after 6 months (Figure 5). Cocktail of protease inhibitors and glycerol had demonstrated a positive effect on the stability of all the extracts (Figure 6).

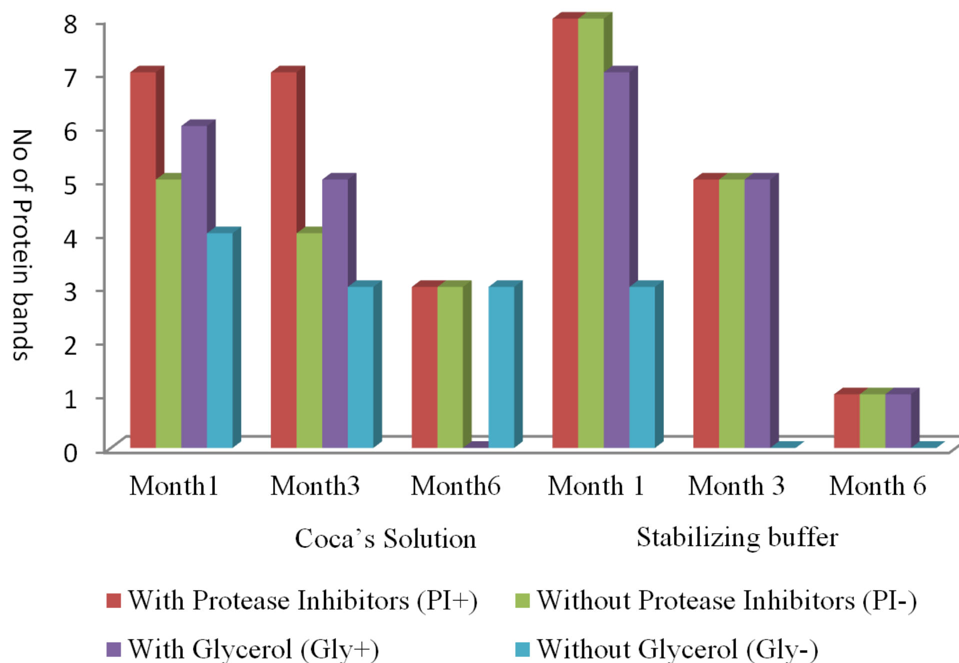


**Figure 5.** Graphical representation of SDS-PAGE analysis for the stability assay of *Triticum aestivum* (wheat) allergenic extracts using coca's solution and stabilizing buffer under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months.

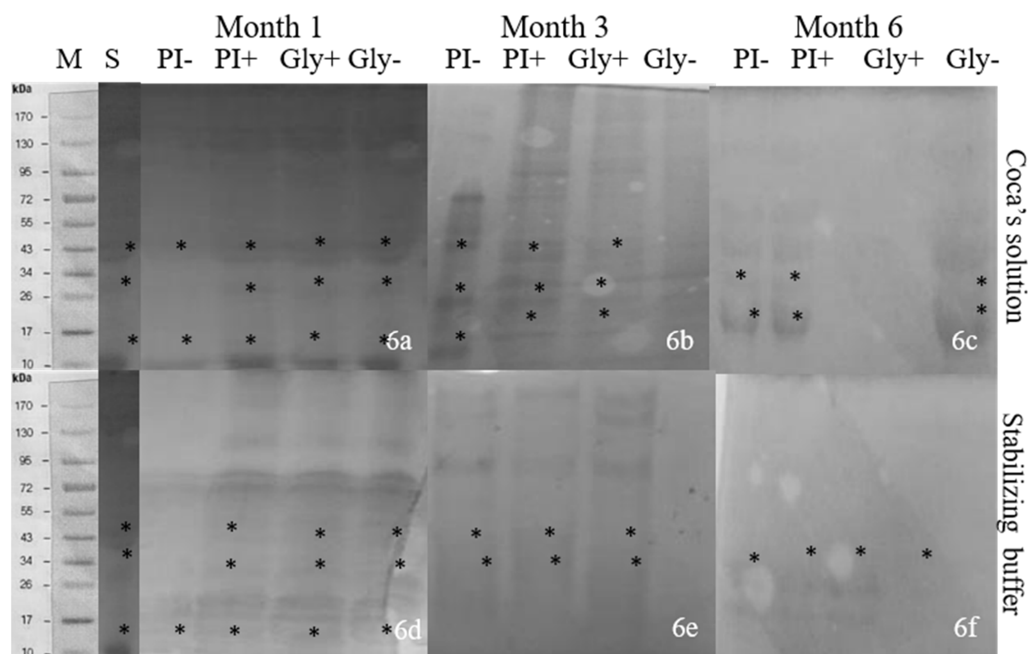


**Figure 6.** SDS-PAGE analysis for the stability assay of *Triticum aestivum* (wheat) allergenic extracts using coca's solution (a–c) and stabilizing buffer (d–f) under four conditions; with protease Inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months. M: prestained protein marker; S: standard extract. *sign shows allergenic bands.

### *3.4. Gallus domesticus (chicken) meat*

After 1 and 3 months of storage with coca's solution, allergenic proteins were moderately comparable with the standard extract (Figure 7). But after 6-month storage, the visibility of protein bands got reduced and substantial protein degradation was observed as shown in Figure 7. In the stabilizing buffer, significant degradation of proteins was seen even after analysis of 1 month storage (Figure 8). After 3 months of storage, protein bands showed complete degradation in all the lanes except with and without protease inhibitors cocktail. Fused bands were observed after 6 months of storage with the stabilizing buffer (Figure 8).

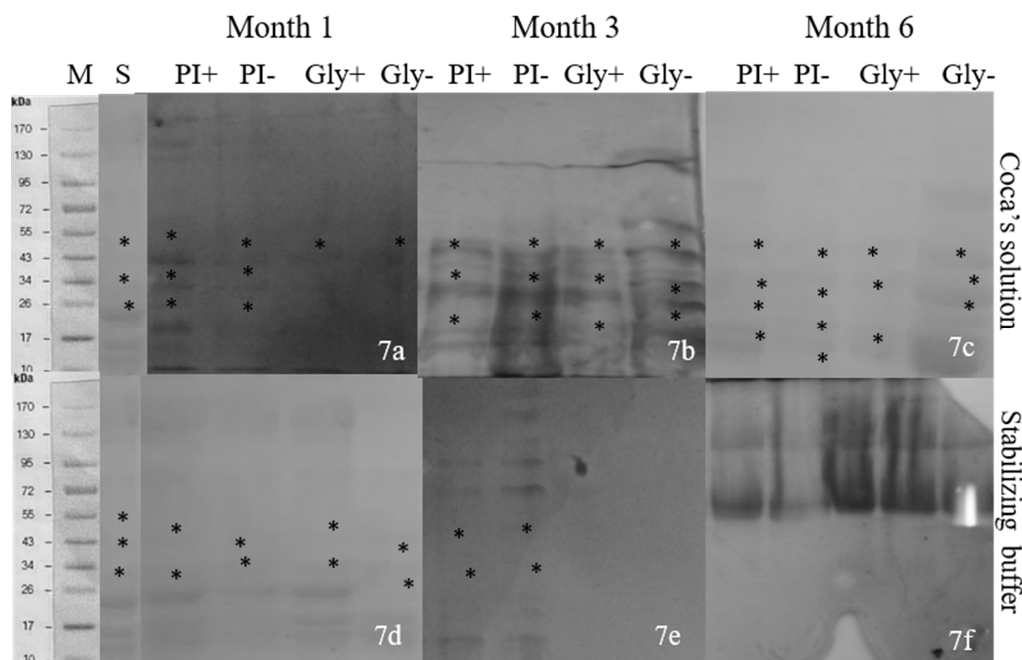


**Figure 7.** SDS-PAGE analysis for the stability assay of *Gallus domesticus* (chicken) meat allergenic extracts using coca's solution (a–c) and stabilizing buffer (d–f) under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months. M: prestained protein marker; S: standard extract. *sign shows allergenic bands.

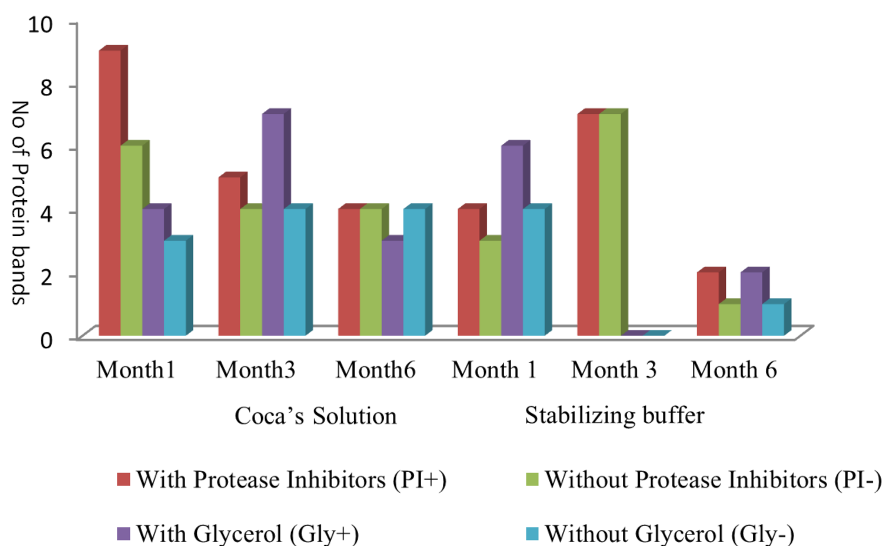


**Figure 8.** Graphical representation of SDS-PAGE analysis for the stability assay of *Gallus Domesticus* (chicken) meat allergenic extracts using coca's solution and stabilizing buffer under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months.

*3.5. Arachis hypogaea (peanut)*

After one month of storage with coca's solution, protein profiling had shown better results when compared with standard extract as seen in Figure 9. Further analysis of allergenic protein stability depicted progressive protein loss in corresponding to the standard extract as seen Figure 5b,c. In case of stabilizing buffer, distinct lane of bands was observed after 1-month storage and it gradually declined in a period of 3 and 6 months (Figure 10).

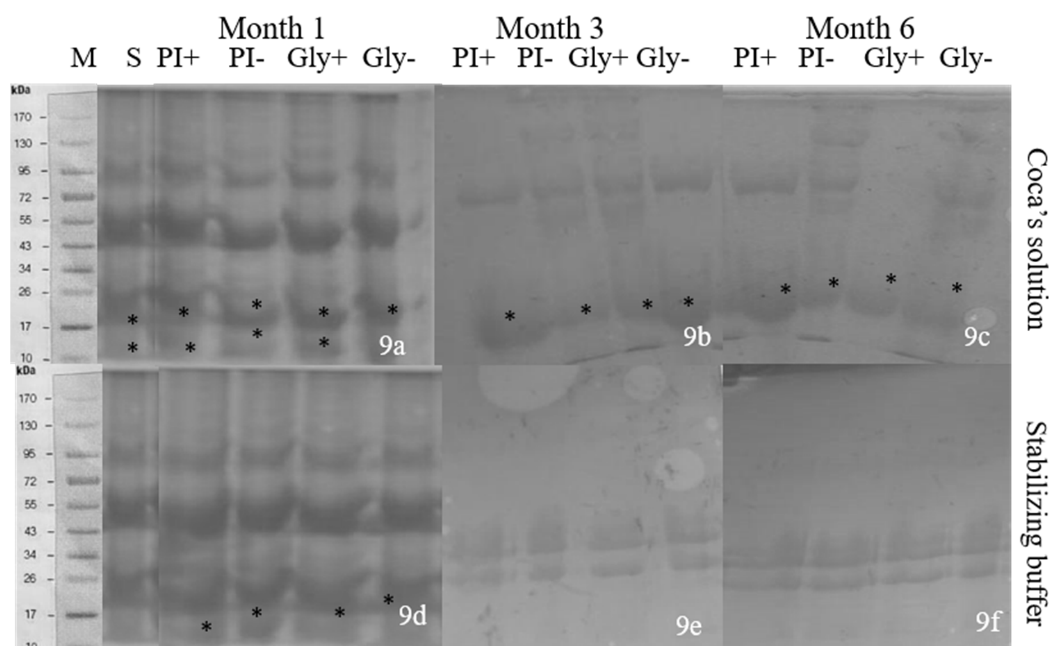


**Figure 9.** SDS-PAGE analysis for the stability assay of *Arachis hypogaea* (peanut) allergenic extracts using coca's solution (a–c) and stabilizing buffer (d–f) under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months. M: prestained protein marker; S: standard extract. *sign shows allergenic bands.

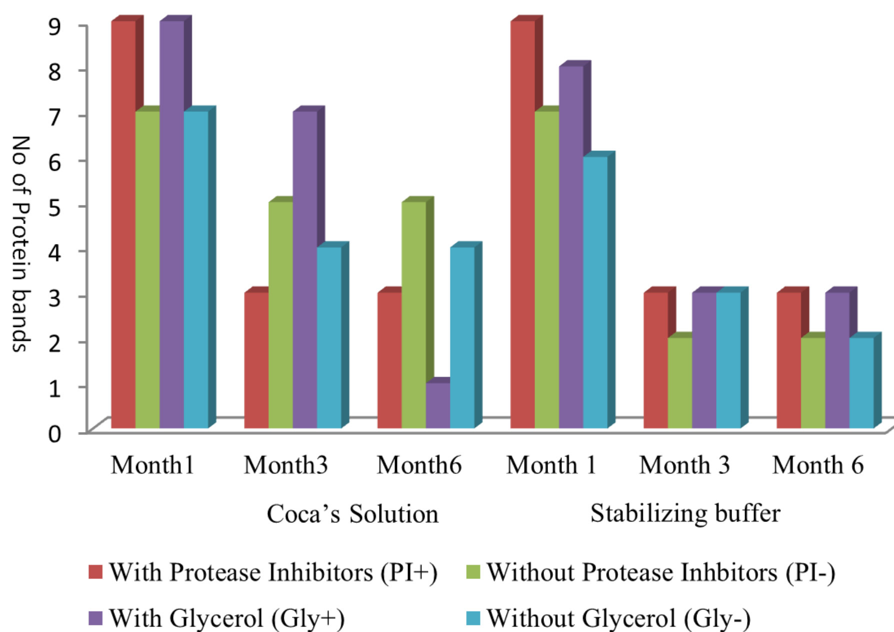


**Figure 10.** Graphical representation of SDS-PAGE analysis for the stability assay of *Arachis hypogaea* (peanut) allergenic extracts using coca's solution and stabilizing buffer under four conditions; with protease inhibitors cocktail (PI+), without protease inhibitors cocktail (PI−), with glycerol (Gly+), without glycerol (Gly−) for up to 6 months.

## 4. Discussion and conclusions

Allergen extract stability is of utmost importance for proper allergy diagnosis and effective immunotherapy. Stability and potency of allergen extracts depend upon source material, extraction procedure, storage conditions, etc. [21]. Allergenic extracts are known to contain proteins which may be heat labile/thermostable structural proteins or enzymes (proteases) [22]. Proteases degrade self and other proteins thereby reducing potency and specific activity of proteins in the extract [23]. Storage conditions and preservatives should prevent protein degradation and maintain the shelf life of the allergen extract. Studies revealed that food, pollen, fungi, and mite extracts behave differently on storage [9,22,24] and, therefore, stability of individual extracts need to be evaluated separately. Different food extracts show variable potency, electrophoretic banding pattern, major allergens, and protein content. In view of these, the present study was undertaken to determine a formulation for storage of *Bos domesticus* (cow) milk, *Gallus domesticus* (chicken) egg, *Trititcum aestivum* (wheat), *Gallus domesticus* (chicken) meat and *Arachis hypogaea* (peanut) extract. Previous studies with phenol and Tween 80 showed deleterious effects on proteins in extracts [22]. Human serum albumin used to prevent adsorption of proteins to vial surfaces was not an effective stabilizer [22]. It is also not preferred due to fear of HIV and diseases associated with blood products.

Coca's solution contains NaCl and $NaHCO_3$ [16] and has been previously for the protein extraction of *Dermatophagoides pteronyssinus* [16]. In present study, coca's solution had shown overall positive results for protein stability of *Bos domesticus* (cow) milk, *Trititcum aestivum* (wheat), *Gallus domesticus* (chicken) meat and *Arachis hypogaea* (peanut) as compared to stabilizing buffer.

Sucrose had been major component of stabilizing buffer and it has been used earlier for stabilizing many biological allergen extracts [22,25]. It acts as a competitive inhibiting substrate for sugar cleaving enzymes, stabilizing the folded state of proteins, or may change protein conformation rendering it less susceptible to proteolytic degradation [22]. In present study, sucrose had maintained potency of *Gallus domesticus* meat allergens similar to standard extract for 6 months more effectively as compared to protease inhibitors cocktail.

Several reports show the self-degradation of allergens by enzymes called proteases in the extracts [21]. Food extracts are composed of these proteases. Therefore, in present study, serine and aspartate protease inhibitors, namely PMSF and DTT, were tested in cocktail for stabilization. The stabilizing effect depends on the specificity of these inhibitors, classes of proteases, and their stability in solution. These protease inhibitors stabilized food extracts for upto 3 months. Although the extract stored for 6 months with a combination of preservative showed mild degradation and a drop in relative potency. Glycerol (50%) offers stabilizing properties to proteins in diagnostic allergen extracts [26]. But glycerol at this concentration has irritant effect at this concentration and cannot be used in therapeutic vaccines. Food extracts stored with lower glycerol concentrations (10%) showed better potency in the present study.

In conclusion, coca's solution had better result in terms of stabilization of proteins as compared to stabilizing buffer. Most of the extracts shown prominent bands for up to one month and then started degradation. Cocktail of protease inhibitors and glycerol (10%) had positive impact as compared to absence of cocktail and glycerol. A combination of coca's solution, protease inhibitors cocktail and glycerol (10%) may have promising role in combating allergy complications and allergen immunotherapy. The recent findings may pave the way to use coca's solution and stabilizing buffers for the stability of various other proteins as well irrespective of their use in stability of mere food allergens. The formulations are reproducible in other protein extract preparations and preservations. The potential allergens obtained in this study can be further confirmed by sera IgE-immunoblot in next phase study.

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**Conflict of interest**

All authors declare no conflicts of interest in this paper.

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