

New Collagen Extracts Conditioned for Applications in Crop Protection Against Pests

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This study demonstrates that collagen hydrolysates obtained by associated enzymatic and chemical processes for high-yield extraction of collagen from leather waste, under mild reaction conditions, are fit for application in agriculture. Collagen polydispersions have the ability to form thin films on the surface of cereal seeds and, on the long term, to release amino acids, including essential amino acids, known as growth stimulators and organic nitrogen sources, ensuring increase of viability and of cereal seed and plant protection. Free amino acids have a stimulating effect on germination, while the amino acids released over time from the film matrix have a nutritional role. The amphoteric nature of collagen films, as well as their permeability to air and water vapours, provides beneficial physiological properties for cereal seeds. The synergy of collagen hydrophilicity, its known biodegradability, bio-active potential and film-forming properties recommend collagen polydispersions for applications in mixtures for seed treatment against pests.

Keywords: collagen extracts, free amino acids, contact angle, seed treatment

Collagen is the most abundant and ubiquitous protein in both humans and animals. The hierarchical structure of collagen allows adaptation and optimization of material at each hierarchical level, to generate the outstanding performance that occurs in applications. For example, the extraordinary resistance to stress of the tendon is due to the combined action of structural elements at nanometric and micrometric level [1, 2] and offers a unique opportunity for modulation and adaptation of properties for certain functions [3, 4].

Development of concepts for collagen-based biomaterials began many years ago and new elements are constantly revealed [5, 6].

At the moment there is a rich palette of materials based on protein extracted from collagen and collagen matrix materials, mainly used in medical, pharmaceutical and cosmetics fields, made using primary collagen resources [7, 8]. In order to use proteins in agriculture, secondary collagen resources such as by-products from natural leather processing industry were identified. Most of the research in recent years has focused on extracting collagen from leather waste [9, 10] and using these extracts in crop fertilization [11, 12], as an alternative for the synthesis amino acids used in fertilizer formulae [13, 14]. There is a recent research studying the possibility of using collagen recovered from the leather industry, as an amendment to agricultural soils [15], but the aim has been to recover collagen from untanned hide waste. However, the largest amount of leather waste resulting from tanneries is chrome tanned leather waste (over 20% of the preserved hides that are processed), which is on the one hand, an environmental problem, and on the other hand, an untapped resource of protein. More recent research focuses on the exploitation of bio-active properties of these collagen extracts in seed treatment to increase germination potential and protect against pests [16, 17].

This paper is focused on characterization of collagen extracts recovered from chromium(III) tanned leather waste, a systematically avoided resource, due to the psychological impact generated by the term "chrome", generally associated with the notion of "heavy metal",

"toxicity", "carcinogenic". Only a small segment of the population knows that modern leather industry, which applies best practices, neither uses nor directly discharges hexavalent chromium, generator of extreme toxic effects. Our own previous research [18, 19] has shown that combined processes for extracting collagen from these by-products may have a very high efficiency in separation of chromium, so that its content in collagen polydispersions would be within the strict limits allowed for drinking water (max. 50 ppb), well below the values reported in other works [20]. The present research aims to approach associated chemical-enzymatic processes for collagen extraction with high yields from leather waste, under mild reaction conditions and obtain bioactive polydispersions through the diverse content of free amino acids, including essential amino acids.

Collagen hydrolysates have the ability to form fine films on the surface of cereal seeds and to release free amino acids which may be growth stimulators and sources of organic nitrogen, with long-term release, ensuring increase of viability and of cereal seed and plant protection. The amphoteric nature of collagen films, as well as their permeability to air and water vapours, provides beneficial physiological properties for cereal seeds. Hydrophilicity synergy of collagen, of recognized biodegradation, of the bioactive potential and film-forming properties, recommend collagen polydispersions as a valuable additive in mixtures for seed treatment against pests.

Experimental part

Materials, equipment and methods

Tanned leather by-products having the following characteristics: 39-55 % volatile matter, 3-7% total ash, 4-6% chromium oxide, 15-16% total nitrogen, pH value of aqueous extract 3.5-5.

Chemical materials: calcium hydroxide from Utchim Ramnicu Valcea, Romania, having the following characteristics: 0.5% volatile matter, 60% calcium oxide, sulfuric acid, concentration of 96%, from S. C. Chimopar S.A. Romania, Alcalase 2.5 L from Novozymes, Denmark, with activity of 2.5 enzymatic units/g, optimal enzymatic

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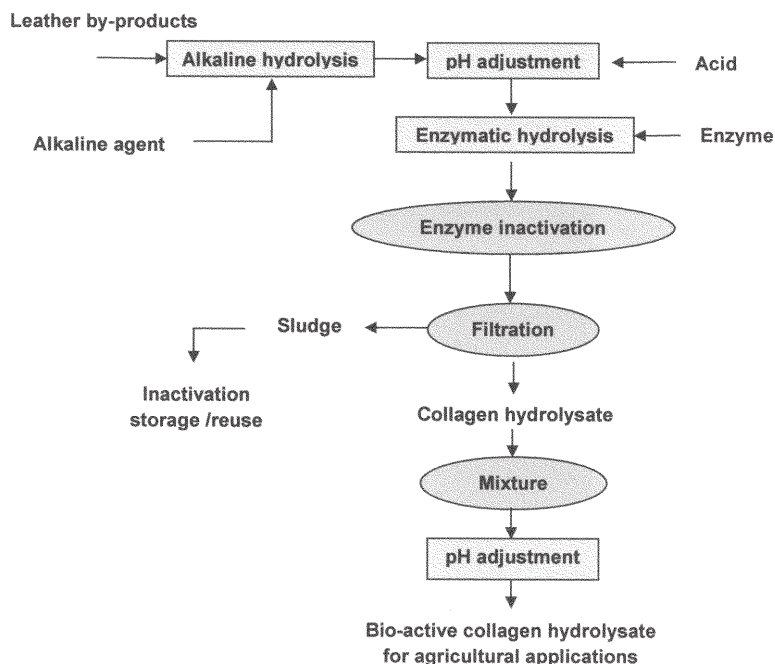


Fig. 1. Workflow for collagen extraction

| No. | Sample | Description |
|-----|--------|---|
| 1 | CH. 1 | Collagen hydrolysate from lot no. 1 |
| 2 | CH. 2 | Collagen hydrolysate from lot no. 2 |
| 3 | CH. 3 | Mixture of collagen hydrolysates from lots no. 1 and no. 2 |
| 4 | CH. 4 | Conditioned mixture of collagen hydrolysates from lots no. 1 and 2, at pH = 7 |

Table 1
DESCRIPTION OF COLLAGEN
HYDROLYSATES SAMPLES

activity at temperature = 60-70°C and pH range = 7-10; acetic acid, concentration of 99 %.

Analytical methods

STAS 8574-92, Finished hides and fur finished hides; SR ISO 5397-96, Finished leathers. Total nitrogen determination; SR EN ISO 4045-02, Finished leathers – Chemical analyses - pH determination; SR EN ISO 13903-05, Animal feeding stuffs. Determination of amino acids content; Sorensen method to determine aminic nitrogen; STAS 8602-90, Finished leathers. Chrome oxide determination.

Chromatography, using a gas chromatograph coupled with a mass spectrometer, AGILENT 7000 GC/MS TRIPLE QUAD Gas Chromatograph, to identify dipeptide, tripeptide and amino acid sequences from collagen hydrolysates and HPLC (Thermo Electron, Finningen Surveier) with Diode Array Detector was used for the qualitative and quantitative determination of aminoacids (according to SR EN ISO 13903).

IR spectral analysis of collagen hydrolysates by FT/IR-4200 (Jasco) with ATR device equipped with diamond reflection crystal, in spectral range: 7800 ÷ 350 cm⁻¹.

Dynamic Light Scattering (DLS), to determine particle size and distribution with ZetaSizer device Nano ZS (Malvern, UK).

The CAM 200 optical system from KSV Instruments was used to measure contact angles under static conditions.

Experimental

Collagen hydrolysates were obtained from tanned leather by-products, by chemical-enzymatic processing, at atmospheric pressure and temperature ≤ 80°C, for a total duration of max. 6 h, followed by processing the resulting product by decanting and filtering, mixing batches and conditioning by adjusting pH at max. 7, according to the workflow presented in figure 1.

Results and discussions

Both intermediary collagen hydrolysates and final ones, obtained after mixing and conditioning, described in table 1, were assessed by chemical and instrumental analyses, to establish chemical and physical characteristics and bioactive properties induced by the free amino acid content.

Results of analyses performed in order to establish the chemical composition of collagen hydrolysates are presented in table 2.

| No. | Characteristics, MU | CH. 1 | CH. 2 | CH. 3 | CH. 4 |
|-----|-------------------------------|-------|-------|-------|-------|
| 1 | Dry substance, % | 8.00 | 8.16 | 8.24 | 8.77 |
| 2 | Total ash, % | 8.50 | 8.95 | 7.89 | 7.87 |
| 3 | Organic substance, % | 91.50 | 91.05 | 92.11 | 92.13 |
| 4 | Total nitrogen, % | 16.13 | 15.69 | 15.78 | 15.17 |
| 5 | Amino nitrogen, % | 1.12 | 1.20 | 1.04 | 1.04 |
| 6 | Average molecular weights, Da | 10800 | 8800 | 12900 | 12900 |
| 7 | Protein substance, % | 90.63 | 88.11 | 88.71 | 85.18 |
| 8 | pH | 9.38 | 9.35 | 8.88 | 6.90 |

Table 2
PHYSICO-CHEMICAL
CHARACTERISTICS FOR COLLAGEN
HYDROLYSATES

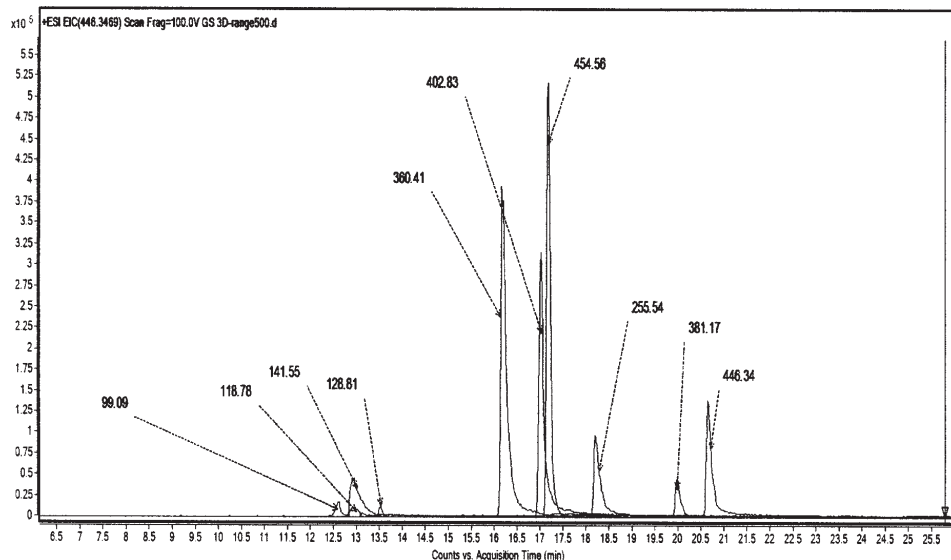


Fig. 2. Chromatography of collagen hydrolysates

Aminic nitrogen content over 1%, corresponding to an average molecular mass below 13000 Da, indicates the possibility of a very high polydispersion of protein fragments. The presence of dipeptide, tripeptide and free amino acid fragments in collagen hydrolysates was investigated by chromatography, using a gas chromatograph coupled with a mass spectrometer.

Figure 2 presents the chromatogram of a collagen polydispersion (CH. 1), with molecular masses of components marked on each peak.

The chromatogram reflects the fact that dipeptide and tripeptide fragments were separated, as well as fragments with mass values close to the mass of amino acids, in accordance with the results of previous research and confirming the reproducibility of the framework model for developing collagen hydrolysates [18].

The IR-ATR spectroscopy method was used for the structural analysis of intermediary collagen hydrolysates and the experimental batch of collagen hydrolysate obtained by their combination.

Corrections of ATR, CO₂ and H₂O, baseline, were performed for each sample. Characteristic infrared absorption bands and their assignments are presented in table 3.

Figure 3 comparatively presents IR spectra of collagen hydrolysate batches collected from processes of hydrolytical disintegration of leather waste (collagen hydrolysates CH. 1 and CH. 2), of the final batch (CH. 3) developed by combining batches of extracted hydrolysate and experimental batch obtained after pH correction (CH. 4).

| Characteristic frequency range, cm-1 | Assignments | Frequency range of samples, cm-1 | | | |
|--------------------------------------|---|----------------------------------|--------------------|---------|---------|
| | | CH. 1 | CH. 2 | CH. 3 | CH. 4 |
| 3800-3000 | ν_{CH} aromatic + $\nu_{CH=CH}$ | 3727.65 3355.45 | 3730.54 3362.20 | 3385.34 | 3333.27 |
| 3100-2600 | ν_{NH} , in free amino acids | 2923.48 | 2925.40 | 2927.33 | |
| 3000-2850 | ν_{CH} , ν_{CH_2} , aliphatic | 2856.94 | 2854.05 | 2855.98 | |
| 2140-2080 | ν_{NH} , in amino acids | 2103.88 | 2116.41 | 2114.48 | |
| 1788-1712 | $\nu_{C=O}$, in aspartic and glutamic acids | | 1740.36 | | |
| 1750-1700 | $\nu_{C=O}$, in oxygenate compounds | | | | |
| 1660-1600 | $\nu_{C=O}$, in -CONH | 1643.94 | 1644.90 | 1645.87 | 1635.26 |
| 1660-1610 | ν_{NH} , in free amino acids | | | | |
| 1475-1425 | δ_{CH_2} , in proline | 1459.77 | 1465.55 | 1463.63 | 1460.73 |
| 1465-1400 | ν_{CN} , in proline | | | | 1410.59 |
| 1450-1264 | δ_{COH} , in aspartic and glutamic acids | | | 1287.17 | |
| 1420-1181 | δ_{COH} , $\nu_{C=O}$, in serine | | | | |
| 1350-1315 | δ_{CH} | 1331.53 | | | |
| 1250-1020 | ν_{CN} | | | 1121.32 | |
| 1114-1094 | ν_{CN} , δ_{CH} , in histidine | 1113.61 | 1113.61 | | 1113.61 |
| 1174-724 | γ_{CH_2} | 1016.22 | 889.908 | 893.765 | |
| 1080-1000 | ν_{CC} + ν_{CN} + ν_{CO} | 891.836 | | | |
| 870-890 | $\nu_{C=C}$ | | | | |

Table 3
SPECTRAL FT / IR-ATR
CHARACTERISTICS OF COLLAGEN
HYDROLYSATES

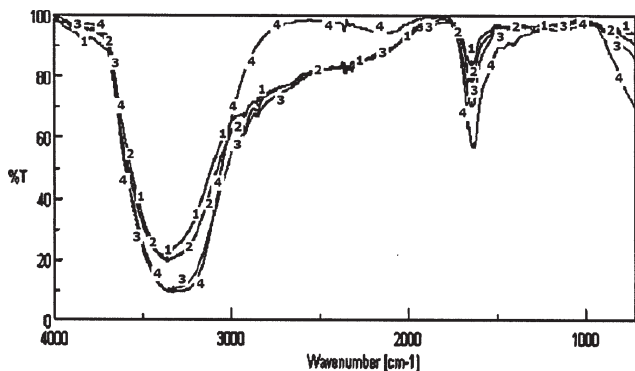


Fig. 3. IR spectral analysis of collagen polydispersions: 1-CH. 1, 2-CH. 2, 3-CH. 3, 4-CH. 4

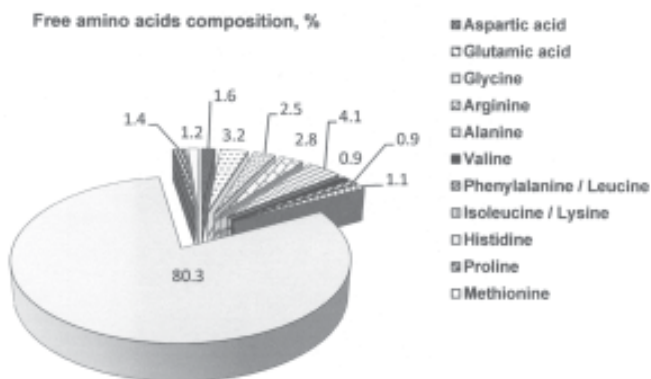


Fig. 4. Free Amino acid distribution of collagen hydrolysates

In these spectra, the bands of ν stretching vibrations and δ vibration distortion occur at wavelengths attributed to the chemical bonds in polypeptides, peptides and free amino acids [21, 22]. The most significant spectral ranges are as follows: 3800–3000 cm^{-1} range, indicates the presence of stretching vibrations ν_{CH} aromatic and $\nu_{-\text{CH}=\text{CH}-}$; 3100–2600 cm^{-1} range, specific for ν_{NH} in free amino acids, 2140–2080 cm^{-1} range, specific for ν_{CH} , ν_{CH_2} , aliphatic, and 2140–2080 cm^{-1} range, general specific for ν_{NH} in amino acids; 1660–1600 cm^{-1} range, specific for $\nu_{\text{C}=\text{O}}$ in $-\text{CONH}$ and 1660–16100 cm^{-1} range, specific for ν_{NH} in free amino acids.

Other vibrations were identified in spectral bands with strict specificity for certain amino acids, such as: $\nu_{\text{C}=\text{O}}$ and δ_{COH} in aspartic and glutamic acids in 1788–1712 cm^{-1} range and respectively 1450–1264 cm^{-1} range; δ_{CH_2} and ν_{CN} in proline, in 1475–1425 cm^{-1} range and respectively 1465–

1400 cm^{-1} range; δ_{COH} and $\nu_{\text{C}=\text{O}}$ in serine in 1420–1181 cm^{-1} range; ν_{CN} and δ_{CH} in histidine in 1114–1094 cm^{-1} range.

IR spectral analysis provides information in accordance with chromatographic analysis, which signaled the dipeptide, tripeptide and free amino acid content of collagen hydrolysates (fig. 2), extracted from semi-processed hide fragments.

Using the high-performance liquid chromatography method, it was established that collagen hydrolysates obtained by hydrolytical disintegration of residual semi-processed hide fragments have a total amino acid content of approximately 10 g/100 mL solution, with an average distribution of amino acids, presented in figure 4.

When applying collagen hydrolysates in seed treatment, the hydrolysate components have a high relative mobility on the seed surface during film matrix formation. Active protein entities can penetrate the coating by faster or slower processes, due to evaporation of the liquid phase on the seed surface. Both the size of high mobility particles upon collagen polydispersion application and that of particles released from the film matrix after complete evaporation of the liquid phase are critical in relation to the thickness and morphology of the seed coat, which has a complex architecture. In other words, the smaller the size of these particles, the higher their chance to penetrate the seed coat.

Particle size and distribution in the representative batches of collagen hydrolysates, CH. 1 and CH. 2, obtained in this study, were analysed by DLS (Dynamic Light Scattering). Results of analyses are illustrated in figure 5 and synthesised in table 4.

It is noticed that, depending on the intensity of the reflected light, the population of very small particles, 1–10 nm, is predominant in both samples, so the chances of seed coat penetration are very high, at least in the period of film matrix formation. The presence of populations of larger particles, in the ranges of 100–1000 nm and 1000–10000 nm, with reduced seed coat penetration, explains the structuring tendency of the film matrix, of importance in the formation of bioactive material deposit which will gradually feed seeds during germination.

An important characteristic of collagen hydrolysates is the contact angle. The contact angle of a liquid drop and a solid surface is a sensitive indicator of changes in surface energy and in the chemical and supramolecular surface structure. Knowing the contact angle allows us to estimate the type of interaction between the surface and the liquid. In this regard, we determined contact angles of collagen

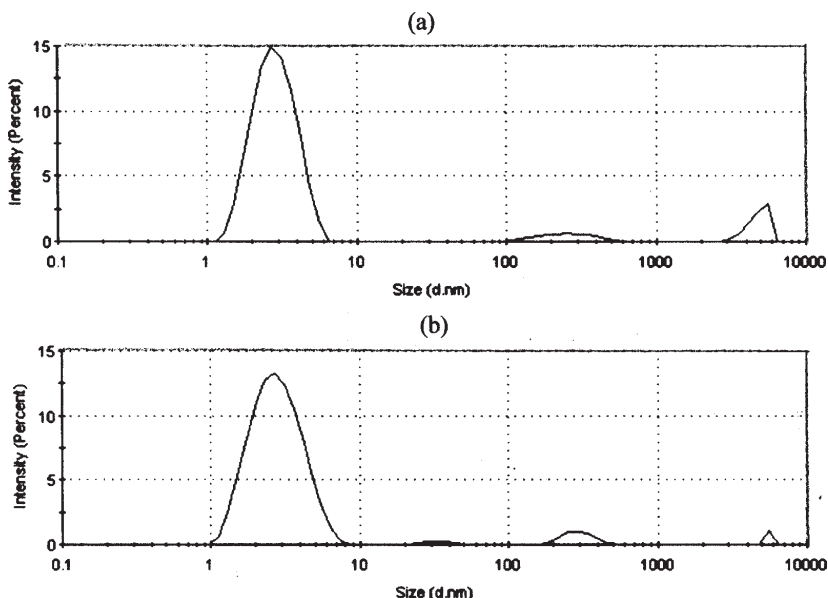


Fig. 5. Particle size in collagen hydrolysates: (a) CH. 1; (b) CH. 2

Table 4

DISTRIBUTION OF PARTICLE SIZE IN COLLAGEN HYDROLYSATES

| No. | Sample | Distribution of particle size, % | | |
|-----|--------|----------------------------------|-------------|--------------|
| | | 1-10 nm | 100-1000 nm | 1000-5000 nm |
| 1 | CH. 1 | 88.0 | 7.3 | 4.7 |
| 2 | CH. 2 | 93.7 | 4.2 | 1.1 |

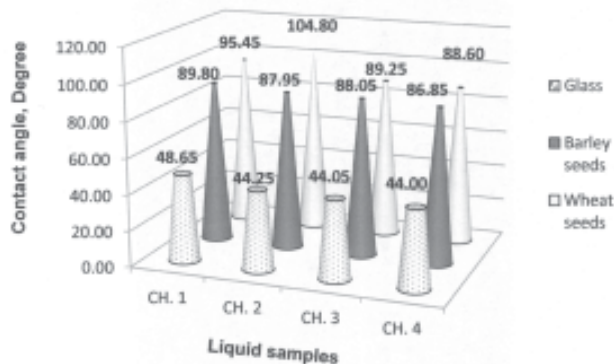


Fig. 6. Comparative contact angles

hydrolysates in relation to glass, as inert control surface, and contact angles of collagen hydrolysates in relation to the surface of cereal seeds.

As seen in figure 6, the contact angle varies in the 44-48° range, relative to glass, but has much higher values and a broader range, 86 - 105°, relative to the seed surface.

The contact angle of collagen hydrolysates in relation to seeds is found to be at least double compared to the contact angle in relation to glass, a consequence of surface roughness, on one hand, and of collagen hydrolysate characteristics, on the other hand, important in this regard being the slight decrease of contact angle upon addition of acetic acid, for conditioned mixture of collagen hydrolysates, CH. 4 compared with the mixture of collagen hydrolysates, CH. 3. It is noticed the slight hydrophobicity of collagen hydrolysates, favourable to the film matrix formation on seed surface.

Conclusions

It was proved that the collagen hydrolysate extracted from solid wastes from tanneries through chemical-enzymatic hydrolysis under atmospheric pressure conditions, at temperatures in the range of $\leq 80^\circ\text{C}$, by stirring, for maximum 6 h, has bioactive properties, due to the diversified content of free amino acids, including essential amino acids, with a total of approximately 10 g/100 mL solution.

It was proved that the collagen hydrolysates predominantly contain very small sized particles, in the 1-10 nm range, able to penetrate the seed coating, but they also contain larger sized particles, situated in 100-1000 nm and 1000-10000 nm ranges, which will ensure the bioactive deposit in the film matrix on the surface.

The wetting ability of collagen hydrolysates, property associated to the contact angle, is favourable to film matrix formation on seed surface.

Acknowledgement: The authors gratefully acknowledge the financial support of the UEFISCDI, Romania, in the framework of National Partnership Program, project "Interdisciplinary research on seed treatment with collagen hydrolysates for quality indicators increasing, pesticide reduction and sustainable development of agriculture production", financing contract no. 122/2012, with co-financing support of Probstdorfer Saatzzucht Romania SRL.

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Manuscript received: 3.03.2014