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Title:	In vitro Intestinal Absorption of ^{3}H-Lignosulfonate using the Caco-2 Monolayer Model
Project No.	6309
Compound(s)	Ca-Lignosulfonate (Ultrazine FG-R 004, Lot DP955) [^{3}H]-lignosulfonate (04-0324-0324)

Summary

Lignosulfonate is a water soluble, anionic biopolymer obtained by acidic sulphite pulping of lignin. Its physicochemical properties such as the high molecular weight (MW average 52 kDa) with broad distribution (1-250 kDa), and very high hydrophilicity suggest very low oral absorption. However, there are only very few experimental data of this colloid. This *in vitro* study preceeded the *in vivo* absorption study in rats and was designed to estimate intestinal absorption.

The Caco-2 cell monolayer permeability was investigated using tritium-labelled Ca-lignosulfonate at concentrations of 1, 3, 10, and 30 mg/ml. Radioactivity in the permeated samples was determined after incubation times of 0.5h, 1h, 1.5h, 2h, and 3h. Absorption of radioactivity was low with 1.7 ± 0.25% per hour and essentially the same for all concentrations. Molecular weight distribution of the absorbed radiolabelled products as determined by size exclusion chromatography revealed, that less than 1% of the transcellular transported radioactivity had molecular weight higher than 200. Most of the radioactivity permeated was tritiated water formed by radiolysis of ^{3}H-lignosulfonate. The apparent permeability coefficient (P_{app}) of lignosulfonate (MW>200) calculated from these data is lower than 0.005×10^{-6} cm/s. From these results, no intestinal absorption of lignosulfonate and no systemic exposure is expected *in vivo*.

Cellular damage caused by lignosulfonate (1, 5, and 50 mg/ml) was measured by the neutral red uptake assay. No decrease in cell viability was observed after 5h incubation with up to 5 mg/ml lignosulfonate. However, cell viability was lower (ca. 90% of control) with 50 mg/ml lignosulfonate. Similarly, monolayer integrity as determined by transepithelial electrical resistance and by permeability measurements of a marker compound (Lucifer yellow) was slightly affected with the highest concentration tested (50 mg/ml), but not with 1 or 5 mg/ml lignosulfonate.

This report consists of Pages I – IV and 1- 26

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Nomeclature / Synonyms

Calcium lignosulfonate

Ca-lignin sulfonate

Ca-lignosulphonate

Ca-lignin sulphonate

Lignosulfonic acid, calcium salt

CAS No: [8061-52-7]

A structural model is shown in Figure 1 (page 18)

Identity:

Ca-lignosulfonate: Ultrazine FG-R; batch FGR-004 (purified and spray dried)

TABLE OF CONTENTS

1. GENERAL INFORMATION

TITLE	In vitro Intestinal Absorption of [^{3}H]-Lignosulfonate using the Caco-2 Monolayer Model
PROTOCOLS	07/01 and 06/04
REPORT	No. 2500301
STUDY DIRECTOR	Dr. Mareike Beck
AUTHOR	Dr. Mareike Beck
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Cell Biology	F. Loechleiter
Analytical Chemistry	B. Rossi
CELL LINE	
Name	Caco-2
Origin	human colon carcinoma
TEST ITEM 1	Ca-Lignosulfonate
Form	spray dried fine brown powder
Supplier	Borregaard LignoTech: liquid source material (DP955) NRD/CF, Sisseln: spray dried DP955 (→ FGR-004)
Identification	Ultrazine FG-R (Trade name from Borregaard LignoTech)
Lot No.	DP955 / FGR-004
TEST ITEM 2	[^{3}H]-Lignosulfonate
Radiolabelling Service	RC Tritec AG / Teufen, CH
Identification/Lot	Code No: 04-03240324
Spec. Activity	25 mCi/mmol or 0.11 mCi/mg
Form	aqueous solution (ca 1 mCi/ml)
ANALYTICAL METHODS	
Methods	LSC, HPLC (SEC)
Samples	Cell incubations
ARCHIVES	Lignosulfonate project folders, NRD/CH Safety

2. INTRODUCTION

Food grade calcium lignosulfonate is developed by DSM Nutritional Products as formulation aid in food applications to replace gelatine in beadlets, e.g. β-carotene beadlets. This is of increasing interest, not only to comply with the requests for kosher and/or halal food, but also in view of the current discussions in the EU concerning allergenicity of fish gelatine.

Ca-lignosulfonate is a water soluble derivative of lignin, produced in the sulphite pulping process of wood. Lignin is unusual compared to other abundant natural polymers due to the low degree of order and the high degree of heterogeneity in its structure. A possible structural model of the lignin constitution is shown in Figure 1A. The structural elements of the amorphous, highly complex, three-dimensional biopolymer derive principally from phenyl-propanoid units, namely coniferyl, p-coumaryl and sinapyl alcohol-type units.

Solubilization of lignin introduces -SO_3H groups (Fig. 1B), whereby sulfonation of phenylpropane units preferably at the α-position is regarded as principal reaction [1]. The starting material for DNP is a spray dried form of a product supplied by Borregaard Industries Ltd. (Sarpsborg, Norway). It is a light brown powder which is soluble in water but not in any common organic solvent. As of lignin, the exact chemical structure of lignosulfonate is not known due to its highly amorphous nature. The molecular weight distribution covers a wide range of lower than 1 kDa to over 250 kDa.

Due to its physico-chemical properties, the oral absorption of lignosulfonate is considered to be very low. However, whether at all, and/or to what extend a systemic exposure to lignosulfonate is given after oral administration is unclear. Radiolabelling of the polymeric lignosulfonate is necessary to overcome analytical problems. Tritium labelling has the advantage that changes in the molecular structure are negligible, leaving absorption properties unchanged compared to the unlabelled lignosulfonate. However, the drawback of using tritium is, that unavoidable radiolysis occurs. The degradation of ^{3}H-lignosulfonate results in formation of tritium labelled low molecular weight components (e.g. tritiated water), which might easily traverse epithelia or cell monolayers. Therefore, radioactivity measurement alone might be misleading with respect to the actual lignosulfonate absorption.

From one previous absorption/excretion study in rats using tritium labelled compound it is known, that about 0.02% of radioactivity is found in blood samples at 48 h after application and 1.4% appeared in urine within 96 h [2]. No original report or data of this study are available and chemical specifications or molecular weight distribution of the tested lignosulfonate are not known.

In our in vitro study we investigated cellular transport through the Caco-2 cell layer using tritium labelled lignosulfonate. A monolayer of Caco-2 cells grown on a collagen coated filter and

suspended between two compartments serves as a model for the epithelium of the small intestine and is used to study kinetics and transport of orally administered compounds across the intestinal epithelial barrier [3]. The results of this *in vitro* study will be used to design an *in vivo* absorption study in rats.

3. MATERIAL AND METHODS

3.1. Cell Culture Media and Material

Cell culture media, buffers, gentamicin, and fetal calf serum (FCS) were purchased from GibcoBRL (Life Technologies). Cell culture dishes were purchased from Costar Corporation. Unless otherwise stated, all other chemicals were from Sigma or Fluka.

3.2. Test Items

3.2.1. Ca-lignosulfonate

Form	spray dried, fine, brown powder
Source/Supplier	via NRD/CF from Borregaard Industries Ltd. (Sarpsborg, Norway)
Storage conditions	4°C
Identification	
Batch/Lot-No	Ultrazine FG-R / FGR-004
Specification	see CoA (page 25)

Ca-lignosulfonate is a brown, amorphous natural polymer, which is soluble in water, but not in any common organic solvent. The molecular weight distribution of lignosulfonate spans a broad region. About 94% of the Ca-lignosulfonate test material ranges between 1 and 250 kDa. It contains 4.8% reducing sugars, mainly mannose and xylose. The methoxyl content is 11.3% and the degree of sulfonation (sulfonic acids per phenylpropane unit) is 0.5. The aqueous solution supplied by Borregaard was spray dried in Sisseln (NRD/CF).

3.2.2. ^{3}H-lignosulfonate

Form	liquid, aqueous solution
Source/Service	RC Tritec AG, (Teufen, Switzerland)
Storage conditions	-20°C
Batch/Lot-No	Code No: 04-0324-0324
Spec. activity (Conc.)	3.5 mCi/mg (~ 1 mCi/ml)
Radiochemical purity	not specified

Radiolabelled [^{3}H]-Lignosulfonate was produced at RC Tritec AG (Teufen, Switzerland) by catalytic (PtO_2) H/T exchange of the spray dried test item (Ultrazine FG-R, FGR-004) dissolved in dimethyl-formamide. The reaction was followed by several washing and recondensation steps in H_2O/MeOH to remove labile tritium and a filtering step to remove the catalyst. The filtrate was dried and resuspended in H_2O to result in (unspecific) tritium labelled [^{3}H]-lignosulfonate at a concentration of 1 mCi/ml and a specific activity of approximately 3.5 mCi/mg.

3.3. Formulation of radiolabelled incubation solution

3.3.1. Preparation of ^{3}H-lignosulfonate stock solution

In concentrated ^{3}H-lignosulfonate solutions with high specific activity, unavoidable, continuous radiolysis occurs. The radiolabelled products of this degradation process are of lower molecular weight. These are expected to permeate the Caco-2 cell monolayer, and could result in misleading high radioactivity levels, not attributable to lignosulfonate.

Therefore, the low molecular weight (MW) fraction of the radiolabelled stock solution was separated from the labelled lignosulfonate of higher molecular weight before preparation of the incubation solution. The separation (or minimization) of the low MW fraction was achieved by several consecutive ultrafiltration steps using Centriplus YM-3 centrifugal filter devices (Millipore, No 4410; MW cut off: 3'000). After each centrifugation (Sigma 4K-15, swing-out rotor No 11140; 300 × g, 25°C, 3 hr), the concentrated sample was rediluted with 10 ml H_2O. The last centrifugation step was performed right before preparation of the incubation solution. The activity of the resulting radiolabelled stock sample was then adjusted to approximately 0.5 mCi/ml.

3.3.2. Incubation solutions

Ingredients	- 34.6 mg/ml Ca-LS in transport medium - 0.5 mCi/ml (~3.5 mCi/mg) ^{3}H-lignosulfonate (after removal of low mol. weight fraction)
Preparation	2 ml ^{3}H-lignosulfonate were added to 13 ml cold Ca-LS solution. → 30 mg/ml the lower concentrations were prepared by consecutive dilution with transport medium*
Concentrations	30 mg, 10 mg, 3 mg, and 1 mg [^{3}H]-lignosulfonate per ml formulation
Spec. activity	ca 67 µCi per ml formulation (~2.2 µCi/mg Ca-LS)

* transport medium: HBSS, 10 mM HEPES/pH 7.4, 3.5 g/L glucose, 0.35 mg/ml $NaHCO_3$, 1 × MEM Non Essential Amino Acids and 50 µg/ml gentamicin

3.4. Caco-2 cells

The human Caco-2 cells (HTB37) were obtained from American Type Culture Collection (ATCC, Rockville) and used between passages 22 and 28. Cells were maintained at 37°C in an atmosphere of 5% CO_2 and 94% relative humidity in vented culture flasks in DMEM medium supplemented with 3.7 g/l $NaHCO_3$,/pH 7.4, 3.5 g/l glucose, 1% non-essential amino acids, 10% FCS, and 50 µg/ml gentamicin. Medium was replaced three times per week and cells were split at 80-90% confluence by trypsinization (0.25% trypsin, 0.1% EDTA).

For the lignosulfonate studies, cells were seeded at a density of 6×10^4 cells/cm^2 on collagen coated Transwell-COL tissue culture inserts (PTFE membranes of 12 mm diameter and 3 µm pore size). The inserts were placed in wells of standard 12-well cell culture plates. Culture medium was replaced three times per week both in the apical (0.5 ml) and the basal (1.5 ml) compartment of the bicameral culture chambers. Measurements of cell parameters and permeability experiment on Caco-2 monolayers were performed after full differentiation between days 21 and 24, postseeding.

3.5. Cell Assays

3.5.1. Caco-2 monolayer integrity

The integrity of the cell monolayer and its barrier function was assessed by measuring the permeability for Lucifer Yellow (LY), a marker compound for paracellular transport and by measuring the transepithelial electrical resistance (TEER). Monolayers showing high LY permeability and/or low TEER values are inadequate for permeability experiments due to incomplete differentiation or physical damage. Monolayer integrity was determined before and after treatment with various concentrations of Ca-lignosulfonate (0.1%, 0.5%, and 5%) in HBSS medium (pH 7.4) containing 10 mM HEPES.

Transepithelial electrical resistance (TEER):

TEER of monolayers were measured using an epithelial voltohmmeter (EVOM) and an Endohm chamber with planar electrodes (World Precision Instruments). Monolayer resistance values were multiplied by membrane area (1.13 cm^2) after subtraction of resistance of bare filter inserts (blank) to calculate TEER expressed as $\Omega \times cm^2$. Intact and fully differentiated monolayers exhibit TEER values above 240 $\Omega \times cm^2$, those with lower TEER values were excluded from permeability/transport studies.

Lucifer Yellow permeation:

LY is a water soluble fluorescent using the paracellular pathway. Intact cell monolayers show very low, standardizable LY transport, while high fluxes of LY across the monolayer indicate

impaired integrity. To determine LY transport, 20 µg/ml of the dye is added to the upper (donor) chamber and quantified in the lower (acceptor) chamber after 1 h incubation. Experiments were done on at least three monolayers. Inserts without cells were always included in the experiments to be able to standardize to 100% permeability across the filter membrane. The fluorescence intensity of the permeated LY was measured spectrofluorimetrically using an microtiterplate reader (HTS 700 Bio Assay Reader Perkin Elmer, Dynatech Microfluor white plates) at excitation and emission wavelengths of 420 nm and 515 nm, respectively, and LY concentrations were calculated using a standard curve determined on reference solutions.
Calculated LY concentrations of samples were converted to values expressing the portion or percentage of LY transport across the monolayer, using the values in samples of inserts without cells as 100% reference.

3.5.2. Cell viability

Neutral red (NR) is a supravital dye that accumulates in lysosomes of viable, uninjured cells. The cytotoxic effects of Ca-lignosulfonate on the Caco-2 cells was measured by NR uptake, according to the procedure described by Fautz et al. [4]. Briefly, Caco-2 cell monolayers were treated for 5 h with various concentrations of Ca-lignosulfonate (0.1%, 0.5%, and 5%) in HBSS/HEPES medium (pH7.4). After treatment, the cells were washed with PBS/pH 7.4. and incubated for 3 h at 37°C with a NR solution (50 µg/ml in PBS), allowing viable cells to take up the dye. Then, the NR solution was removed, the cells were washed, and the dye in the cells was extracted with 1% (v/v) acetic acid in a 1:1 (v/v) H_2O/ethanol mixture. The OD_{540} was measured spectrophotometrically on 1:4 diluted samples. The cell viability was expressed in (%) relative to untreated control cells.

3.6. Caco-2 Permeability Assay

Caco-2 monolayers were used at passage 25 after full differentiation (day 24, postseeding). To verify monolayer integrity, transepithelial electrical resistance (TEER) was checked at the beginning and at the end of the experiments.
[^{3}H]-lignosulfonate solutions at concentrations 1, 3, 10, and 30 mg/ml (2.2 µCi/mg) were prepared as described in section 3.3.2. in transport medium (Hanks' Balanced Salt Solution (HBSS), 10 mM HEPES/pH 7.4, 3.5 g/L glucose, 0.35 mg/ml $NaHCO_3$, 1 × MEM Non Essential Amino Acids and 50 µg/ml gentamicin).
For equilibration, cell monolayers were washed and preincubated with transport medium for at least 30 min at 37°C before adding the Ca-lignosulfonte to the apical side of the cell monolayer. Transport medium alone was added to the basolateral chambers as acceptor solution. All

concentrations were tested on 3 replicate monolayers. Aliquots of the incubation solutions, removed at the beginning of the experiment, were stored at -80°C until analysis.

Incubation times were 30 min, 60 min, 1.5 h, 2 h, and 3 h. As the permeability of lignosulfonate was expected to be low, and therefore sink conditions were expected to maintain over the 3 h period, incubations were performed for each time interval separately. Analysis of incubation solutions and permeated samples is described in section 3.7. After treatment, cells were washed with fresh transport medium before storing at -20°C for balance of radioactivity, if necessary.

The appearance of radioactivity on the receiver side was plotted versus the time and the flux was calculated from the slope of the regression line obtained from the linear part of the curve. Transport data are expressed as mean ± SD and apparent permeability coefficients (P_{app}) in [cm/s] were calculated using the following equation:

$$P_{app} = \frac{\Delta Q}{\Delta t} * \frac{1}{A} * \frac{1}{c_0}$$

where $\Delta Q/\Delta t$ is the flux or amount of compound transported per time interval from the donor to the receiver compartment expressed in [µg/s]

A is the surface area of the monolayer in [cm^2] and

c_0 is the initial (t = 0) concentration or radioactivity in the donor compartment in [µg/ml] or [µCi/ml], respectively.

3.7. Analytical Methods

Ca-LS in dilution in aqueous solution can be quantified spectro-photometrically using the UV characteristics with a minimum at 260 nm and a maximum at 280 nm (see Figure 2).

3.7.1. Determination of Radioactivity

Radioactivity of incubation solutions and permeation samples was determined directly by counting aliquots mixed with 10 ml scintillation cocktail 'ultima gold' in a liquid scintillation counter (TriCarb 3100, Packard) using external standardization for quench correction.

3.7.2. Size Exclusion Chromatography

Size exclusion chromatography (SEC) was used to determine the molecular weight distribution of the polymeric Ca-lignosulfonate. Briefly, the pH of a sample aliquot was adjusted to approximately 8 by addition of NH_4-acetate before injection. HPLC conditions were as follows:

Column:	TKS Super SW3000; 4.6 × 300 mm
Flow:	0.2 ml/min
Detection:	UV at λ = 280 nm
Solvent:	10 mM NH_4-acetate, pH 7.0

As simultaneous radiodetection was not feasible, the radioactivity of ^{3}H-lignosufonate was analyzed by LSC of eluting fractions. For comparison and depiction, the corresponding area % of the MW-fraction in the chromatogram was determined.

4. RESULTS AND DISCUSSION

4.1. Characterization of Lignosulfonate Incubation Solutions

Ca-Lignosulfonate in aqueous solution displays a characteristic UV-maximum at 280 nm. For sterility reasons, cell incubation solutions were filtered (0.2 µm pore size), which did not alter the UV-characteristics of the lignosulfonate solutions (Figure 2). With increasing concentration the colour of the cell incubation solutions turned from light yellowish (3 mg/ml) to brown (30 or 50 mg/ml).

Size exclusion chromatography (SEC) was used to determine the molecular weight distribution of Ca-lignosulfonate (Fig. 3A). The elution profile shows a broad distribution with retention times (RTs) between 10 and 22 min, reflecting the molecular weight range of about 1-250 kDa. All molecules larger than the pore size are unretained and elute together after around 10-11 min. Absolute molecular weight determination is not possible, since no commercial lignosulfonate molecular weight standards are available, but for comparison, egg-albumin (MW 43 000) and p-aminobenzoic acid (MW 137.14) elute after 12.3 min and 21.45 min, respectively (Fig. 3B & 3C). However, a drawback of using non-lignosulfonate standards is the difference in hydrodynamic volumes due to different shapes and extensions.

Radiolabelled ^{3}H-lignosulfonate as received from Tritec AG was fractionated by size exclusion chromatography (SEC), and radioactivity in eluting fractions was determined. A significant portion (>25%) of the radioactivity was found to be of low and very low molecular weight with longer than 20 min retention time on the column. Small molecules present in the Ca-lignosulfonate product, eg mannose and xylose (MW ca 150-180), contribute to this radiolabelled low molecular weight fraction, and a major part of it is assumed to be tritiated water, most likely resulting from radiolysis of ^{3}H-lignosulfonate.

As sugars and small molecules like tritiated water permeate easily the Caco-2 monolayer, they had to be removed or reduced as much as possible from the labelled lignosulfonate (of higher molecular weight). This was achieved by repeated ultrafiltration before preparation of the incubation solutions. After the last filtration step, less than 3% of the radioactivity in the ^{3}H-stock-solution, which was used to prepare the incubation solutions, was of low molecular weight with retention times longer than 20 min in SEC (Figure 4). Tritiated water (HTO) elutes with a sharp peak in the fraction at 25 min.

Radioactivity of the ^{3}H-lignosulfonate incubation solutions was determined by liquid scintillation counting. Radioactivity levels are listed in Table 1. The molecular weight distribution of lignosulfonate and of the corresponding radiolabelled components in the incubation solution of highest concentration (30 mg/ml) was analyzed at the day of incubation and are shown in Fig 5.

Good correspondence was observed in the MW distribution of radioactivity and UV absorbance, demonstrating the position of the tritiumlabel on the Ca-LS molecules. However, about 6% of the radioactivity elutes after longer than 22 min compared to only 2.5% of the UV absorbance in this molecular weight region, indicating that radiolysis has already occured and formed some tritiated water. Further degradation was observed when measuring the incubation solutions after 3 weeks of storage at -20°C (Figure 6). While lignosulfonate itself shows high stability in aqueous solution with respect to the molecular weight distribution, the tritiumlabel is transferred in radiolytic processes over time resulting in formation of tritium water.

4.2. In vitro Lignosulfonate Absorption

Monolayers of differentiated Caco-2 cells grown on a permeable filter support were used as model for the intestinal epithelium. ^{3}H-Lignosulfonate at various concentrations (1, 3, 10, and 30 mg/ml) was introduced to the apical side, which models the lumen of the intestine, and the radioactivity in the receiver compartment was measured after different incubation times. The apical to basolateral transepithelial passage of radioactivity is shown in Figure 7. Radioactivity in the basolateral compartment increased with incubation time at all concentrations. The dependence was linear with exception of the 1 hr value, which was higher for all concentrations. For experimental reasons, the 1 hr incubation could not be started in parallel with the others in the morning but only in the afternoon of the same day. During this 'waiting' period of several hours, the respective cells had been incubated with transport medium alone which might have caused the altered permeability. Therefore, further evaluation of the data was performed without these outliers. The results are summarized in Table 2.

The relative transepithelial transport of radioactivity was 1.7% per hour on average, and approximately the same for the three lower lignosulfonate concentrations tested (range 1.73-1.91% per hour), while it was slighly lower for the highest concentration (1.31% per hour). For comparison, the permeation across the filter support alone without cell monolayer was 37% per hour (see Fig 7B).

From these radioactivity data, the apparent permeability coefficient (P_{app}) was calculated to be $(2.1 \pm 0.3) \times 10^{-6}$ cm/s. Given the high molecular weight of lignosulfonate (52 kDa MW average; see CoA) this value is surprisingly high and a major part the observed radioactivity was suspected to be caused by radiolabelled molecules of smaller molecular weight, which might have formed by radiolysis of the ^{3}H-lignosulfonate and passed through the cell monolayer.

Therefore, samples collected at the receiver side after the incubation with 3 and 30 mg/ml ^{3}H-lignosulfonate were analyzed additionally by fractionation using SEC. Radioactivity in the eluting fractions was determined by liquid scintillation counting in order to characterize the

radioactivity that has passed through the cell layer with respect to the molecular weight. The results are listed in Table 3A (30 mg/ml) and 3B (3 mg/ml) and visualized in Figure 8.

In all analyzed samples, 99.5% or more of the radioactivity had low to very low molecular weight eluting from the column after longer than 20 min, and most of the radioactivity eluted together with tritiated water after around 24-26 min. On average, only 0.3% and 0.03% of the radioactive labelled permeate after 30 mg/ml and 3 mg/ml incubation, eluted before 20 min. In the 20-22 min fraction also elutes p-aminobenzoic acid with MW 137.14 (RT 21.45 min). Thus, taking p-aminobenzoic acid as reference, extremely low Caco-2 monolayer permeability of ^{3}H-lignosulfonate with MW higher than 200 was observed.

The calculated ^{3}H-lignosulfonate (MW>200) permeability coefficients from these results are 0.002×10^{-6} cm/s and 0.005×10^{-6} cm/s for lignosulfonate incubations with 3 mg/ml and 30 mg/ml, respectively (summarized in Table 4). From these values, no absorption and thus no oral availability of lignosulfonate is expected *in vivo*.

4.3. Effect of Lignosulfonate on Caco-2 Monolayer Integrity and Cell Viability

Influence of lignosulfonate on monolayer integrity was measured after 5h incubation with increasing concentrations of lignosulfonate (0, 0.1, 0.5, and 5%) using a fluorescent dye. Lucifer yellow (LY), a marker of paracellular transport, usually shows very low Caco-2 monolayer permeability [3]. After 5h incubation with the transport buffer only, LY transport of 0.43% per hour was measured. This low permeation remained after incubation with 1 mg/ml, and with 5 mg/ml lignosulfonate (Table 5). However, cell monolayer permeability for LY increased more than 3-fold (1.53% per hour) after treatment with 50 mg/ml lignosulfonate, indicating that at high lignosulfonate concentration, the monolayer integrity was slightly impaired. This was confirmed when measuring transepithelial electrical resistance (TEER) of the monolayer. TEER decreased from 346 Ω cm^2 to 246 Ω cm^2 after incubation with the highest lignosulfonate concentration (5%), while at 0.1% and 0.5% lignosulfonate TEER values were 338 Ω cm^2 and 390 Ω cm^2, respectively (Table 6). This influence on monolayer integrity could also explain that the permeability coefficient for lignosulfonate at 30 mg/ml is higher compared to 3 mg/ml, although still negligible low.

Cell viability was measured by neutral red uptake. No influence of 1 mg/ml or 5 mg/ml lignosulfonate on cell viability was observed, while after treatment with 50 mg/ml lignosulfonate neutral red uptake decreased (89 $\pm$ 8.3%) compared to control (Table 7).

Thus, lignosulfonate showed no effect on epithelial barrier function and no cytotoxic effect at concentrations up to 5 mg/ml in our experiments. However, at high concentrations, ligno-sulfonate affected both cell viability and monolayer integrity.

5. SUMMARY / CONCLUSION

In vitro intestinal absorption of lignosulfonate was studied using the Caco 2 model.

Cell toxicity of the polymer was measured by cellular neutral red uptake after a 5h incubation with 0, 1, 5, and 50 mg/ml lignosulfonate. Cell viability was unaffected by the lower concentrations while it decreased to ca. 90% with 50 mg/ml lignosulfonate. Monolayer integrity was assessed by measuring transepithelial electrical resistance (TEER) and permeability of a marker compound (LY). No effect was observed with lignosulfonate of up to 5 mg/ml, indicating that epithelial barrier function was maintained, while TEER was lower and LY permeability increased slightly with 50 mg/ml lignosulfonate.

Apical to basolateral Caco-2 monolayer permeability was investigated using tritium-labelled Ca-lignosulfonate at concentrations of 1, 3, 10, and 30 mg/ml. Radioactivity in the permeated samples was determined after incubation times of 0.5h, 1h, 1.5h, 2h, and 3h. Transport of radioactivity was linear during the observed incubation interval, and it was low ($1.7 \pm 0.25\%$ per hour) and essentially the same for all concentrations.

However, tritiated water (HTO) is formed by radiolysis of ^{3}H-lignosulfonate, and the radioactivity levels at the basolateral receiver side were misleading, as HTO is readily absorbed. Molecular weight distribution of the absorbed radiolabelled products as determined by size exclusion chromatography revealed, that less than 1% of the transcellular transported radioactivity had molecular weight higher than 200. Most of the radioactivity permeated was tritiated water formed by radiolysis of ^{3}H-lignosulfonate. The apparent permeability coefficient (P_{app}) of lignosulfonate (MW>200) calculated from these data is lower than 0.005×10^{-6} cm/s.

From the results of this study, no intestinal absorption of lignosulfonate and no systemic exposure is expected *in vivo*.

6. TABLES AND FIGURES

Table 1: Radioactivity levels of incubation solutions

Radioactivity in ^{3}H-lignosulfonate incubation solutions as determined by liquid scintillation counting on diluted aliquots ($n \geq 4$).

	Lignosulfonate concentration	Radioactivity (^{3}H)
	(mg/ml)	(µCi/ml)
c1	1	2.61 ± 0.09
c2	3	7.33 ± 0.25
c3	10	24.08 ± 0.32
c4	30	69.01 ± 2.94

Table 2: Caco-2 permeability of radioactivity after incubation with ^{3}H-lignosulfonate

Radiolabelled lignosulfonate (LS) was added to the apical side of the Caco-2 cell monolayer, and radioactivity in the basolateral/receiver side was quantified by liquid scintillation counting of aliquots.

	LS conc.	Radioactivity dose per incubation (^{3}H)	Permeation of radioactivity		Permeability coefficient (P_{app})
	(mg/ml)	(dpm)	(dpm/min)	(% per hr)	(cm/s)
c1	1	2'898'500	838	1.73	2.13×10^{-6}
c2	3	8'141'000	2'586	1.91	2.34×10^{-6}
c3	10	26'725'000	8'118	1.82	2.24×10^{-6}
c4	30	76'604'000	16'776	1.31	1.61×10^{-6}

Table 3: MW distribution of radioactivity transported across Caco-2 monolayers after incubation with ^{3}H-lignosulfonate

Basolateral solutions of the Caco-2 model after apical addition of ^{3}H-lignosulfonate were fractionated according to molecular weight by size exclusion chromatography. Radioactivity in eluting fractions was measured by liquid scintillation counting and expressed as percent of total injected.

(A) MW distribution of radioactivity (in percent of total inject in SEC) after transepithelial transport in monolayer incubations with 30 mg/ml ^{3}H-lignosulfonate

	% radioactivity in					
HPLC fraction	inc. solution	permeate after incubation time (hr)				
(min)		0.5	1	1.5	2	3
2	0.04	0.00	0.00	0.00	0.00	0.00
4	0.05	0.00	0.00	0.00	0.00	0.00
6	0.03	0.00	0.00	0.00	0.00	0.00
8	0.02	0.00	0.00	0.00	0.00	0.00
10	1.53	0.00	0.00	0.00	0.00	0.00
12	37.64	0.00	0.00	0.00	0.00	0.00
14	34.00	0.01	0.02	0.03	0.05	0.08
16	16.45	0.06	0.11	0.14	0.17	0.24
18	3.40	0.01	0.04	0.07	0.05	0.10
20	0.83	0.07	0.03	0.05	0.06	0.08
sum 0-20 min	94.00%	0.15%	0.21%	0.30%	0.35%	0.50%
22	0.34	0.23	0.33	0.43	0.43	0.55
24	2.46	34.49	32.68	35.30	30.30	30.56
26	2.53	53.68	54.99	53.03	56.91	56.63
28	0.32	9.48	9.44	10.06	8.74	8.10
30	0.20	1.69	2.00	0.58	2.79	3.05
32	0.07	0.18	0.18	0.13	0.29	0.39
34	0.02	0.04	0.07	0.07	0.09	0.10
36	0.02	0.03	0.05	0.05	0.06	0.06
38	0.03	0.01	0.03	0.03	0.03	0.04
40	0.02	0.01	0.02	0.02	0.02	0.02
sum 21-40 min	6.00%	99.85%	99.79%	99.70%	99.65%	99.50%
total dpm inject	39'174	15'218	37'671	28'008	35'541	48'236

Table 3 (continued)

(B) MW distribution of radioactivity (in percent of total inject in SEC) after transepithelial transport in monolayer incubations with 3 mg/ml ^{3}H-lignosulfonate

	% radioactivity in					
HPLC fraction	tritiated water	permeate after incubation time (hr)				
(min)		0.5	1	1.5	2	3
2	0.00	0.00	0.00	0.00	0.00	0.00
4	0.08	0.00	0.02	0.00	0.00	0.00
6	0.00	0.00	0.00	0.02	0.02	0.00
8	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	0.00	0.00	0.02	0.00
12	0.00	0.00	0.00	0.00	0.00	0.00
14	0.00	0.00	0.00	0.00	0.02	0.00
16	0.00	0.00	0.03	0.04	0.02	0.05
18	0.00	0.00	0.00	0.00	0.03	0.05
20	0.00	0.07	0.02	0.00	0.00	0.05
0-20 min	0.08%	0.07%	0.06%	0.06%	0.10%	0.16%
21	0.00					
22	0.00	35.52	0.26	0.19	0.24	0.33
23	0.10					
24	6.96	52.80	4.58	3.99	4.68	3.61
25	86.78					
26	5.40	7.81	94.19	94.94	94.32	94.95
27	0.03					
28	0.10	3.51	0.59	0.56	0.62	0.69
29	0.13					
30	0.00	0.11	0.15	0.15	0.17	0.19
32	0.05	0.18	0.09	0.02	0.10	0.07
34	0.00	0.00	0.03	0.08	0.05	0.00
36	0.03	0.00	0.02	0.00	0.02	0.00
38	0.43	0.00	0.00	0.00	0.00	0.00
40	0.00	0.00	0.03	0.00	0.00	0.00
21-40 min	99.92%	99.93%	99.94%	99.94%	99.90%	99.84%
total dpm inject	3'966	2'818	6'556	5'182	5'848	5'759

Table 4: Caco-2 permeability of ^{3}H-lignosulfonate

		c2	c4
Incubation			
LS concentration	(mg/ml)	3	30
applied radioactivity	(dpm)	8'141'000	76'604'000
Transepithelial transport			
radioactivity	(dpm/hr)	155'200	1'006'600
portion of radioactivity with MW > 200	(%)	0.03	0.30
Permeability coefficient (P_{app}) of ^{3}H-lignosulfonate with MW > 200	(cm/s)	0.0021×10^{-6}	0.0046×10^{-6}

Table 5: Influence of lignosulfonate on Lucifer Yellow permeation

After lignosulfonate incubation, cells were washed and Lucifer yellow in the basolateral compartment was measured fluorimetrically 1h after apical addition of 20 µg/ml.

Lignosulfonate concentration	**LY permation of cell monolayers** (%/hr)*					**Permeation coefficient**
(mg/ml)	A	B	C	D	**Mean** ± SD	(cm $s^{-1} \times 10^6$)
0	0.54	0.40	0.41	0.36	**0.43** ± 0.08	**0.28** ± 0.05
1	0.45	0.43	0.41	0.26	**0.39** ± 0.08	**0.25** ± 0.05
5	0.49	0.41	0.41	0.43	**0.44** ± 0.04	**0.28** ± 0.02
50	1.44	1.35	1.51	1.82	**1.53** ± 0.21	**0.98** ± 0.13

* LY permeation through filter membranes without celllayer was measured as control to determine the maximal transport (100% per hour)

Table 6: Influence of lignosulfonate on transepithelial electrical resistance

Transepithelial electrical resistance (TEER) of Caco-2 monolayers after 5 h incubation with lignosulfonate. TEER values are given in Ω cm^2

Lignosulfonate concentration	**TEER of Caco-2 monolayers** (Ω cm^2)				
(mg/ml)	A	B	C	D	**Mean** ± SD
0	362	335	347	342	**346** ± 12
1	334	320	359	339	**338** ± 16
5	400	380	408	373	**390** ± 17
50	242	247	236	260	**246** ± 10

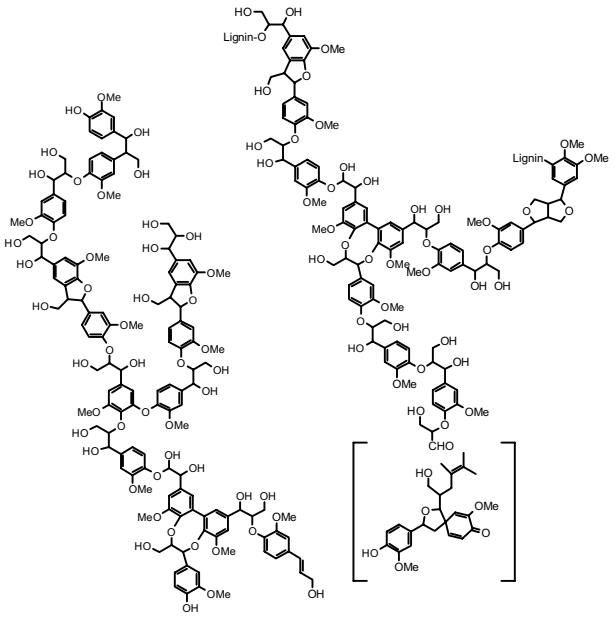
Table 7: Influence of lignosulfonate on cell viability

Cell viability was measured by neutral red uptake assay after 5 h incubation with lignosulfonate.

Lignosulfonate concentration	**NR uptake of Caco-2 cells** (OD_{540})					**relative cell viability**
(mg/ml)	A	B	C	D	**Mean** ± SD	(%)
0	0.66	0.69	0.74	0.69	**0.69** ± 0.03	**100** ± 4.9
1	0.74	0.71	0.72	0.69	**0.72** ± 0.02	**103** ± 3.5
5	0.78	0.69	0.65	0.70	**0.71** ± 0.05	**102** ± 7.6
50	0.69	0.62	0.55	0.62	**0.62** ± 0.06	**89** ± 8.3

Figure 1: Lignin structure and Lignosulfonate

(A) Depiction of a part of the currently accepted macrostructure of the lignin polymer in wood as constructed from various spectroscopic and chemical analyses of various lignin functionalities of wood (modified from [5]).Theoretically, a scheme of lignin constitution can only reflect the average distribution of possible linkages.



(B) The acidic sulfite pulping process modifies the lignin structure mainly by introduction of -SO_3H groups (→ Lignosulfonate, soluble). Sulfonation of phenylpropane-units preferably at the α-position is regarded as principal reaction (after Gruber, 2000; [1]).

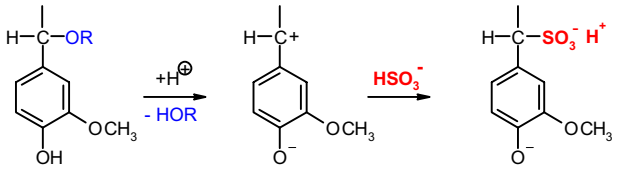


Figure 2: UV absorbance of Lignosulfonate

UV-Absorbance spectra of 0.1 mg/ml Ca-Lignosulfonate in HBSS/HEPES-medium (pH 7.4). A 40 mg/ml solution was sterile filtered and spectra were measured of 1:400 diluted aliquots before and after filtration.

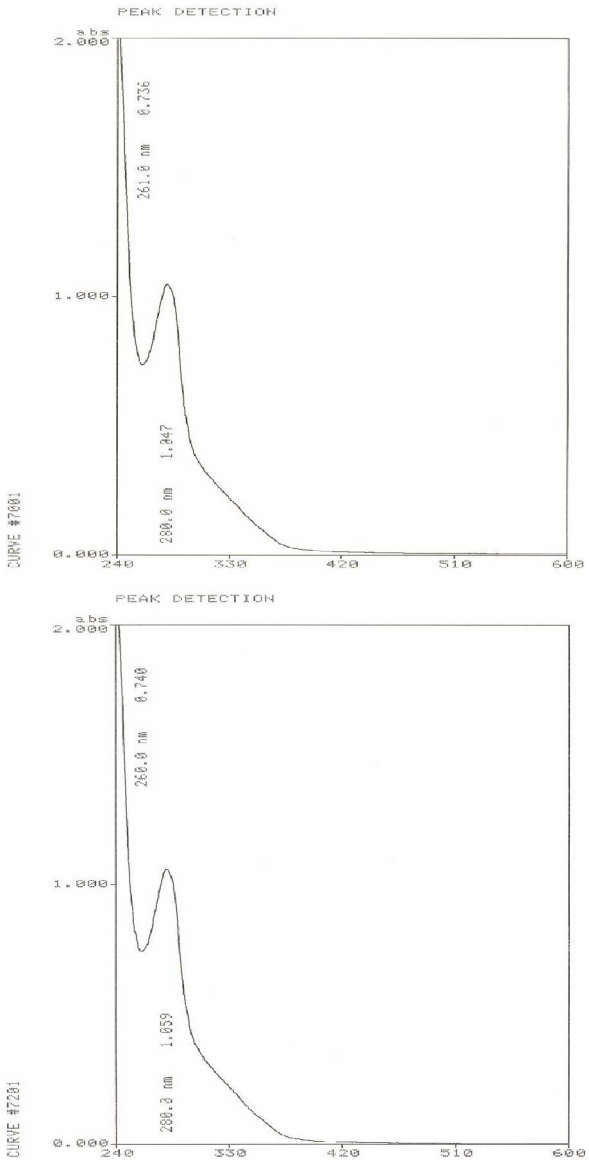


Figure 3: MW distribution of Lignosulfonate

(A) Molecular weight distribution of an aqueous solution of Ca-lignosulfonate using size exclusion chromatography (SEC). For comparison, elution profiles of (B) egg albumin (43kDa) and (C) p-aminobenzoic acid (MW 137.14) are shown.

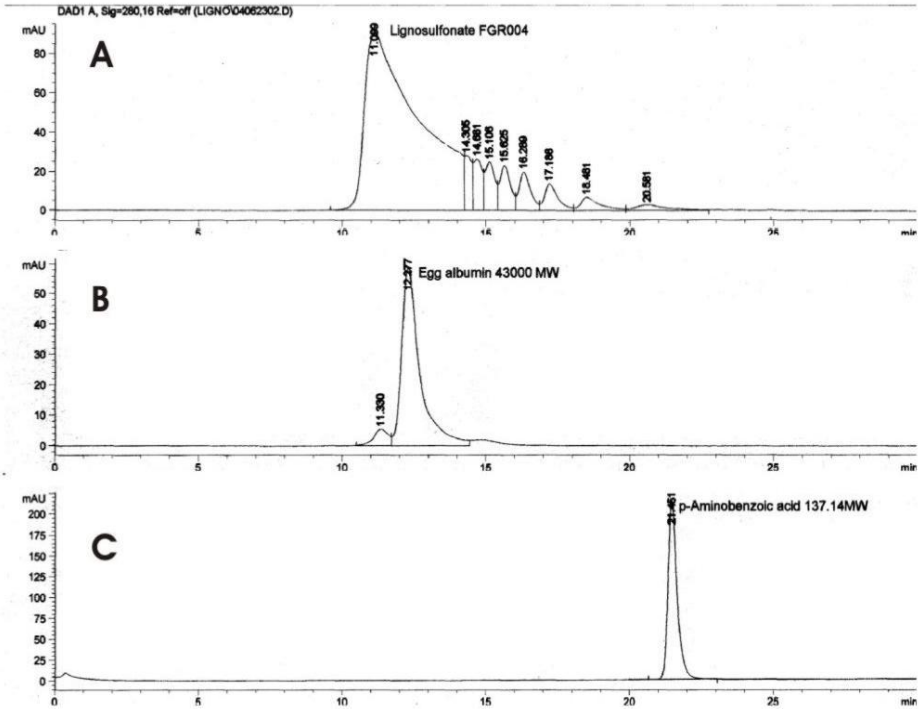


Figure 4: MW distribution of ^{3}H-Lignosulfonate stock after filtration

Molecular weight distribution of radiolabelled components in the ^{3}H-lignosulfonate stock solution.

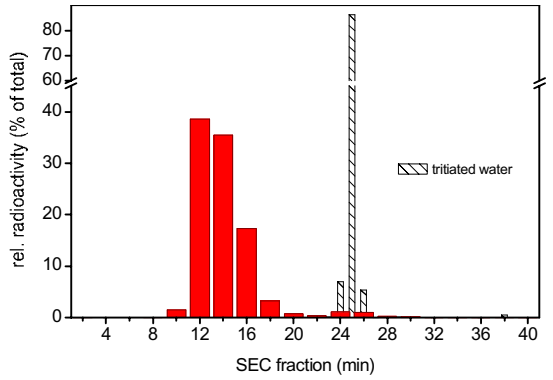


Figure 5: MW distribution of ^{3}H-lignosulfonate incubation solution

Molecular weight distribution of ^{3}H-lignosulfonate incubation solution (c4=30 mg/ml) as detected by UV at 280 nm in SEC (green) and radioactivity in eluting fractions (red).

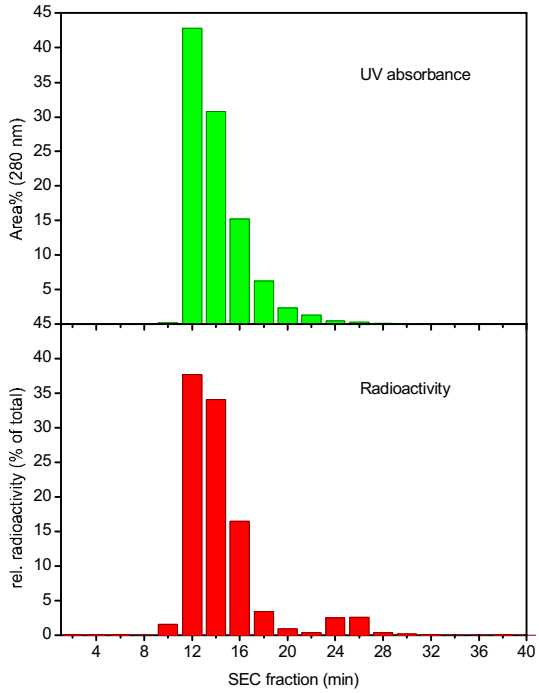


Figure 6: Stability of incubation solution

Formation of tritiated water and/or other tritium labelled degradation products of low molecular weight by radiolysis of ^{3}H-lignosulfonate incubation solution during 3 weeks storage at -20°C.

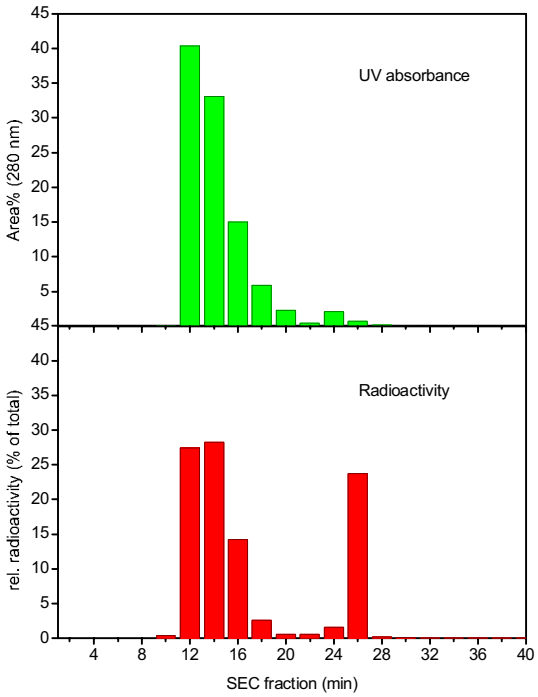
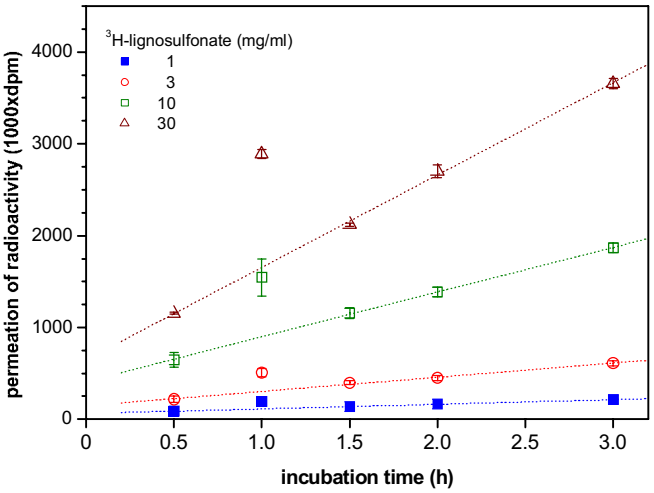


Figure 7: Time course of radioactivity transport across Caco-2 monolayer

(A) Time course apical to basolateral Caco-2 permeation of radioactivity after incubation with different concentrations of ^{3}H-lignosulfonate. The plotted data are the mean ± S.D (n=3).



(B) Time course of relative permeation of radioactivity in percent of given. At all concentrations approximately the same relative amount is transported (ca 1.7% per hour). The insert shows the permeation across the filter support alone without cells (37% per hr).

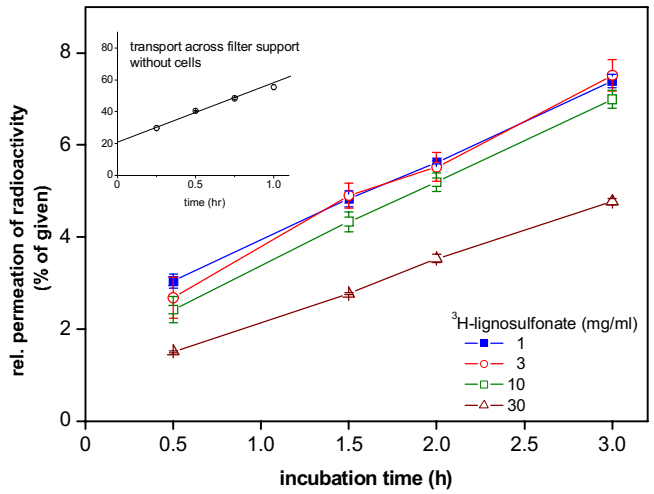
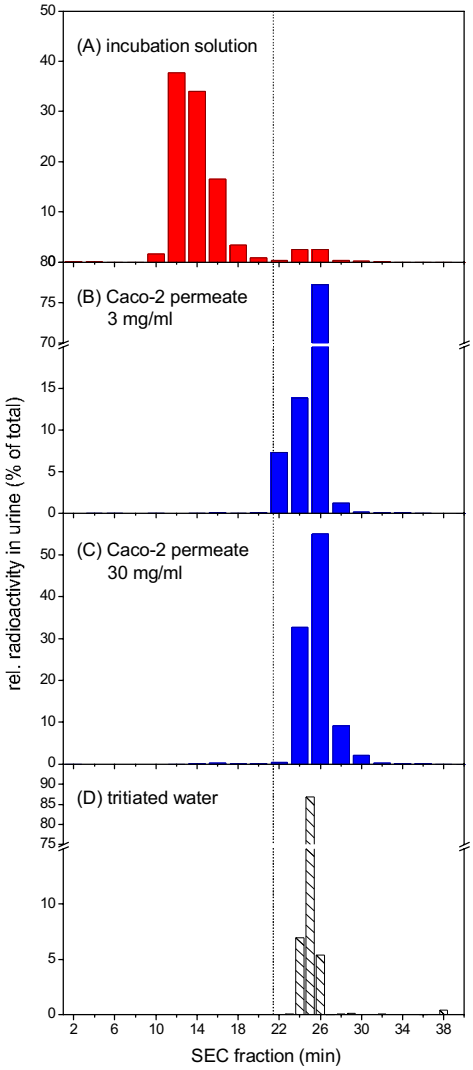


Figure 8: MW distribution of radioactivity permeated through intestinal cell layer

The MW distribution of the radioactivity in the incubation solution (A) is shown in comparison to MW distribution of samples collected from the basolateral side after apical addition of (B) 3 mg/ml and (C) 30 mg/ml ^{3}H-lignosulfonate. The SEC elution profile of triatited water radioactivity is also shown (D).

The dotted line at 21.5 min separates on its right the very low molecular weight fractions (MW<137.14; p-aminobenzoic acid) from the low to very high molecular weight fractions on its left side (fractions 1-21.5 min).



Report No. 2500301 **11-July-2006, Beck M**

7. CERTIFICATE OF ANALYSIS*: SPRAY DRIED CA-LIGNOSULFONATE

* copy of first page of CoA from August 26, 2005

Borregaard LignoTech

CERTIFICATE OF ANALYSIS
from
Borregaard LignoTech Research & Development

Ultrazine FG-R

Batch no.	**Specification**	**FGR-004**
Dry Solids		95.5 %
Molecular weight average, Mw	40,000 Da – 65,000 Da	52,000 Da
Sample within range 1,000 Da – 250,000 Da	> 90 %	93.9 %
K solid	> 11.5	11.7
Colour in 0.5 % solution	< 0.40 AU	0.31 AU
Phenolic OH	1.5 % – 2.5 %	2.0 %
pH in 10 % solution	2.7 – 3.3	2.9
Viscosity at 38 % DS and 35 °C	100 mPas – 600 mPas	200 mPas
Ash	< 14.0 %	6.8 %
Sulphite, SO_3^{2-}	< 0.5 %	0.2 %
Free oxalate	< 0.5 %	Not detected
Reducing Sugars	2.0 % - 5.0 %	4.8 %
Calcium	< 5.0 %	3.2 %
Methoxyl	10 % – 13 %	11.3 %
Degree of sulfonation	0.3 – 0.7	0.5
Total heavy metals	< 10 ppm	< 10 ppm
Heavy metals (reported in ppm)		
Arsenic	< 0.05	< 0.04 ppm
Mercury	< 0.05	< 0.01 ppm
Cadmium	< 1	0.1 ppm
Lead	< 2	1.2 ppm
Chromium	< 5	0.8 ppm
Copper	< 5	3.9 ppm
Nickel	< 5	0.8 ppm
Zinc	< 50	20 ppm
Iron	< 150	58 ppm

Page 1 of 3

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Web-link: http://www.cellulose-papier.chemie.tu-darmstadt.de/Deutsch/Vorlesungen_und_Veranstaltungen/Vorlesungen/Nachwachsende_Rohstoffe/PDF/10_Klassischer_Holzaufschluss.pdf

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