

ENTRAPMENT OF FLAXSEED EXTRACT INTO XANTHAN-CHITOSAN COMPLEX

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This paper aimed at developing a procedure for the entrapment of flaxseed extract into xanthan-chitosan polyionic complex and studying the kinetic aspects of its *in vitro* release. The total phenolic content, radical scavenging activity and the concentration of delivered secoisolariciresinol (SECO) were determined. The release of SECO from the Xa-CS complex was studied both in aqueous hydrochloric acid solution (pH = 2.0) and in phosphate buffer (pH = 7.4). The study revealed two distinct periods in SECO delivery process in an alkaline medium: an initial period, located in the range of 0 to 8 h, characterized by a high release rate with a delivered SECO amount of approximately 66.5% relative to the incorporated polyphenol and a second period, from 8 to 24 h (up to 81.5% released), when the release process was described by a low and constant rate with a linear profile of the kinetic curve, characteristic of zero-order kinetics. Under acidic conditions, the delivery took place in the first 9 h, with approximately 90.5% of the entrapped SECO being released.

Keywords: flaxseed extract, chitosan, xanthan, lignans

INTRODUCTION

Flaxseed (*Linum usitatissimum*) is the most valuable oily seed and the richest dietary source, which contains a considerable amount of essential oils and phenolics, namely lignans. The free lignans are compounds with a parent structure containing two cinnamyl units linked together by a 8,8'-(β , β') carbon-carbon single bond in the side chains.¹

An extract from flaxseed contains secoisolariciresinol diglucoside (SDG) and the hydroxycinnamic acids p-coumaric acid glucoside and ferulic acid glucoside.² SDG appears to be readily soluble in aqueous methanol or ethanol, but alkaline hydrolysis is subsequently needed to release free SDG. A higher degree of acid hydrolysis of SDG leads to secoisolariciresinol (SECO). SECO and SDG cannot be directly determined in the flaxseed extract, because they are bound in the solid matrix through glycosidic and esteric linkages.

The SDG extraction is usually performed by alkaline hydrolysis, which provides the breaking of ester linkages, while acid hydrolysis able to break both ester linkages and glycosidic bonds is applied for the release of free aglycone SECO.^{1,3}

SECO and SDG are active antioxidants, which have a number of potential benefits, including the reduction of the serum cholesterol level, of the occurrence of diabetes and cardiovascular disease, and decreased formation of breast, prostate and colon cancer.⁴⁻¹²

The effectiveness of phenolic compounds depends on preserving the stability, bioactivity and bioavailability of the active ingredients. The instability at light, temperature, the unpleasant taste of most polyphenols and the difficulty in the dosage formulation are important limitations of their application.

The administration of phenolic compounds requires the formulation of a finished product able

to maintain the structural integrity of the polyphenol until the consumption or the administration, mask its taste, increase its water solubility and bioavailability, and convey it precisely towards a physiological target. These aspects can be developed and improved by the encapsulation/entrapment of polyphenols using polymers.

The lyophilized extracts of yerba mate (*Ilex paraguariensis*) were encapsulated in calcium alginate beads, with and without a chitosan layer.¹³ The effect of the encapsulating system on the mechanical properties of the beads and the influence of the matrix on the active compound stability and diffusion properties were investigated. Delivery systems containing chitosan for the controlled release of antioxidants, such as catechin, tea catechins, olive leaf extract, and polyphenols separated from spruce wood bark, have been developed.¹⁴⁻¹⁷ The encapsulation/entrapment of polyphenols and the technologies employed are summarized in a review by Fang *et al.*¹⁸

The natural polymers used for the entrapment in this study are chitosan (CS) and xanthan (Xa), which are water-soluble, biodegradable and biocompatible and can provide new directions of using, applying and delivering bioactive compounds in environmentally friendly products to be followed in industry.

EXPERIMENTAL

Materials

CS ($M_w = 94.8$ kDa, with a polydispersity index of 3.26 and deacetylation degree of 79.7%) was purchased from Vanson, Inc. (Redmond, W.A. USA). Xa was obtained from BioChemika, Australia (degree of substitution per side chain of 0.73 and 0.75 for acetate and pyruvate groups, respectively, as determined by proton ¹H NMR). Folin-Ciocalteu's reagent (2M) was from Sigma-Aldrich, Australia. All other reagents were of analytical grade and used as received.

Extraction procedures

The flax seeds were air-dried, ground in an electric mill (Retsh Grindomix, GM 200) and reduced to a fine powder of 0.5 mm. The ground raw material was lyophilized to remove the water content and to avoid thermal exposure and then extracted with hexane in a Soxhlet installation in order to remove the lipophilic compounds.

The extraction of phenolic compounds was carried out on the defatted samples using ethanol/water 70/30 (v/v) as solvent. Two procedures were carried out: the first one was performed in an ultrasound bath at 40 °C

during 4 hours and the second one was performed in an oil bath at 60 °C during 4 hours. In both cases, the extracts were concentrated under vacuum to 50 mL and freeze-dried.

For the determination of phenolic acids, the concentrated extracts were submitted to successive liquid-liquid extractions with ethyl acetate. The organic phases were evaporated to dryness, diluted in methanol and followed by HPLC determination.

Alkaline and acidic hydrolysis was performed on the freeze-dried extracts. 0.5 g of the extract was solubilized in ethanol and followed by alkaline or acidic hydrolysis for 2 hours at 80 °C. The concentration of NaOH/HCl in the sample during the hydrolysis was 2M. The extracts obtained were neutralized and filtered through a 0.45 µm filter and then freeze-dried.

Determination of total phenolic compounds in extract

The total phenolic content of the plant extracts was determined using Folin-Ciocalteu's reagent according to a previously developed protocol.¹⁹ About 1 mL of plant extracts was mixed with 500 µL of FCR, 2 mL of 10% sodium carbonate and 5 mL of water. The mixture was shaken thoroughly and was allowed to stand for 90 minutes. Then the absorbance at 765 nm was determined against a blank, which contained all reagents without the samples or the gallic acid under the same conditions. The total phenolic content is expressed as the number of equivalents of gallic acid (GAE).

Radical scavenging activity

The radical scavenging activity of the natural extracts was evaluated by the reduction of the diphenyl-1-picrylhydrazyl (DPPH) radical. The antioxidant activity of the extracts was expressed as EC50, equivalent amount of an extract that neutralizes 50% of the radical. The colorimetric assay was performed according to a modified method developed by Almela *et al.*²⁰

Different dosages of a 0.5 mg/mL methanolic solution of the freeze-dried extracts (25, 50, 100, 200, 300, 400, 500 µL each) were added in screw capped glass vials containing 2 mL of DPPH. All the tubes were adjusted to 3.1 mL with MeOH. After a reaction time of 30 min, the absorbance was measured at 517 nm. The inhibition percentage of the free radical DPPH (% I) was calculated according to the following formula:

$$\% I = \left[\frac{(A_0 - A)}{A_0} \right] \times 100$$

Methanolic solutions of ascorbic acid, gallic acid, caffeic acid and catechin were tested as reference antioxidants. The different quantities of the extracts tested, expressed as micrograms, were plotted on a dose-inhibition curve.

High performance liquid chromatography (HPLC) analysis of flaxseed extractions

Chromatographic analyses of polyphenols were carried out on a Dionex UltiMate 3000, liquid chromatography apparatus, coupled to a diode array detector. Eluent A was 1% aqueous acetic acid, and eluent B was 1% acetic acid in methanol, and the flow rate was kept constant throughout the analysis at 1.2 mL/min. Injections were accomplished with a 15 μ L fixed loop. The column was an Agilent Zorbax, C18 RP (4.6x250 mm, 5 μ m particle size) and the temperature was maintained at 30 °C. The elution gradient used was: from 10% B to 40% B in 30 min. Chromatograms were recorded at 280 nm, and the identification was based on retention times and calibration curves in comparison with the standard compounds.

For the separation and identification of lignans, the same mobile phases, column and equipment were used and the gradient was changed as follows: 10% B 0-10 min, 10-40% B 10-30 min, 40% B 30-35 min, 40-55% B 35-45 min, 55% B 45-49 min, 55-10% B 49-50 min. The flow rate was of 1.0 ml/min and the chromatograms were recorded at 280 nm.

Xanthan-chitosan complex preparation

The chitosan (CS) solution was prepared by dissolving 6.5 g CS powder in 300 mL 0.1 N HCl, neutralized with 0.1 N NaOH, followed by the addition of distilled water until a total volume of 1 L and pH = 6.0 were reached. Xanthan (Xa) solution was prepared under stirring by dissolving 6.5 g of dried powder into 1 L distilled water. The Xa-CS hydrogels were prepared as capsules in the following way: 880 mL of the previously degassed Xa solution were added dropwise through a syringe needle using a peristaltic pump (flow rate: 3 mL/min) to 400 mL of CS solution. The obtained hydrogel capsules were kept for maturation under mild magnetic agitation for 30 min at room temperature. After filtration, the hydrogel capsules were washed with distilled water until a neutral pH was reached, and freeze-dried (Alpha 1-4

LSC, Christ, Germany). The characterization of the Xa-CS complex was previously reported.²¹

Encapsulation of flaxseed extract and kinetical study

The entrapment of the lignan extract (alcoholic freeze-dried extract) was made by dissolving 1.1597 g of lyophilized product in 14 mL ethylic alcohol: water 4/3 v/v, over which 0.2501 g xanthan-chitosan complex was added. The absorbance of the lignan solution in the Xa-CS complex was complete within an hour. The complex obtained was lyophilized and for the *in vitro* kinetic study, flat-faced Xa-CS-lignans complex 100 mg tablets, 12 mm in diameter, were prepared by compression using a Carver laboratory press (Model 3912, USA) at a 2-tonne force, for 1 min.

Two tablets were obtained, with a mass of 0.6185 g, to be used for releasing in acid medium, and of 0.6280 g to be used for releasing in basic medium. The total amount of SECO in the samples was of 2.600 μ g/g, as determined by HPLC.

The kinetics of the SECO release process was performed in a thermostatic bath at 37 °C under stirring at 150 rotations/min. The release of active ingredients occurred in 20 mL aqueous hydrochloric acid solution, pH 2.0, and 20 mL phosphate buffer solution, pH 7.4 – pH values found in the gastrointestinal tract. At determined time intervals, 1 mL of sample was taken for the HPLC analysis, was completed with 1 mL eluent. The SECO delivery was determined by HPLC.

RESULTS AND DISCUSSION

Total phenolic content

The content of extracted polyphenols (Figure 1) depends on the extraction agent used. Therefore, the highest content in polyphenols was acquired for the alkaline and acidic extractions (234.5 and 278.6 mg GAE/100 g, respectively). On the other hand, the content of free polyphenols in the alcoholic extract was of 157.25 GAE/100 g.

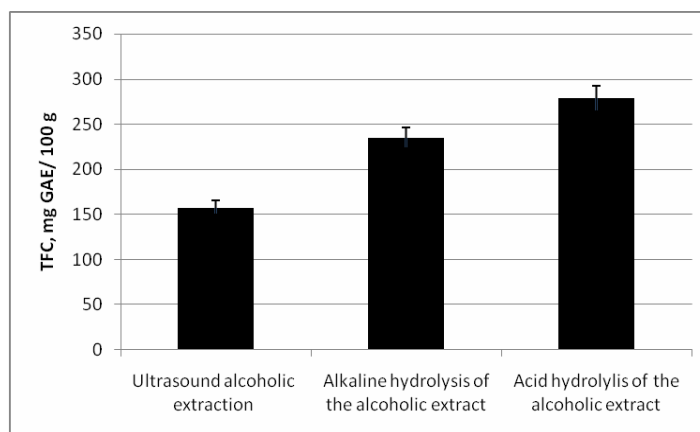


Figure 1: Total phenolic content of flaxseed extracts

HPLC analysis

The chromatographic profiles of the standard polyphenols and lignans, SECO, matairesinol (MATA), lariciresinol (LARI) and vegetal

extracts are presented in Figures 2-4. The analytical polyphenolic composition of the samples is illustrated in Table 1.

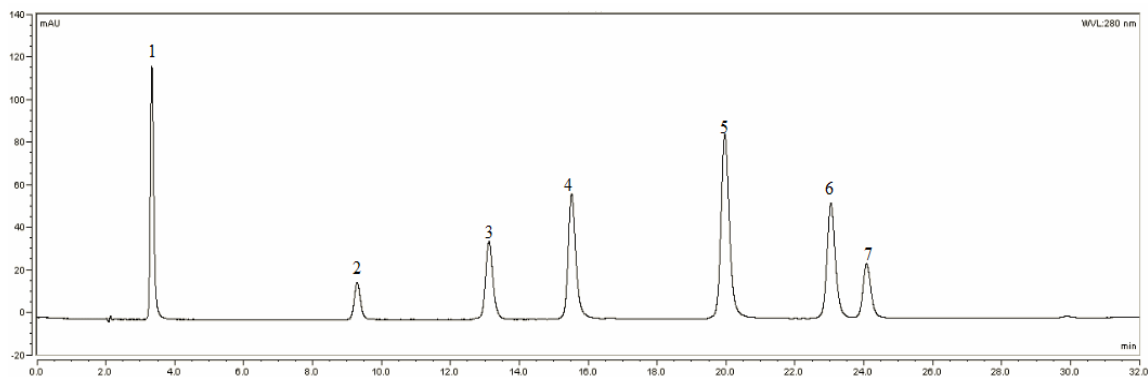


Figure 2: Typical chromatogram at 280 nm obtained for standard polyphenols: 1 – gallic acid, 2 – catechin, 3 – vanillic acid, 4 – caffeic acid, 5 – syringic acid, 6 – p-coumaric acid, 7 – ferulic acid and 8 – sinapic acid

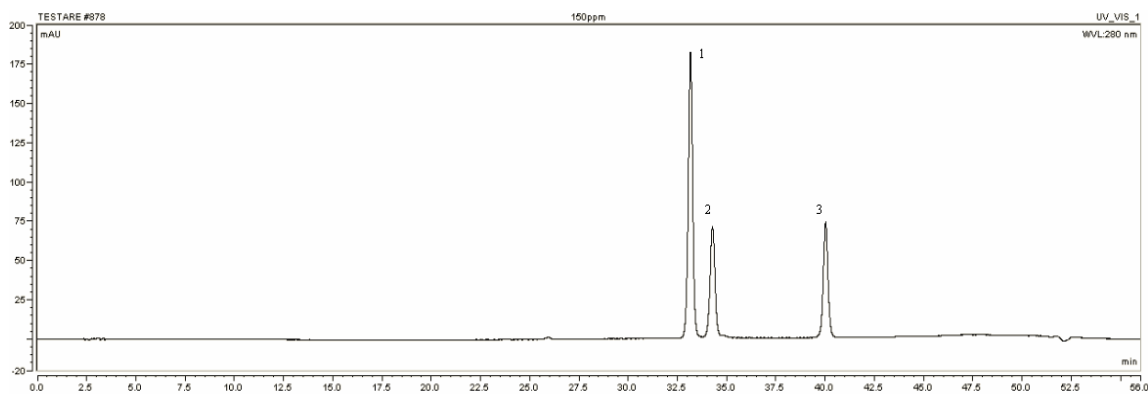


Figure 3: Chromatographic profile of lignans: 1 – LARI, 2 – SECO, 3 – MATA

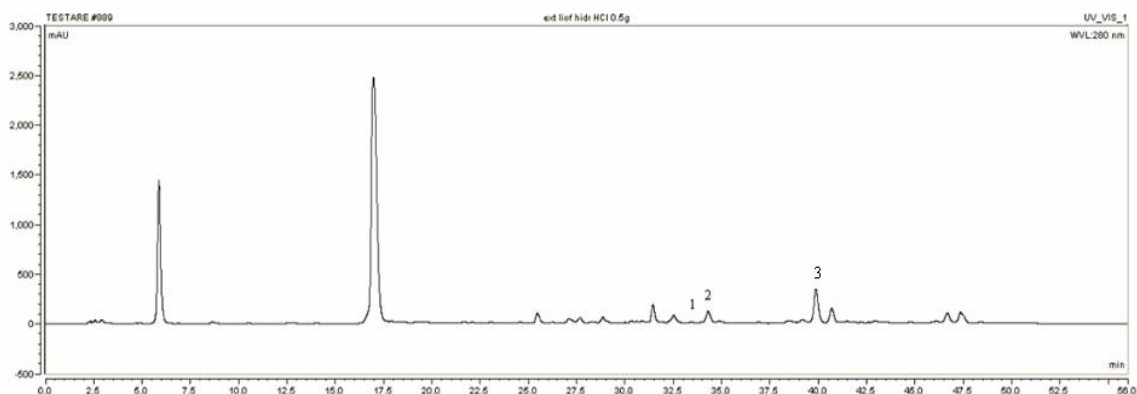


Figure 4: Chromatographic profile of acid hydrolyzed flaxseed extract: 1 – LARI, 2 – SECO, 3 – MATA

As can be noticed from the table, phenolic acids (gallic acid, p-coumaric acid and ferulic acid)

were characteristic of the alcoholic extract, while the major lignans specific to flaxseed extracts

were identified after the hydrolysis step. Also, higher amounts of phenolic acids were found in the hydrolysates.

It is well known that lignans and other phenolic compounds, such as *p*-coumaric acid and ferulic acid, are also present in extracts in

glycosidic and esteric forms as a part of oligomers.¹ Hydrolysis, by which both ester linkages and glycosidic bonds are broken, is often done to simplify the subsequent chromatographic analysis of the extracts and to liberate the bound compounds from a complex matrix.³

Table 1
Concentration of phenolic compounds (mg/100 g dried plant) in the investigated samples

Extract	SECO	LARI	MATA	Gallic acid	<i>p</i> -coumaric acid	Ferulic acid	Sinapic acid
Alcoholic extract	-	-	-	0.98	0.79	0.45	-
Acid hydrolysis	21.37	5.14	53.34	-	-	2.53	3.34

DPPH assay

DPPH radical scavenging has been widely used to assess the antioxidant ability of various plant extracts and natural products. This method is based on the capacity of DPPH radical to react with hydrogen donor species, such as phenolics present in natural extracts. Upon receiving a proton from the donor species it loses its color and becomes yellow. As the concentration of phenolic compounds increases, their DPPH radical scavenging activity also increases.

The radical scavenging activity of the flaxseed extracts was compared with that of some standard polyphenols (gallic acid, catechin, caffeic acid) and ascorbic acid, one of the common synthetic antioxidants used in food industry.

The fall in absorbance was measured at 517 nm for different quantities of standards and

extracts, and the results were plotted on a dose-inhibition curve.

Linear regression showed a good fit to experimental data and high R^2 values were observed for almost all the samples.

Figure 5 shows the inhibition % of DPPH for different quantities of standard compounds and vegetal extracts. The complete neutralization of DPPH radicals was obtained for ascorbic acid, gallic acid and catechin, while caffeic acid provided only 80% inhibition.

As concerns the flaxseed extracts, an inhibition of 90-100% was achieved for the hydrolyzed compounds, while the alcoholic extract exerted only a 60% inhibition of the DPPH radical. The EC₅₀ values for the standards and vegetal extracts are reported in Table 2.

Table 2
EC₅₀ values obtained for the standard compounds and vegetal extracts

Samples	EC ₅₀ , µg	R ²
Ascorbic acid	4.6±0.87	0.998
Gallic acid	1.7±0.32	0.907
Caffeic acid	42.1±1.02	0.993
Catechin	12.7±0.98	0.999
Acidic hydrolysis	170.75±1.98	0.994
Alkaline hydrolysis	179.15±2.3	0.997
Alcoholic extract	240.3±2.98	0.998

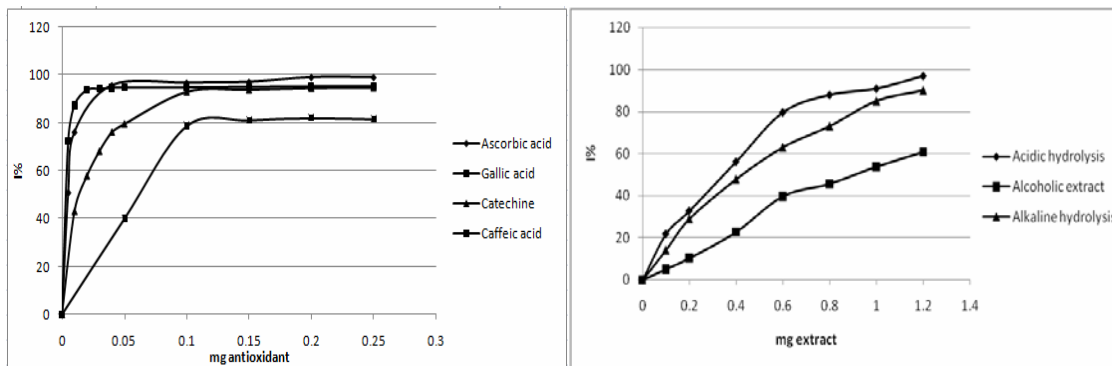


Figure 5: Inhibition effect of standard polyphenols and natural extracts on DPPH reagent

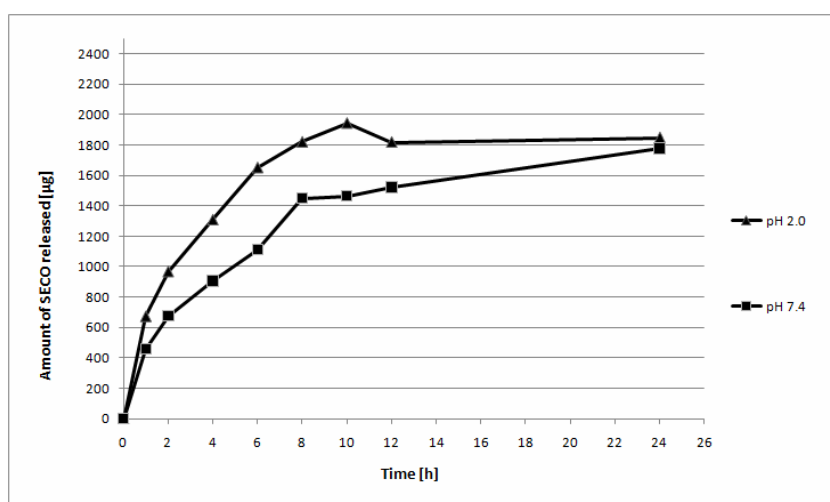


Figure 6: Kinetics of SECO release

***In vitro* release of SECO from complex**

Figure 6 presents the kinetic curves of SECO release, indicating that lignan which is found predominantly (over 95%) in the extracts of flaxseeds, in acidic medium (pH 2.0) and basic medium (pH 7.4).

The release of SECO in two different eluting media occurred differently. The maximum quantity of SECO released in acid medium is 90.5% and 81.5%, respectively, in alkaline medium. At pH 7.4, the study revealed two distinct periods in SECO delivery process: an initial period, located in the range of 0 to 8 h, characterized by a high release rate with a delivered SECO amount of approximately 66.5%, relative to the incorporated polyphenol, and a second period, from 8 to 24 h (up to 81.5% released). In this last stage, the kinetic curve is linear, which indicates zero-order release kinetics.

In acid medium, the maximum amount of polyphenols is released after 8 hours from the beginning of the release kinetic.

The release profile data were fitted with a kinetic model that describes the release mechanism of SECO from the Xa-Cs complex. The diffusion parameters were evaluated with the semi-empirical equation, which may be used to describe the Fickian and non-Fickian release behavior of controlled release systems.

The equation is shown below:

$$M_t/M_p = kt^n$$

where M_t/M_p represents the fraction of released SECO at time t , k is a constant related to structural and geometric characteristic of the tablet and n is an empirical parameter describing the release mechanism. In this study, the diffusional exponent n and the characteristic constant k have been evaluated from the slope and

intercept of the plot $\ln(M_t/M_p)$ versus $\ln(t)$ as presented in Figures 7 and 8. The diffusional exponent, n , is an important indicator of the transport mechanism of a SECO through the complex. When $n = 0.5$, the rate of diffusion is much lower than the rate of relaxation and the process is called Fickian diffusion; if $n = 1.0$, the diffusion process is much faster than the relaxation process and the controlling step is the velocity of an advancing front, which forms the boundary between the swollen outside layer and the glassy core. When n is different from these values, the diffusion and relaxation rates are

comparable and the process is called non-Fickian diffusion or anomalous diffusion mechanism.²² As can be seen from the plots of $\ln(M_t/M_p)$ versus $\ln(t)$, the data show that at pH 2.0, the parameters are: $n = 0.47$ for the diffusion exponent and $k = 0.31$ for the characteristic constant, while at pH 7.4, $n = 0.5$ and $k = 0.21$, in the first stage, in the second stage the value are for $n = 0.24$ and $k = 0.38$. The release process of SECO entrapped in the Xa-Cs complex, in acid and alkaline media, is Fickian diffusion in the first stage, and a non-Fickian process in the second stage at pH 7.4.

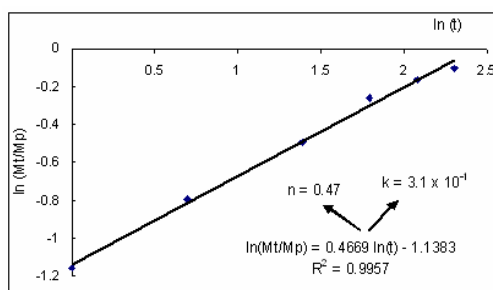


Figure 7: Plot of $\ln(M_t/M_p)$ versus $\ln(t)$ for pH 2.0

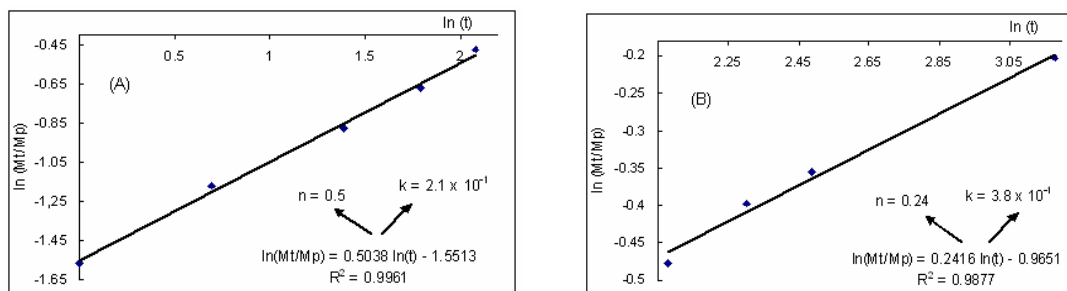


Figure 8: Plot of $\ln(M_t/M_p)$ versus $\ln(t)$ for pH 7.4: A – the first stage, B – the second stage

CONCLUSION

The content of extracted polyphenols from flaxseed depends on the extraction agent and on the parameters used. The highest content of polyphenols was acquired for the alkaline and acidic extractions (234.5 and 278.6 mg GAE/100 g, respectively). The content of free polyphenols in the alcoholic extract was of 157.25 GAE/100 g. The DPPH radical scavenging of the flaxseed extracts was compared with that of some standard polyphenols (gallic acid, catechin, caffeic acid) and ascorbic acid, one of the common synthetic antioxidants used in food industry. The complete neutralization of DPPH radicals was obtained for ascorbic acid, gallic acid and catechin. The hydrolyzed compounds of the flaxseed extract achieved an inhibition of 90-100%, while the

alcoholic extract exerted only a 60% inhibition of the DPPH radical. The alcoholic extract of flaxseed was entrapped into xanthan-chitosan polyionic complex. The *in vitro* release of SECO from the Xa-CS complex was investigated under simulated conditions, mimicking the gastrointestinal tract environment (pH 2.0 and pH 7.4). The delivery process of SECO in an alkaline medium revealed two distinct periods: an initial period, characterized by a high release rate (66.5% relative to the incorporated SECO), and a second period (up to 81.5% released). At pH 2.0 the delivery took place in the first 9 h, with approximately 90.5% of the entrapped SECO being released.

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