Water quality is extremely important for enzymatic activity, as it is a type of catalysis that occurs in an aqueous environment. The three-dimensional protein structure of enzymes is highly dependent on the possibility of hydrogen bonding between active functional groups in the structure of amino acids (hydroxyl groups of serine, tyrosine or threonine or carboxyl groups not included in the peptide bond of aspartic or glutamic acid) or intra / inter-protein hydrogen bonding in the case of the α-helix or β-sheet structure of the polypeptide chain, all of which are favored by the aqueous medium [1].

Proteases, amylases, lipases and cellulases are enzymes commonly used in detergents to improve their washing properties to reduce the cost of this process - reducing the temperature of the washing process, water consumption and, at the same time, the impact on the environment - reducing the concentration of potentially toxic ingredients on the environment [2]. Enzymes produced by biotechnological processes have the advantage of being detergent-compatible enzymes with an optimal temperature of the washing process, water consumption and, at the same time, the impact on the environment - reducing the concentration of potentially toxic ingredients on the environment - reducing the environmental impact by using more biodegradable components related to organic and inorganic components. The purpose of our study was to evaluate the impact of environmental factors such as the variability of water hardness in the public water supply network and the temperature applied to the amylolytic, lipolytic and proteolytic enzyme activity of 6 detergents available on the market. The determinations were performed by modified methods from the European Pharmacopoeiae 8th Edition in determination the enzymatic activity of the pancreas powder. The determinations show a significant influence of increased water hardness on lipase and protease activity in detergent (p <0.01 *) between extreme hardness values, while amylolytic activity does not vary significantly with increasing water hardness.

Keywords: enzyme laundry detergent, amylase, lipase, protease, water hardness

Water hardness caused by the presence of calcium and magnesium ions, can influence the enzymatic activity, metal ions acting as enzyme modulators - activating effect or, on the contrary, inhibiting effect (poisons) by altering the electrical charges in the active center, becoming more or less capable of binding the substrate, actively participating in oxidation-reduction processes or forming insoluble complexes with the reaction products. On the other hand, metal ions can cause protein-enzyme denaturation by dehydration [10], so the catalytic effect is altered. Therefore, obtaining stable alkaline pH enzymes over a wider temperature range and a catalytic activity independent of water hardness is one of the desires of the detergent chemical industry [11-13].

Although enzyme detergents are industrially manufactured since the beginning of the last century, they seem to be a future solution to saving the planet’s resources (water, electricity, etc.) therefore the purpose of this study is to determine the enzymatic efficacy of detergents with declared enzyme content under the conditions of variable amounts of metallic ions in the washing water, starting from a controversy in the literature: on the one hand they are environmentally friendly by the lower content of chemicals, on the other hand detergent components such as sodium dodecyl sulfates are environmental pollutants.
by denaturing enzymes from living organisms that come into contact with polluted water [14].

Taking into account all these facts, the aim of the present work was to study if there is any influence of the water hardness on the proteolytic, amylolytic and lipolytic activity of enzymatic detergents, during a cleaning cycle in the washing machine.

**Experimental part**

Water samples were obtained from different counties - from the public water supply system or water from the hydrophore and used domestically.

Six liquid enzyme detergents on the Romanian market were selected, which were coded as D1-D6.

Determination of the enzymatic activity of detergents was performed by methods described in European Pharmacopoeia 8th Edition for pancreatic powder and adapted for detergent determinations [15].

The standard substance was prepared from 150 mg of pancreatic powder (10,000 units of lipase, 8,000 units amylase, 600 units of protease activity quantified by Ph Eur. method) of pharmaceutical purity suspended in distilled water so that it was diluted 50 times.

The detergent sample was diluted 100 times as recommended by manufacturers directly in U1 Ultra Thermostatic Water Bath with water of different hardness.

**U, Ultra Thermostatic Water Bath**

Determinations were performed using U, Ultra Thermostatic Water Bath (fig. 1) produced by Medingen with the following technical characteristics: power = 650 W, thermostat, motor 1450 rpm and flow rate 4 L/min.

Determination of amylolytic activity is based on iodometric titration of excess starch left unmodified in the presence of amylase from the detergent. The detergent was diluted directly into U, Ultra Thermostatic Water Bath with water of various hardness.

**Results and discussions**

Water hardness from several water sources was analyzed by a complexonometric method and the values obtained were between 2.82-22.81 °dH. Four water sources with low hardness of 2.82 °dH, medium low 5.23 °dH, medium high 11.51 °dH and high 22.81 °dH were selected for analysis. The influence of temperature on enzymatic activity is shown in table 1.

Enzymatic activity increases with increasing temperature up to one point (considered to be the optimal temperature), after which this activity decreases. Detergent manufacturers recommend the use of enzyme detergents at 20°C, which would reduce electricity consumption. From the graphical representation of enzymatic activity as a function of temperature it is observed that at 20°C the enzymes are less active, the highest activity being around 37°C. Unlike in vivo active enzymes, in the case of genetically engineered enzymes, the enzymatic activity does not decrease drastically as temperature increases, the enzymatic activity does not become 0 not even at temperatures above 60°C (fig. 2). We note, however, a significant increase in the variability of the protease enzyme activity at high temperatures, which can be explained by a direct influence of temperature increase on the structure of the substrate (denaturation of proteins with temperature rise), casein being much more sensitive to temperature increase compared to starch or oily emulsion.

This variability of enzymatic activity with temperature is best described by a 3rd order polynomial equation, which raises the question of interpreting these data - the enzymatic activity should increase with temperature,
decrease and then increase again. For the mathematical
determination of the optimum temperature for enzymatic
activity, the order II derived function of the mathematical
equation expressing the variation of the enzymatic activity
as a function of temperature was calculated. Even though
a quadratic equation has two distinct solutions, we must
consider that the water temperatures in the public water
supply system have positive values and that the optimal
temperature values above 50°C are biologically improbable.
Thus, in the case of amylase, the calculated mathematic
value of optimum temperature differs from that in vivo [16]
and is 34°C and for lipases and proteases is 33 and 27°C,
respectively. Obtaining enzymes with amylolytic activity
in genetically modified bacterial cultures provides thermic
resistance and a reduced sensitivity to pH variations, but
even in these cases the presence of EDTA chelants in the
environment reduces enzymatic activity by up to 22%. A
drawback identified by industrial producers is the difficult
and expensive isolation and purification of these varieties
[17]. Literature data show that lipase activity is maximal
at a temperature of 40°C and at a pH between 4.3-7.2. In
the case of detergent lipase, maximum catalytic activity
is also influenced by the alkaline pH value in the
environment, which can increase the sensitivity of the
enzyme to low temperature variations [18, 19]. Data from
the literature show that the activity of proteases of bacterial
origin in detergents is optimal in the range of 30-40°C and
alkaline pH (pH = 9-11) seems to favor the catalytic
process [20, 21].

Water hardness influence on the amylolytic activity of
detergents
The results obtained in determining the activity of the
amyloses in the detergents are shown in table 2.

| Temperature (°C) | Amylase activity | Lipase activity | Protease activity |
|-----------------|------------------|-----------------|------------------|
|                 | Average U/L (DSR%) | Average U/L (DSR%) | Average U/L (DSR%) |
| 10              | 3.7732 (4.36)    | 24.012 (5.85)   | 2.883 (6.71)     |
| 20              | 6.9979 (6.45)    | 48.285 (10.32)  | 6.0042 (8.78)    |
| 37              | 16.0498 (5.77)   | 30.1265 (9.92)  | 12.4615 (7.73)   |
| 50              | 14.2237 (9.26)   | 11.0237 (8.69)  | 2.8834 (10.82)   |
| 60              | 4.9434 (7.16)    | 7.9355 (10.86)  | 14.135 (10.78)   |
| 70              | 4.1233 (9.58)    | 8.5049 (12.23)  | 12.996 (73.26)   |
| 90              | 4.2558 (10.21)   | 11.2585 (9.13)  | 12.5921 (75.35)  |

Table 1
THE INFLUENCE OF THE WATER TEMPERATURE
ON THE ENZYMATIC ACTIVITY OF DETERGENT D1

Although there are differences in the capacity of
enzymes in different detergents to hydrolyze starch, the
presence of varying amounts of calcium and magnesium
ions in the medium does not appear to significantly
influence the catalytic activity.

Amylase is a metal enzyme containing calcium ions in
the active center, and moreover, a 2013 study by Ghollasi
M et al indicates that obtaining isolated enzymes from
Bacillus megaterium mutant species to which an additional
binding site for calcium ion was introduced by replacing a
histidine residue with glutamic acid, exhibited an increased
thermostability and a wide range of pH in which they are
active (3.5-9.0), which would be of great importance for
the detergent industry [22].

Yadav JK shows that there is a compromise that
detergent manufacturers make between increased
enzymatic activity (Ca²⁺ at low concentrations 1.0-2.0 mM is
α-amylase activator) and enzyme thermostability (Ca²⁺ at
high concentrations is enzyme inhibitor, but in at the same
time the enzyme becomes thermostable) therefore, as
can be seen from figure 3, enzymatic activity of amylase
increases generally with increasing water hardness but at
the highest value (22.81 °dH) the activity decreases [23].

Studies demonstrating the influence of calcium ion on
α-amylase activity use EDTA to complex calcium, its
absence from the environment inactivates enzymes, even
in the case of apparently calcium independent amylase, it
is supposed to be so strongly bonded to the protein structure
that it cannot be removed by EDTA [24].

Binding of calcium ion to the α-amylase protein structure
increases the thermal stability and against various
surfactants and tensoactive substances of the enzyme by
reducing the flexibility of the protein structure, therefore
there is a hypothesis of saturation of the enzymatic structure
with calcium ions a prior to its addition to detergents, which
would explain, on the one hand, the low influence of the water hardness on the enzymatic activity and, on the other hand, the 10-fold higher catalytic activity of amylase at 90°C than the lipase or the detergent proteases (fig. 3) [25].

Water hardness influence on the lipolytic activity of detergents

The results obtained in the determination of lipase activity are shown in table 3.

| Water hardness (°dH) | Amylase activity | Lipase activity |
|----------------------|------------------|-----------------|
|                      | Average U/L (DSR%) |                   |
|                      | D₁ | D₂ | D₃ | D₄ | D₅ | D₆ |
| 2.82                 | 189691 | 329587 | 235175 | 156701 | 230928 | 107216 |
|                      | (9.41) | (12.90) | (9.34) | (11.31) | (11.46) | (8.64) |
| 5.23                 | 140206 | 329587 | 206185 | 123711 | 206168 | 148454 |
|                      | (12.05) | (10.46) | (9.38) | (13.64) | (10.20) | (11.09) |
| 11.51                | 164948 | 373196 | 181443 | 149485 | 247423 | 177732 |
|                      | (13.05) | (6.07) | (11.99) | (12.34) | (16.66) | (8.40) |
| 22.81                | 111237 | 330928 | 173193 | 148454 | 214433 | 173196 |
|                      | (11.66) | (12.01) | (12.69) | (13.12) | (8.38) | (10.76) |

There are statistically significant differences ($p < 0.01^*$) between the enzymatic activity at extreme values of the water hardness for the detergents D₁, D₄, D₅ and D₆ regarding the lipolytic; D₆ has the highest enzymatic activity and the catalytic activity of lipase in detergents D₁ and D₂ is not significantly influenced by the water hardness (fig. 4).

It is well known that lipase is an enzyme activated by calcium ions from the environment because the formation of insoluble salts (soaps) with reaction products, fatty acids, modifies the chemical equilibrium. This problem is
extremely important in the case of lipid-containing pharmaceutical products or foodstuff in emulsified form, the control of digestion by pancreatic lipase of lipid droplets in the intestinal tract raising the issue of the addition of chelants which are suitable for use in vivo such as lyso-lecithin, and β-lactoglobulin [26] or cosurfactants such as Tween 20 [27].

A study by Hu M et al in 2010 shows that there is a minimal concentration of Ca²⁺ in the environment that is required for the lipase enzyme activity; thus, in the absence of Ca²⁺, the enzymatic activity is <12%, while at a concentration of 20 mM Ca²⁺ lipase activity is higher than 95% [26].

In vivo the influence of calcium ions on lipase activity also depends on the nature of the hydrolyzed substrate: in the case of short chain fatty acids (eg milk triglycerides), Ca²⁺ does not activate lipase because the resulting soaps are water soluble, the activating effect being only for the triglycerides of long-chain saturated fatty acids as is the case of sunflower oil triglycerides (Emulsio oleosa being used as the substrate in the determination) or unsaturated and omega 3 or 6 polyunsaturated fatty acids [28].

The enzymatic process catalyzed by lipases and phospholipases is extremely complex - besides the hydrolysis reactions of triglycerides and phospholipids, they also catalyze transacylating and transphosphorylation reactions [29]. Lipases are ubiquitously spread enzymes in nature, but only those produced by microorganisms are used in the manufacture of detergents, cosmetics, surfactants, etc. [30]. To eliminate the shortcomings of microbial cultures (low production yield and hard to be controlled industrially), recombinant heterologous proteins in a host system technique are currently applied [31].

Water hardness influence on the proteolytic activity of detergents

The results obtained in determining the proteolytic activity are presented in table 4.

From figure 5, it is observed that detergent D₂ practically has no proteolytic activity and that the enzymatic activity depends on the hardness of the water - the higher the water hardness the highest the enzymatic activity of the analyzed detergents, the difference between the proteolytic enzymatic activity at the extreme hardness values being statistically significant (p < 0.01*).

Proteases are enzymes active in vivo over a very wide pH range - from pepsin active in stomach (pH = 1.5-3.0) to trypsin and alkaline-active chymotrypsin, thus obtaining commercially active proteolytic enzymes at pH = 8 is not an impediment, but proteases have to be stabilized to prevent inactivation (proteolysis) of other enzymes in detergents. Manufacturers recommend either reducing the water content of detergents or adding stabilizers such as borate/propylene glycol. Since numerous proteases contain calcium in the active site and the added anti-scaling agents can eliminate calcium, its presence in the wash water will increase proteolytic activity [32, 33].

A study by Stoner MR et al shows that calcium ion binding on the protein structure, as with amylases, increases the stability of enzymes and calcium chelators, by forming micelle aggregates, compete with the enzyme for binding Ca²⁺. In this competition for Ca²⁺ binding, account must be taken on the stronger interactions between the protein structure and the calcium ion as compared to chelates, but also that chelating agents are in much higher

| Water hardness (°dH) | D1 | D2 | D3 | D4 | D5 | D6 |
|---------------------|----|----|----|----|----|----|
| 2.82 (7.23)         | 25192 | 9221 | 63462 | 39038 | 25385 | 24808 |
| 5.23 (11.96)        | 34904 | 12279 | 30577 | 31442 | 21346 | 30865 |
| 11.51 (8.43)        | 66923 | 17596 | 75288 | 90865 | 628845 | 82869 |
| 22.81 (8.27)        | 11385 | 20192 | 172789 | 199039 | 130962 | 130962 |

Table 4

THE INFLUENCE OF WATER HARDNESS ON PROTEASE ACTIVITY OF DIFFERENT DETERGENTS
concentrations than enzymes in detergents, therefore the authors recommend addition of CaCl$_2$ in concentrations of $10^{-5}$ M in the detergent composition required for proteolytic activity [34].

The role of proteases in detergents is to remove stains of biological origin [35]; determining the effectiveness of detergents in removing stains is done by visual methods (in the case of colored stains - blood, cocoa), turbidity, densitometric or colorimetric methods. However, it is important that detergents containing enzyme proteases are not recommended for use in wool and silk fabrics because they can irreversibly degrade the secondary keratin structure [36, 37].

A review from 2017 identifies detergents, and especially enzyme detergents, as a pathognomonic cause of atopic dermatitis, causing skin dehydration, skin pH modification, proinflammatory cytokine release and even mediated IgE hypersensitivity [38]. The market for enzyme detergents has much evolved - if in the 1970s type I, IgE mediated allergies were fairly prevalent especially in people with atopic terrain, the prevalence of these allergies nowadays is rare, caused by long exposure or high concentrations of detergent (asthma and allergies to workers in the detergents industry - occupational hazard) [39, 40].

Conclusions

The enzymatic activity of washing detergents is clearly influenced by temperature and various water quality. Higher water hardness significantly influences the lipase and protease activity in detergent, while amylolytic activity does not show significant changes.

Acknowledgment: The authors thank SC SITRONIC SRL for the technical assistance provided by the design and realization of U1 Ultrathermostat Water Bath.

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Fig. 5. Water hardness influence on protease enzymatic activity of different detergents
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Manuscript received: 21.03.2018