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Regulatory Document

DSM

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Authors:	Beck M* and Rossi B
Title:	Absorption, Distribution and Excretion of Tritium Labelled Lignosulfonate after Single Oral Administration to Rats
Project No.	6309
Compound No.	LSFG (Lignosulfonate, Food Grade), Ultrazine FG-R 004, Lot DP955

Summary

Absorption, distribution and excretion of ^{3}H-labelled calcium lignosulfonate was studied in male and female rats following a single oral dose of 10 mg/kg bw. Lignosulfonate was administered as an aqueous solution by gavage, and the molecular weight distribution of the polymer in the application solution was analyzed by size exclusion chromatography.
Radioactivity levels in blood samples from 3 male rats taken at 1h, 2h, 4h, 6h, and 24h post dose were extremely low, reaching the highest level of 0.0015% of the dose per g blood at 6h. This level remains almost unchanged until sacrifice at 48h.
Urine and faeces were collected from 0-24h and from 24-48h, and animals were sacrificed after 48h. Radioactivity in excreta, liver, terminal blood, gastrointestinal tract, skin/fur, and residual carcass was determined. Additionally, urine and plasma were analyzed by HPLC for characterization of the molecular weight distribution of radiolabelled components.
The excretion balance resulted in an overall recovery of 98.4 ± 0.7% (n=6) of the radioactivity after 48h. The majority of the administered radioactivity was excreted via faeces, 90% of which within the first 24h. Apart from a slightly slower faecal excretion in females, no significant gender difference could be observed in tissue, blood and excreta levels.
After 48h, only 0.8% of the administered radiolabelled lignosulfonate is found in urine (0.05%), liver (0.08%), blood (0.01%), and remaining carcass (0.66%).
The results demonstrate, that lignosulfonate absorption after oral administration is extremely low, and systemic exposure is below 1%.

This report consists of Pages I – IV and 1- 31

Distribution
Karin Feltes, VML

Approved

Name	Signature	Date
Main Author Mareike Beck	Mareike Beck	22.11.05
Principal Scientist / Competence Mgr Jochen Bausch		22.11.05
Research Center Head Luis Pasamontes		24.11.05
Project Manager Bernd Mussler		24.11.05

NOMECLATURE / SYNONYMS

Calcium lignosulfonate;

Ca-ligninsulfonate

Lignosulfonic acid, calcium salt

Name used internally (DNP/Borregaard): Ultrazine FG-R

CAS No. 8061-52-7

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

ABBREVIATIONS:

Ca-LS	calcium lignosulfonate
DMF	dimethyl-formamide
HTO	tritiated water
LSC	liquid scintillation counting
LSFG	lignosulfonate, food grade
(L)MW	(low) molecular weight
RT	retention time
SEC	size exclusion chromatography

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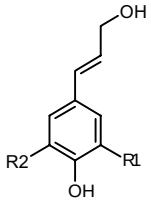
1. INTRODUCTION

Food grade calcium lignosulfonate (LSFG) 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.

After cellulose and hemicellulose, lignin is one of the most abundant class of chemicals in vascular terrestrial plants. It is essential for mechanical support, defense, and water transport. The structural elements of this amorphous, highly complex, three-dimensional network derive principally from phenylpropanoid units, namely coniferyl, p-coumaryl and sinapyl alcohol-type units.



Lignin monomers:

R1 = R2 = H	p-coumaryl alcohol
R1 = R2 = OCH_3	sinapyl alcohol
R1 = OCH_3, R2 = H	coniferyl alcohol

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 process of digestion of wood with acidous calcium bisulphite solution (sulphite pulping) solubilizes lignin as calcium lignosulfonate (Ca-LS), which is then separated from the cellulose (pulp) by filtration. The filtrate is purified by ultrafiltration and evaporated.
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 stucture 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.

To be able to relate effects to the test item in toxicity studies, it is of fundamental significance to assess whether at all and/or to what extend a systemic exposure to lignosulfonate is given after oral administration. As a food additive lignosulfonate is not meant to have nutritional benefit,

and the oral absorption of lignosulfonate is considered to be very low. The extremely low permeation of lignosulfonate in our Caco-2 monolayer model supports this prediction (report in preparation). However, measurement of lignosulfonate blood or tissue levels is not possible due to its physico-chemical properties.

Therefore, radiolabelling of the polymer was necessary to resolve the analytical problems. Besides beeing a comparatively rapid and economical method, tritium labelling has the advantage that changes in the molecular structure are negligible, and thus absortion properties, remain unchanged compared to the unlabelled lignosulfonate. However, the drawback of using tritium is, that unavoidable radiolysis occurs and subsequently tritiated water is formed, which is readily absorbed leading to systemic radioactivity levels, e.g. in blood and urine.

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, but it is not unlikely that the test item also contained tritium-labelled low molecular weight components. These are probably absorbable and thereby might have constituted the radioactivity found in blood and urine.

We performed absorption, distribution, and excretion studies using unspecifically tritium labelled [^{3}H]-lignosulfonate.

2. OBJECTIVES

The objective of this study was to determine radioactivity levels in blood, tissues and excreta 48 h after single oral administration of ^{3}H-lignosulfonate to rats at a dose level of 10 mg/kg bw.

To assess the appropriate radioactive dose for analytics, 3 male rats were given a dose of 200 µCi/kg bw in a pilot study (Protocol No.: VFHS 03-2004). In the main study (Protocol No.: VFHS 01-2005), three female and three male rats were treated with a slightly increased radioactive dose (250 µCi/kg bw).

3. MATERIAL AND METHODS

3.1. Study Design

Protocol No.: VFHS 03-2004 (Pilot)

Three male rats received a single dose of ^{3}H-lignosulfonate by oral gavage at a dose level of 10 mg/kg bw (200 µCi/kg bw).

Multiple samples (0.5 ml) were taken from the cannulated vena jugularis at the following time points: 1h, 2h, 4h, 6h, and 24h post dose. Urine and faeces were collected for 0-24h and 24-48h. Rats were sacrificed and dissected at 48 h, post dose.

Total radioactivity was determined in blood, urine and faeces. Additionally, radioactivity levels in gastrointestinal tracts (stomach, small intestine, colon), in liver, skin/fur, and rest carcass were determined before and after drying.

To further characterize the ^{3}H-lignosulfonate absorbed, urine samples were fractionated into several molecular weight fractions, and radioactivity of each fraction was determined separately.

Protocol No.: VFHS 01-2005

Three male and three female rats were kept individually in metabolic cages. All animals received a single dose of ^{3}H-lignosulfonate by oral gavage at a dose level of 10 mg/kg bw (250 µCi/kg bw).

Urine and faeces were collected for 0-24 h and 24-48 h. Rats were sacrificed 48 h after dosing, terminal blood was collected and animals dissected.

Excretion of ^{3}H-lignosulfonate was determined by measurement of total radioactivity in faeces and urine.

Radioactivity levels in blood, plasma, liver, stomach, intestinal tract, colon, skin/fur, and rest carcass were determined before and after drying of sample aliquots. Additionally, the correspondent urine samples (0-24 h; 24-48 h) of the 3 animals of each gender were pooled and radioactivity in separated molecular weight fractions was determined.

3.2. Animals

3.2.1. Test System

Species/Strain	Wistar albino rats (SPF)
Source	RCC, Füllinsdorf, Switzerland
Number/Sex	3 female + 6 male
Body weight/age	≥ 200g , ♀ ~ 8-10 weeks, ♂ ~ 6-8 weeks
TVB-No.	75244

3.2.2. Animal Husbandry

Acclimatization	
Room	Bldg. 205/05 and 205/332
Accommodation	Rats were housed in groups of 3 in macrolon cages type 3, and individually in metabolic cages at least 24 h prior to administration.
Conditions	Temperature: 22 ± 2°C Rel. humidity: 50-60% Light: 12 h/day from 6 am to 6 pm (300 Lux) Air changes: 18-21/h
Diet	Kliba diet # 3436; ad libitum
Drinking water	Tab water ad libitum
Experimental Phase	
Room	Bldg. 205/332
Accommodation	Rats were housed individually in metabolic cages
Conditions	Temperature: 22 ± 2°C Rel. humidity: 50-60% Light: 12 h/day from 6 am to 6 pm (300 Lux) Air changes: 18-21/h
Diet	Kliba diet # 3433; ad libitum
Drinking water	Tab water ad libitum
Duration	48 h

3.2.3. Surgical Procedure

In case of multiple blood sampling (Protocol VFHS 03-2004), rats were surgically prepared by cannulating the vena jugularis under anaesthesia (Isoflurane : N_2O:O_2). The catheter (silicon tubing I.D. 0.508 mm over a PE 50 tubing I.D. 0.58 mm, total length 15 cm) was fixed in the vena jugularis, passed underneath the skin to the neck. The catheter was filled with heparinized saturated sucrose solution and closed with a metal pin.

3.3. Test Items

Name	Ca-lignosulfonate	^{3}H-lignosulfonate
Type	spray dried, fine, brown powder	liquid, aqueous solution
Source/Supplier	via VFP from Borregaard Industries Ltd. (Sarpsborg, Norway)	RC Tritec AG, (Teufen, Switzerland)
Storage conditions	4°C	-20°C

Identification	Ultrazine FG-R	04-0324-0324
Batch/Lot-No	DP 955/FG-R004	
Specification	see CoA (Aug 2005)	
Spec. activity/Conc.		3.5 mCi/mg (~ 1 mCi/ml)
Radiochemical purity		not specified

Ca-lignosulfonate is a brown, amorphous natural polymer, which is soluble in water, but not in any common organic solvent. The structure of lignosulfonate is not totally known, but the structural elements derive principally from OH-sulfonated coniferyl, p-coumaryl and sinapyl alcohol-type units. The molecular weight distribution of lignosulfonate spans a broad region. About 94% of the test material (Ultrazine FG-R 004/DP 955) 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 (VFP).

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 dissolved in DMF. 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 approx. 3.5 mCi/mg.

3.4. Formulation

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

The low molecular weight (LMW) fraction of the radiolabelled ^{3}H-lignosulfonate obtained from Tritec AG was separated from the labelled lignosulfonate (of higher molecular weight) before preparation of the application solution. This 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.

Due to continuous radiolysis in the concentrated sample with high specific activity, the last centrifugation step was performed right before preparation of the application solution. The activity of the resulting radiolabelled stock sample was then adjusted to approximately 1 mCi/ml.

3.4.2. Application solution

Protocol No.: VFHS 03-2004 (Pilot)

Ingredients	- 2.63 mg/ml Ca-LS in water - 1 mCi/ml (~3.5 mCi/mg) ^{3}H-lignosulfonate (after removal of low mol. weight fraction)
Preparation of dosing formulation	0.25 ml ^{3}H-lignosulfonate were added to 4.75 ml cold Ca-LS solution.
Concentration	2.5 mg lignosulfonate per ml formulation
Spec. activity	50 µCi per ml formulation (~20 µCi/mg Ca-LS)

Protocol No.: VFHS 01-2005

Ingredients	- 4 mg/ml Ca-LS in water - 1 mCi/ml (~3.5 mCi/mg) ^{3}H-lignosulfonate (after removal of low mol. weight fraction)
Preparation of dosing formulation	0.6 ml ^{3}H-lignosulfonate were added to 6 ml cold Ca-LS solution and filled up to 12 ml with H_2O.
Concentration	2 mg lignosulfonate per ml formulation
Spec. activity	~50 µCi per ml formulation (~25 µCi/mg Ca-LS)

3.5. Treatment

Route of administration	Orally by gavage
Lignosulfonate dose level	Nominal dose of 10 mg/kg bw
Dose volume - radioactivity	
Protocol 03-2004 (Pilot):	4 ml / kg bw or 200 µCi/kg bw
Protocol 01-2005:	5 ml / kg bw or 250 µCi/kg bw
Frequency of administration	single oral dose

3.6. Experimental Procedures

In the pilot study three male rats (R1-R3), in the main study three female (F1-F3) and three male (M4-M6) rats were included. Rats R1-R3 were surgically prepared by cannulating the vena jugularis as outlined in section 3.2.3.

All rats were kept individually in metabolic cages, and urine control samples (cU) of R1, F1 and M4 were collected over a period of 24 hr (-24-0 h) before application.

All animals received a single oral gavage dose of 10 mg lignosulfonate per kg body weight. The syringe was weighed before and after administration to determine the individual amount precisely. The application solution was prepared as outlined in section 3.4.2.

After administration, an aliquot of the application solution was used for analysis, another aliquot of 0.1 ml was diluted 1:10 with H_2O and used to spike cU. The rest of the application solution was shock frozen in liquid nitrogen before storage at -80°C until analysis.

Blood samples of about 0.5 ml each were collected from the cannulated vena jugularis of R1-R3 at 1h, 2h, 4h, 6h, and 24h. Samples were transfered to EDTA tubes to prevent coaggulation.

For each animal, urine and faeces were collected in the intervals 0-24 h, and 24-48 h. During collection, vessels for excreta were cooled with solid carbon dioxide/ethanol. As radioactivity in faeces was expected to be high, while in contrast, radioactivity in urine was expected to be very low, special care was taken to avoid crosscontamination. For one 24 h collection interval, 5 ml of each control urine (cU_R, cU_F and cU_M) were kept in equal vessels and spiked at the beginning of the collection period with 50 µl of 1:10 diluted application solution (0.25 µCi), which approximately corresponds to the expected 0.05-0.1% of a rat's dose per ml urine. These control samples served to differentiate between radiolysis of the application solution in urine during sample storage versus radiolysis occuring in the gastrointestinal tract before oral absorption and/or during systemic circulation.

After homogenization of faeces with water and determination of total weights, all urine and faeces samples were stored at -80°C until analysis.

Animals were sacrificed by exsanguination from the vena cava caudalis under isoflurane anesthesia after 48 h. The terminal blood was collected in EDTA tubes to prevent coaggulation, and aliquots of about 1 g were removed for direct measurement of radioactivity. From the remaining blood samples of F1-F3 and M4-M6 EDTA-plasma was prepared immediately by centrifugation (1400 $\times$ g , 15 min, 4°C). Whole blood, plasma and the cellular fraction samples were stored in separate tubes.

Animals were dissected and weights of removed organs and tissues were determined. First, the intestinal tract (stomach, small intestine and colon including its contents) was removed carefully to prevent contamination of the rest carcass with high levels of radioactivity. Liver and skin/fur were removed, the rest carcass homogenized. Remaining radioactivity was determined in cage washes.

Weights of all samples were determined before samples were shock frozen in liquid nitrogen, and stored at -80°C until analysis.

Aliquots of urine, faeces, blood, and tissues were analyzed for total radioactivity by liquid scintillation counting (LSC) and after combustion in an oxidizer. Additionally, urine samples were further analyzed by fractionating and subsequent LSC of the single fractions.

3.7. Analytical Methods

3.7.1. Analysis of the application solution

Ca-LS in high 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). However, a lot of compounds show absorption in this spectral region, therefore also the so called nitrosolignin method described by Jayme and Pohl [3] was used for Ca-LS quantification. In principle, the reaction of lignosulfonate with nitric acid results in a colour change shifting the absorbance of lignosulfonate to higher wavelengths with two maxima around 350 nm and 440 nm.

The homogeneity and activity of the application solution was determined directly by counting diluted aliquots mixed with 10 ml scintillation cocktail 'ultima gold' in a liquid scintillation counter.

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

Column:	TKS Super SW3000; 4.6 $\times$ 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 fraction in the chromatogram was determined.

In the ^{3}H-lignosulfonate stock solution, no UV absorbance could be detected due to low concentration, i.e. high specific radioactivity (~3.5 mCi/mg). In this case, SEC was applied for fractionation and the molecular weight distribution was assessed by relating the radioactivity in the fractions to the retention time (fraction number) on the column.

3.7.2. Analysis of Biological Samples

3.7.2.1. Determination of Radioactivity

Sample aliquots were analyzed directly or after solubilization with the appropriate solubilizer ('Solvable', Perkin Elmer; or 'Soluene', Packard) by mixing with scintillation cocktail 'ultima gold' and counting in a liquid scintillation counter.

Radioactivity in most samples was also determined after drying. Sample aliquots were put on a "combusto cone" and placed on a "combusto pad". After drying over night the samples are combusted in an oxidizer (Packard Oxidizer model 307). The ^{3}H is collected as tritiated water and, after addition of Monophase solution, counted in a liquid scintillation counter. In some cases the radioactivity in lyophilized samples was determined in addition. After lyophilization, samples are dissolved in 'Soluene' before mixing with 'ultima gold'.

3.7.2.2. Molecular weight distribution of radioactivity in urine and plasma

Individual or pooled urine and plasma samples for each gender and time interval were fractionated by HPLC (SEC) and radioactivity of the fractions was determined by LSC according to the procedure described in section 3.7.1. in order to characterize absorbed radioactivity with respect to its molecular weight.

4. RESULTS AND DISCUSSION

4.1. Characterization of Radiolabelled ^{3}H-Lignosulfonate Solution

Size exclusion chromatography (SEC) was used to determine the molecular weight distribution of lignosulfonate (Fig. 3A). The chromatogram 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. 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).

Radiolabelled ^{3}H-lignosulfonate as received from Tritec AG was fractionated by SEC, and radioactivity in eluting fractions was determined (Fig. 4). 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, eg mannose and xylose (MW ca 150-180), contribute to this radiolabelled low molecular weight (LMW) fraction, and a major part of it is assumed to be tritiated water, most likely resulting from radiolysis of ^{3}H-lignosulfonate. Tritiated water (HTO) elutes with a sharp peak between 24-26 min.

As these small molecules are very likely to be absorbed easily, and thereby leading to radioactivity levels in blood and plasma, they were removed as much as possible from the labelled lignosulfonate (of higher molecular weight) by repeated ultrafiltration before preparation of the application solution. The molecular weight distribution of radioactive components in the last filtrate as well as in the resulting ^{3}H-lignosulfonate are also shown in Figure 4. More than 50% of the radioactivity in the filtrate showed the retention time of tritiated water. In contrast, after the last filtration step, less than 3% of the radioactivity in the ^{3}H-stock-solution, which was used to prepare the application solutions, was of low molecular weight (RT>20 min in SEC.)

After preparation of the ^{3}H-lignosulfonate application solutions, radioactivity measurements revealed good homogeneity with lower than 1% of variation between independent samples.

The molecular weight distribution of lignosulfonate and of the corresponding radiolabelled components in the application solution are shown in Figure 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. Less than 3% of the radioactivity elutes after longer than 22 min. Some difference in the shape of the MW distribution curve was observed comparing the application solutions of the pilot study and the main study. This probably reflects the gel properties of the column changing with time and use. However, the molecular weight distribution profile detected at 280 nm, is almost identical to what is observed with unlabelled

lignosulfonate (Fig 3A) with retention times between 10 and 22 min, clearly demonstrating the identity of the unlabelled and radiolabelled lignosulfonate.

The properties of the application solutions are summarized in the following table:

	Pilot study	Main study
Lignosulfonate concentration	2.5 mg/ml	2.0 mg/ml
Radioactivity	50.58 µCi/ml	51.07 µCi/ml
Radiolabel in LMW fraction (RT>22min)	2.25%	2.71%
Dosing	4 ml/kg bw	5 ml/kg bw

The stability of the application solution was checked after 1 week of storage both at 4°C and -80°C. As depicted in Fig. 6A the MW distribution in the lignosulfonate solution remained stable independent of the storage temperature. Also, no significant radiolysis could be observed after 1 week. However, after 3 weeks storage at -20°C roughly 25% of the radioactivity elutes from the SEC column at retention times of tritiated water (Fig 6B), while the MW distribution profile of lignosulfonate remains almost unchanged.
In summary, 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. Application

A single dose of 10 mg/kg bw ^{3}H-lignosulfonate was administered to rats by oral gavage. Three male rats (R1-R3) were included in the pilot study, and three female (F1-F3) and three male (M4-M6) were studied in the main trial. The application data with animal weights and given doses are summarized in Table 1.

4.3. Blood and Plasma Levels

Radioactivity levels in multiple blood samples taken from R1-R3 at 1h, 2h, 4h, 6h, and 24h are extremely low, reaching the highest level of 0.0015 $\pm$ 0.00015% of the dose per g blood ca. 6h after administration. The radioactivity corresponds to about 36.6 ng equivalent lignosulfonate per g blood, and the level remains almost unchanged until sacrifice at 48h. No significant interindividual differences were observed, and the profile of the mean values is shown in Figure 7.

Radioactivity levels in terminal blood (48h) of the rats in the main study are in the same range of 0.0017% and 0.0013% of the dose per g blood for females and males, respectively. Total radioactivity in 48h blood (based on an average total volume of 7-8g) does not exceed 0.012% of the given dose for females and 0.01% for males. After plasma preparation the radioactivity is almost evenly distributed between plasma and cellular fraction (radioactivity plasma:blood ~ 0.57).

Interestingly, if radioactivity is determined after solubilization of the blood instead of counted after oxidizer combustion, the levels are 50-60 fold higher with 0.092% and 0.074% per g blood for females and males, respectively. This means, that most of the radioactivity in blood samples is removed during the drying process preceding combustion and therefore must be attributed to tritiated water rather than to lignosulfonate. This is confirmed, when plasma is analyzed by counting the radioactivity of molecular weight fractions prepared by SEC. Only about 1% of the radioactivity is found in fractions of higher molecular weight eluting from the column before 22 min, while 98.5% coelute with tritiated water after ca. 25 min (Fig 8).

4.4. Excretion Balance

Radioactivity in excreta, blood, tissues and organs was determined by LSC either directly or after solubilization of the samples. Values for all individuals are listed in table 2a (R1-R3), as well as in table 3a for female (F1-F3), and table 4a for male rats (M4-M6) of the main study.

Full recovery of radioactivity was achieved with 98.4 $\pm$ 0.7% of the given dose after 48h. Recovery in the pilot study was not as good (88.4 $\pm$ 4.8%) most probably due to incomplete collection of faeces.

After 48h, the majority of radioactivity (74.6 $\pm$ 1.1%) is recovered in faeces. While total faecal excretion over 48h is not significantly different between males and females, it seems to be slightly slower in females as can be seen in the different distribution within the two collecting intervals.

Faecal excretion (radioactivity in % of dose):

	Females	Males	All
0-24h	61.9 $\pm$ 0.9	70.6 $\pm$ 2.5	66.2 $\pm$ 5.1
24-48h	12.2 $\pm$ 1.9	4.4 $\pm$ 1.7	8.3 $\pm$ 4.6
sum (0-48h)	74.1 $\pm$ 1.3	75.0 $\pm$ 1.0	74.6 $\pm$ 1.1

In contrast, radioactivity in urine over 48h is very low, with 3.1% and 2.6% for females and males, respectively. Amounts are only slightly higher in the first 24h interval.

Urinary excretion (radioactivity in % of dose):

	Females	Males	All
0-24h	1.67 ± 0.13	1.31 ± 0.31	1.49 ± 0.29
24-48h	1.46 ± 0.08	1.25 ± 0.18	1.35 ± 0.17
sum (0-48h)	3.12 ± 0.11	2.56 ± 0.49	2.84 ± 0.44

Similar results for urinary excretion have also been obtained in the pilot study, with 1.4% of the radioactivity in each of the two 24h collection intervals.

Levels in tissues and organs of the animals do not show any significant gender differences or interindividual variance. In the gastrointestinal tract with stomach, small intestine and colon (including the contents), radioactivity accounts for 2.1 ± 0.3% of the dose after 48h.

4.4 ± 0.5% of the given radioactivity is associated to the skin/fur of the animals (pooled samples taken from neck, belly and back), the mayor part of it most probably due to (external) contamination of the fur with excreta. Minor amounts of 0.04 ± 0.01% are also recovered in cage washes.

Apart from urine, which contains radioactivity that has been absorbed by the rats, systemic radioactivity was determined in blood, and in liver as well as in the homogenized remaining carcass. Levels in blood and liver were low with 0.6 ± 0.1% and 0.7 ± 0.1% of the dose, respectively, while 13.1 ± 0.7% of the given radioactivity was recovered in the remaining carcass.

The mean values of radioactivity recovered after 48h are compiled in the following table:

	radioactivity (% of dose)
faeces	74.56
gastrointestinal tract	2.10
cage wash	0.04
skin/fur	4.42
urine	2.84
blood	0.61
liver	0.73
remaining carcass	13.06
sum	98.37

4.5. Radioactivity Levels After Removal of Water

A relatively high amount of the radioactive dose was found in blood, liver and remaining carcass. Together with urinary radioactivity it adds up to approximately 17.2% of the dose which has been absorbed by the rats. In view of the hydrophilic nature together with its undefined, but high molecular weight it is unlikely that lignosulfonate is absorbed to that extend. Tritiated water formed by radiolysis of ^{3}H-lignosulfonate on the other hand might give rise to misleading high radioactivity levels in the tissues. Therefore, the radioactivity found in the body was suspected to origin - at least to some extend - from tritiated water formed before absorption or to be associated to smaller molecules, like mannose and xylose, also present in the lignosulfonate test material.
Looking at the radioactivity concentrations per gram of these samples rather than at the amounts gives further support for this hypothesis.

	radioactivity (% of dose per g sample)
urine	0.070
blood	0.083
liver	0.085
remaining carcass	0.077
average	0.079 ± 0.007

The concentration of radioactivity in all the above listed samples is in the same (low) order of magnitude. This would be expected if tritiated water is formed by radiolysis, as it will be readily absorbed and distributed evenly within the body without preference for a specific tissue.
To test, whether in fact and to what extend tritiated water causes the systemic exposure to radioactivity after oral administration of ^{3}H-lignosulfonate, aliquots of most samples were dried to reduce the water content as much as possible before determining the radioactivity levels again. Alternatively, liquid samples as urine and plasma were analyzed by determining the radioactivity of different molecular weight fractions separated by SEC.

4.5.1. Analysis of urine

High UV absorption of many other components in urine as well as the low concentrations of ^{3}H-lignosulfonate in these samples did not allow analytical UV detection in a HPLC run. Therefore, pooled urine samples for each gender and time interval were fractionated by SEC and radioactivity of the fractions was determined by LSC in order to characterize the excreted urinary radioactivity with respect to its molecular weight.

As p-aminobenzoic acid with a molecular weight of 137.14 elutes after 21.5 min (see Fig. 3), the 22 min fraction was used as a limit to separate potential lignosulfonate components from the very-low molecular weight fraction with longer retention times on the column.

The MW distribution of radioactivity in urine is shown in figure 9 for the rats of the main study and in figure 10 for R1-R3 of the pilot study. 96.8% of radioactivity in 0-24h urine, and 100% of the 24-48h urine elute with the very low molecular weight fractions after 22 min, clearly indicating that almost all of the urinary radioactivity is present as tritiated water mainly. Roughly 3.2% of the 0-24h urinary radioactivity was found in the fractions between 16 and 21 min. No difference could be observed between males and females.

The results correspond well with the results from the pilot trial, where ca 3.8% of the urinary radioactivty eluted in the fractions before 22 min (Fig 10).

Some radiolysis also ocurred in urine spiked with the original application solution and handled in parallel with the urine samples (Fig. 9A). Approximately 8% of the radioactivity coelutes with tritiated water, compared to less than 3% of the radioactivity in application solution itself. Interestingly, also the portion of highest molecular weight around the 11 min fraction increases in the spiked urine sample, which might indicate a tendency of the lignosulfonate molecules to conglomerate or polymerize in urine.

100% of the radioactivity of urine spiked with tritiated water elutes in the 23-26 min fractions.

Portion of radioactivity eluting from SEC column before and after 22 min (MW~140):

	MW≥140	MW<140	radioactivity in % of dose	
application solution	0.973	0.027		
control urine spiked with applic. sol.	0.919	0.081		
control urine spiked with tritiated water	0.000	1.000	total	MW≥140
0-24h urine	0.032	0.968	1.49	<0.05
24-48h urine	0.000	1.000	1.35	0.00

In summary, only a very small portion of urinary radioactivity can be associated with compounds of higher MW, i.e. with lignosulfonate. Thus, after 48h, less than 0.05% of the lignosulfonate dose is excreted in urine.

4.5.2. Analysis of dried samples

Radioactivity in faeces, tissues and organs was determined again in dried samples. Individual values for the rats are listed in tables 3b and 4b.

The mean values in % of dose administered and the values comparing the radioactivity of the dried samples with the values of 'untreated', wet samples are compiled in the following table:

Radioactivity in dried samples:

	% of dose	% of 'wet' samples
faeces	71.04	95.3
skin/fur	0.44	9.9
stomach *	0.01	3.0
small intestine *	0.03	3.6
colon *	0.11	11.8
blood	0.01	1.8
liver	0.08	10.4
remaining carcass	0.66	5.0

* tissues incl. contents

More than 95% of the radioactivity in faeces remains upon drying, indicating that only little tritiated water is present, and ^{3}H-lignosulfonate is excreted mainly unchanged in faeces.

In contrast, a striking difference in radioactivity levels between the dried and untreated tissue and organ samples can be observed. Only small fractions of the radioactivity is retained in the dry tissues.

The highest retention (11.8% of wet value) was observed in colon. To a major degree this is due to one female rat (F1) and could be explained by slower excretion in this animal as radioactivity was determined in all GI tracts including the contents. One could assume, that some of the remaining radioactivity in colon samples would have been excreted as faeces after 48h. Relatively high retention of 9.9% was also observed in skin/fur, which can be explained by contamination of the animals fur by excreta.

In blood and in the remaining carcass only 1.8% and 5% of the radioactivity is retained after drying, respectively. Retention in liver seems to be higher (10.4%).

However, the levels in dried tissues are very low and without the skin/fur, total radioactivity in the animal tissues add up to only 0.9% of the dose.

In summary, the major part of the radioactivity found after oral administration of ^{3}H-lignosulfonate in organs and tissues are caused by tritiated water, as removal of water by air drying or lyophilization of the tissues reduces radioactivity levels significantly. Therefore it can be concluded, that the radioactivity levels found in the dried samples are the maximum lignosulfonate levels expected after oral administration.

5. SUMMARY

Absorption, excretion and tissue distribution of lignosulfonate was studied in male and female rats after single oral administration of radiolabelled ^{3}H-lignosulfonate.

After 48h, complete recovery of radioactivity was achieved (98.4 ± 0.7%). The majority (74.6%) of the administered radioactivity was excreted via faeces, only 2.8% via urine, and ca. 20.9% of the radioactivity was recovered in skin/fur, gastrointestinal tracts, blood, liver and carcass.

Apart from slightly slower faecal excretion of females, no further sex difference was observed, and also the overall interindividual variability was very low.

Tritiated water (HTO) is formed by radiolysis of ^{3}H-lignosulfonate causing misleading radioactivity levels in urine, blood and tissues, as HTO is readily absorbed and distributed in the body. Removal of water by drying tissue or organs or by separating the very low molecular weight fractions in urine by SEC resulted in significantly lower radioactivity levels. These in turn reflect levels of radiolabelled lignosulfonate.

	direct measurement (LSC)	**after 'subtraction' or removal of water**
Faeces 0-24h	*66.24*	*63.44*
Faeces 24-48h	*8.32*	*7.61*
Total Faeces	**74.56**	**71.04**
Stomach	*0.30*	*0.01*
Small Intestine	*0.87*	*0.03*
Colon	*0.93*	*0.11*
Total GI (incl. contents)	**2.10**	**0.15**
Skin/Fur	**4.42**	**0.44**
Blood	*0.61*	*0.01*
Liver	*0.73*	*0.08*
Remaining carcass	*13.06*	*0.66*
Total 'intrinsic'	**14.40**	**0.75**
Urine 0-24h	*1.49*	*0.05*
Urine 24-48h	*1.35*	*0.00*
Total Urine	**2.84**	**0.05**
Recovery	98.37	
systemic radioactivity (absorbed / excreted)	**< 18.0%**	**< 1.0%**

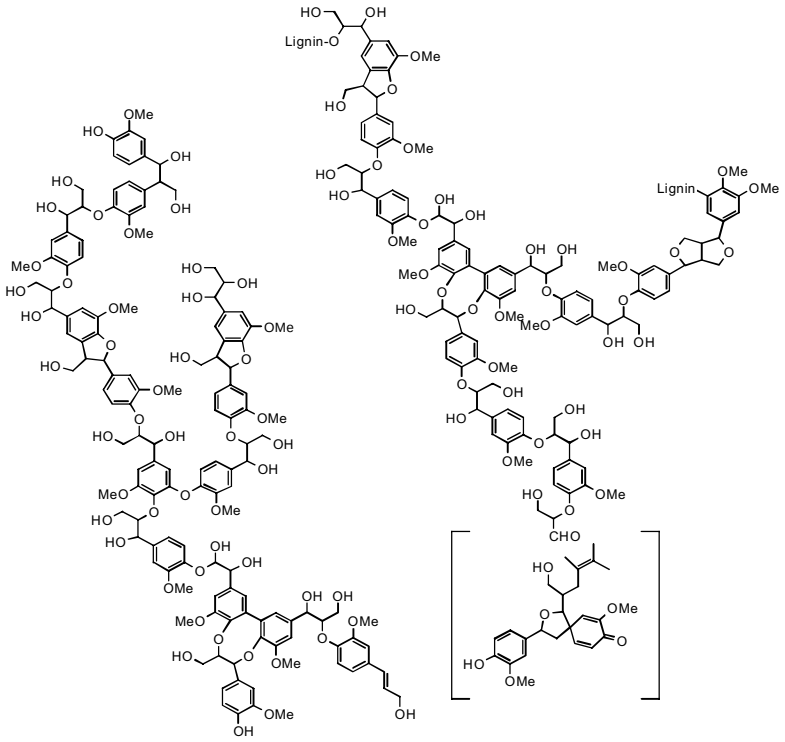
Only 0.8% of the administered lignosulfonate dose is found in urine, blood, liver and carcass 48h after oral administration of ^{3}H-lignosulfonate. As complete removal of water might not have been achieved, the actual levels might even be lower.

It can be concluded, that lignosulfonate absorption is very low after oral administration.

FIGURES AND TABLES

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 [4]).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; [3]).

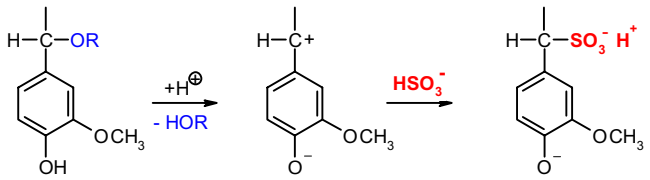


Figure 2: UV absorbance of Lignosulfonate

UV-Absorbance spectrum: 0.1 mg/ml Ca-Lignosulfonate in aqueous buffer (HEPES/pH 7.4).

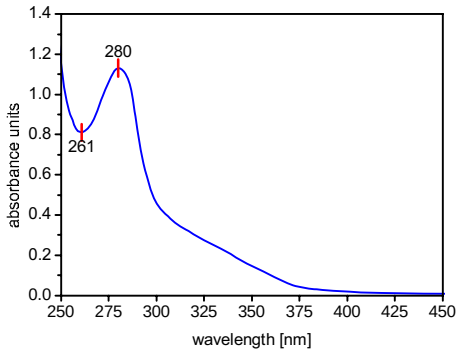


Figure 3: MW distribution of Lignosulfonate

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

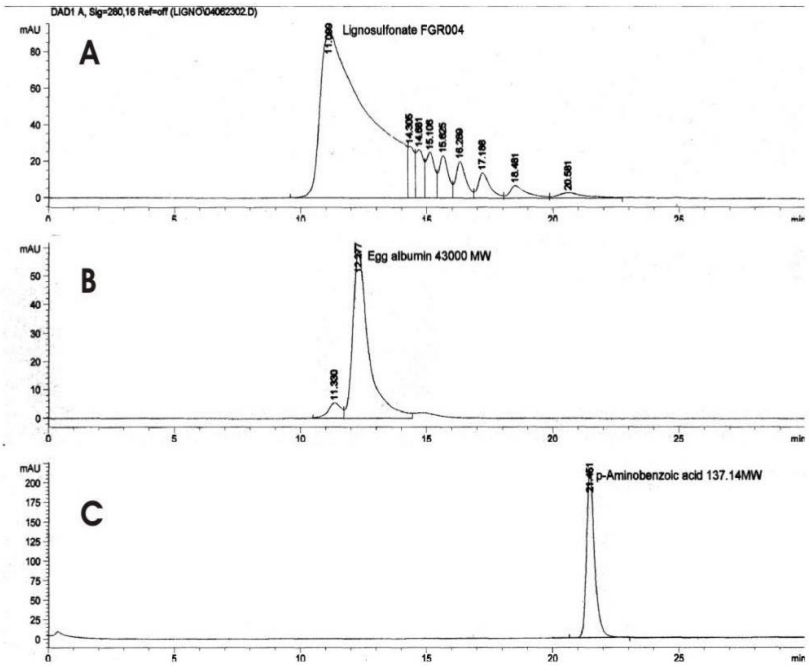


Figure 4: MW distribution of ^{3}H-Lignosulfonate before and after filtration

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

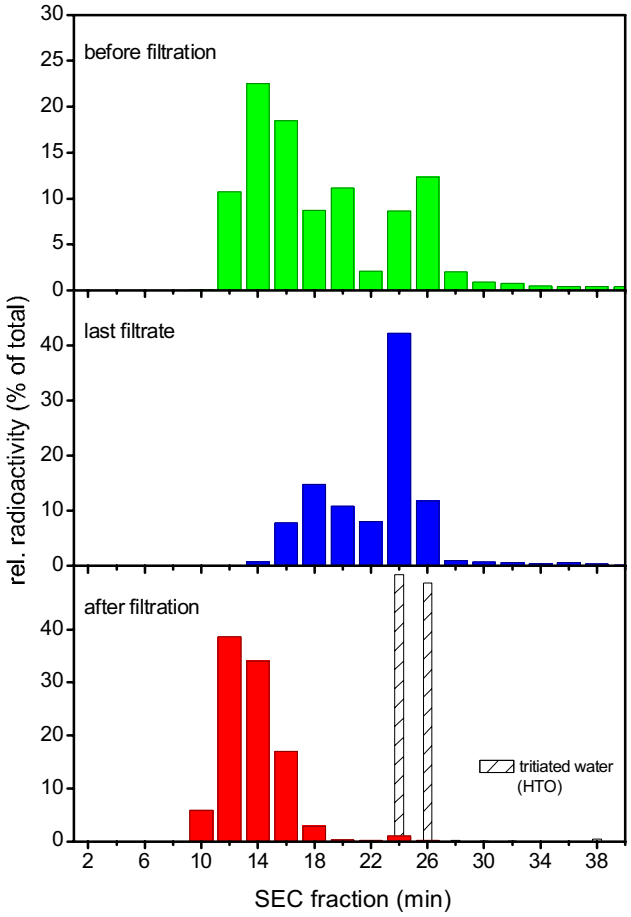


Figure 5: Application solutions

Molecular weight distribution of lignosulfonate application solutions as detected by UV (280 nm) in SEC (green) and radioactivity in eluting fractions (red).

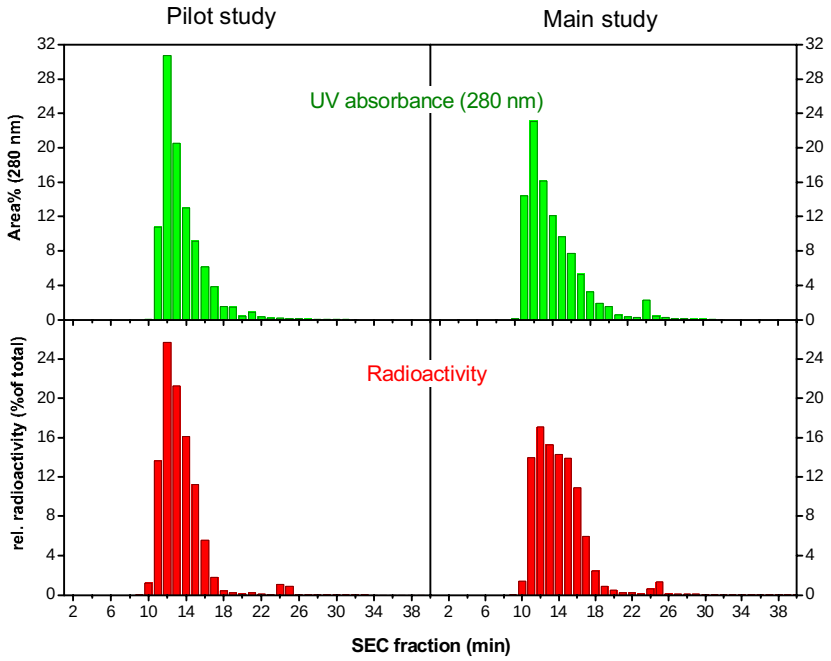
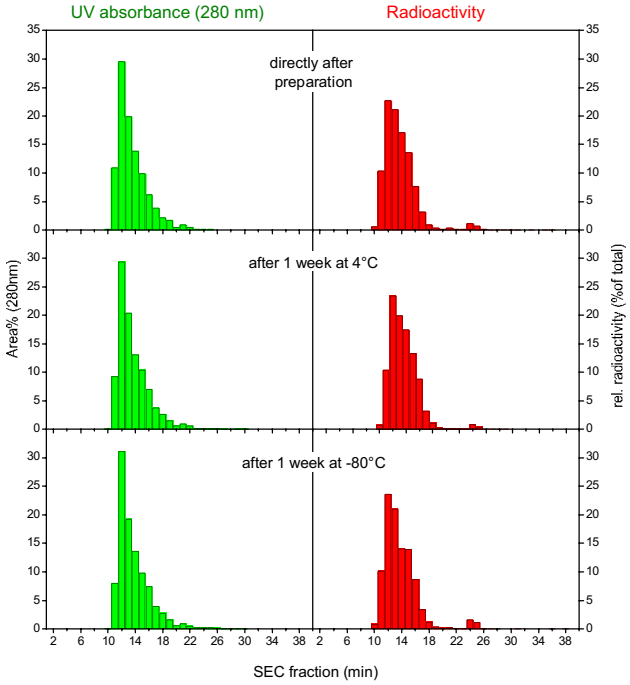


Figure 6: Stability of application solution

(A) Stability of application solution during storage.



(B) Formation of tritiated water by radiolysis in ^{3}H-lignosulfonate solution during longer storage periods.

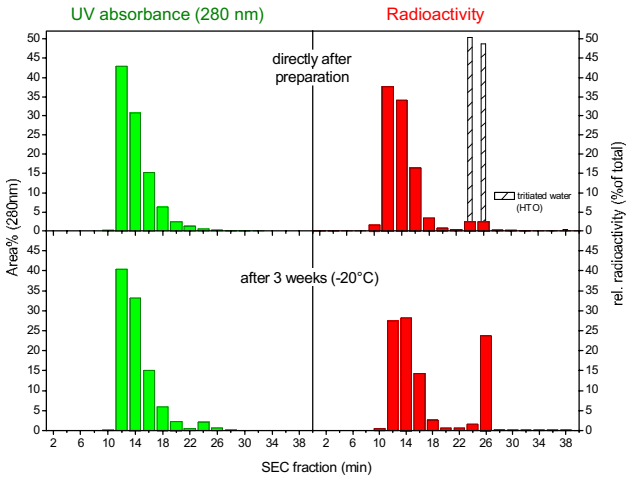


Figure 7: Radioactivity in blood

Radioactivity levels in blood after single oral administration of ^{3}H-lignosulfonate to male rats. Data are shown in dpm and in % of dose per gram blood.

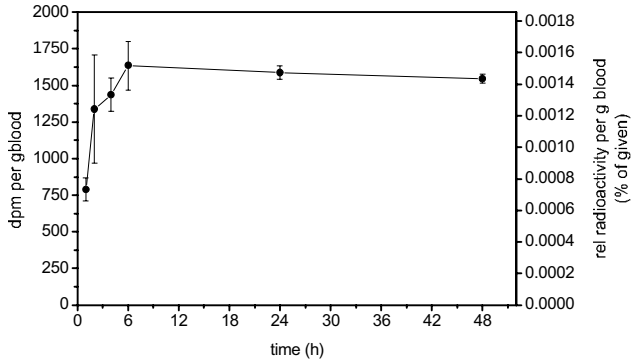


Figure 8: MW distribution of plasma radioactivity

Distribution of plasma radioactivity with respect to molecular weight (SEC).

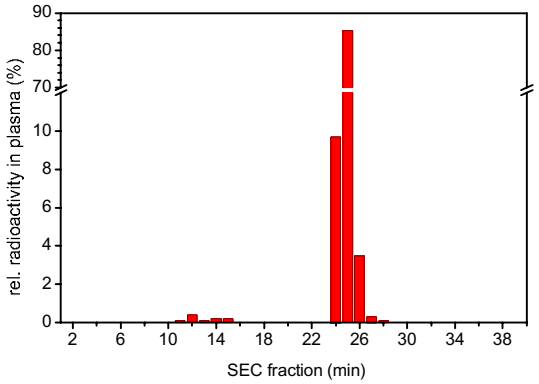


Figure 9: MW distribution of urinary radioactivity (Main study)

The molecular weight distribution of the radioactivity in the application solution and in spiked control urine (A) in comparison to the distribution of the radioactivity in pooled female and male urine samples for the collection intervals 0-24h (B) and 24-48h (C).

The dotted line at the 22 min fraction 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 1-22 min).

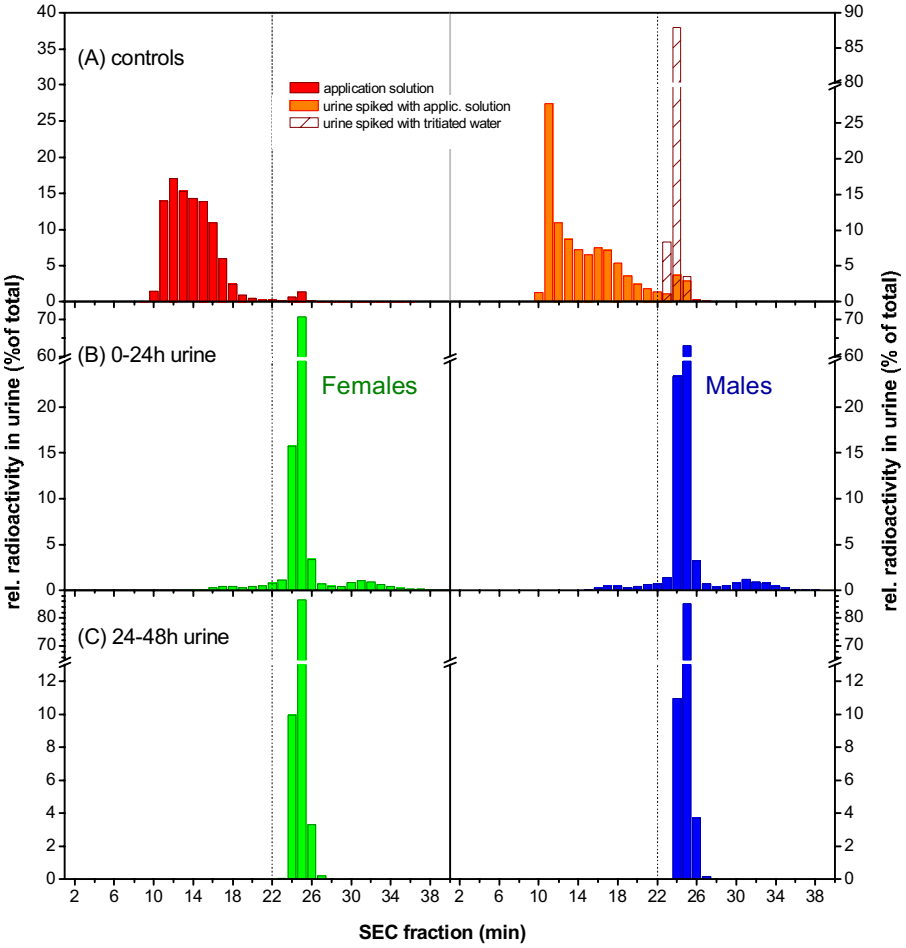


Figure 10: MW distribution of urinary radioactivity (Pilot study)

The molecular weight distribution of the radioactivity in the application solution and in spiked control urine (A) in comparison to the distribution of the radioactivity in urine samples of the collection intervals 0-24h (B) and 24-48h (C).

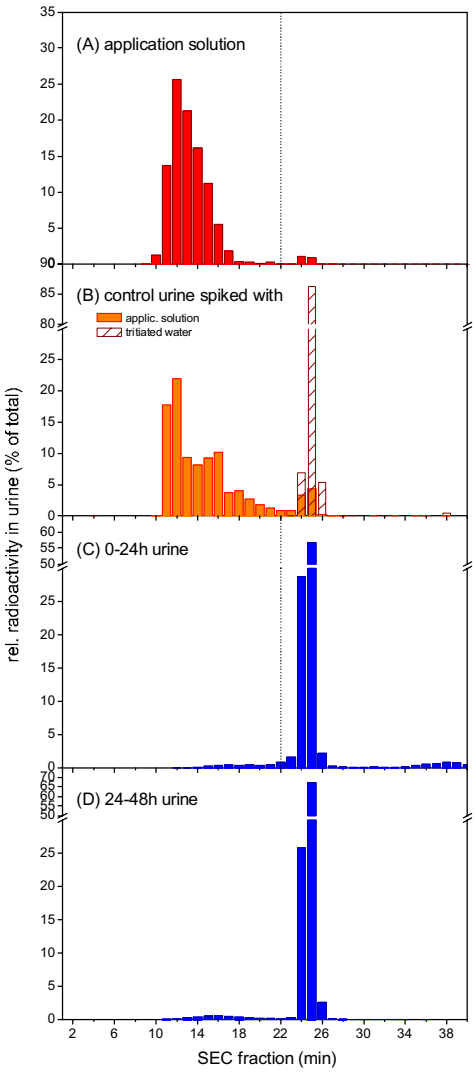


Table 1: Animal weights and doses

(a) Pilot study

Rat	R1	R2	R3	mean	SD
animal weight (kg)	0.248	0.248	0.249	**0.248**	0.001
lignosulfonate (mg/kg bw)	9.7	9.7	9.7	**9.7**	0.02
tritium dose (µCi/kg bw)	195.6	195.9	195.0	**195.5**	0.4

(b) Main study

Rat	F1	F2	F3	mean females	SD
animal weight (kg)	0.254	0.247	0.228	**0.243**	0.013
lignosulfonate (mg/kg bw)	10.3	10.2	10.1	**10.2**	0.1
tritium dose (µCi/kg bw)	261.4	257.3	257.0	**258.5**	2.5

Rat	M4	M5	M6	mean males	SD
animal weight (kg)	0.283	0.278	0.301	**0.287**	0.012
lignosulfonate (mg/kg bw)	10.2	10.2	10.2	**10.2**	0.05
tritium dose (µCi/kg bw)	259.4	257.3	257.3	**258.0**	1.2

Table 2: Radioactivity levels in male rats 48 h after single oral administration of ^{3}H-lignosulfonate (Pilot trial)

(a) Radioactivity (in percent of given dose) in unchanged excreta, GI tract, blood and tissues 48h after oral administration of ^{3}H-lignosulfonate.

Animals		R1	R2	R3	mean	SD
Urine	0-24h	1.72	1.41	1.12	1.42	0.30
	24-48h	1.43	1.25	1.52	1.40	0.13
	total (0-48h)	3.14	2.66	2.64	**2.81**	0.28
Cage wash		0.05	0.07	0.02	**0.05**	0.02
Faeces	0-24h	52.5	57.5	60.8	56.9	4.2
	24-48h	7.1	7.0	9.9	8.0	1.6
	total (0-48h)	59.6	64.5	70.7	**64.9**	5.6
GI tract (tissue incl. contents)	stomach	0.23	0.32	0.25	0.27	0.05
	small intestine	1.53	1.55	1.42	1.50	0.07
	colon	0.33	0.34	0.27	0.31	0.04
	total GI	2.09	2.21	1.94	**2.08**	0.14
'Intrinsic'/ 'systemic'	whole blood	0.84	0.72	0.73	0.76	0.06
	liver	0.90	0.96	0.83	0.90	0.07
	remaining carcass	13.29	13.45	13.37	13.37	0.08
	total 'intrinsic'	15.02	15.14	14.93	**15.03**	0.11
skin/fur		3.93	3.31	3.27	**3.50**	0.37
Recovery		83.8	87.8	93.5	**88.4**	4.8

(b) Radioactivity (in percent of given dose) in excreta, GI tract, blood and tissues after removal of water by drying or by separating low molecular weight fraction (urine).

Animals		R1	R2	R3	mean	SD	% of 'wet'
Urine	0-24h	0.06	0.06	nd	0.06	0.00	4.08
	24-48h	0.01	0.08	nd	0.05	0.05	3.40
	total (0-48h)	0.07	0.14		**0.11**	0.05	**3.74**
Faeces	0-24h	54.7	58.0	58.5	57.0	2.1	100.2
	24-48h	6.5	5.9	8.6	7.0	1.4	87.5
	total (0-48h)	61.1	63.9	67.0	**64.0**	2.9	**98.7**
GI tract (tissue incl. contents)	stomach	0.008	0.009	0.008	0.009	0.001	3.24
	small intestine	0.085	0.069	0.069	0.074	0.009	4.95
	colon	0.051	0.036	0.023	0.037	0.014	11.74
	total GI	0.14	0.11	0.10	**0.12**	0.02	**5.75**
'Intrinsic'/' systemic'	whole blood	0.012	0.011	0.010	0.011	0.001	1.43
	liver	0.083	0.084	0.075	0.080	0.005	8.98
	remaining carcass	0.79	0.76	0.74	0.76	0.02	5.70
	total 'intrinsic'	0.88	0.85	0.83	**0.85**	0.03	**5.68**

nd not determined

Table 3: Radioactivity levels 48h after oral administration of ^{3}H-lignosulfonate to female rats (Main study)

(a) Radioactivity (in percent of given dose) in unchanged excreta, GI tract, blood and tissues 48h after oral administration of ^{3}H-lignosulfonate.

Female animals		F1	F2	F3	mean	SD
Urine	0-24h	1.54	1.80	1.67	1.67	0.13
	24-48h	1.46	1.37	1.54	1.46	0.08
	total (0-48h)	3.00	3.17	3.20	**3.12**	0.11
Cage wash		0.03	0.04	0.05	**0.04**	0.01
Faeces	0-24h	60.9	62.1	62.7	61.9	0.9
	24-48h	14.4	10.7	11.7	12.2	1.9
	total (0-48h)	75.3	72.8	74.4	**74.1**	1.3
GI tract (tissue incl. contents)	stomach	0.35	0.29	0.33	0.32	0.03
	small intestine	0.86	0.97	0.77	0.87	0.10
	colon	1.43	0.83	0.83	1.03	0.35
	total GI	2.64	2.09	1.93	**2.22**	0.37
'Intrinsic'/ 'systemic'	whole blood	0.61	0.79	0.57	0.66	0.12
	liver	0.71	0.78	0.64	0.71	0.07
	remaining carcass	12.96	13.92	12.80	13.23	0.60
	total 'intrinsic'	14.29	15.48	14.01	**14.59**	0.78
skin/fur		4.05	5.00	3.67	**4.24**	0.68
Recovery		99.3	98.6	97.2	**98.4**	1.1

(b) Radioactivity (in percent of given dose) in excreta, GI tract, blood and tissues after removal of water by drying or by separating low molecular weight fraction (urine).

Female animals		F1	F2	F3	mean	SD	% of 'wet'
Urine	0-24h	SEC on pooled			0.05		3.18
	24-48h	SEC on pooled			0.00		0.00
	total (0-48h)		**0.05**		**0.05**		**1.70**
Faeces	0-24h	58.6	59.5	61.6	59.9	1.6	96.8
	24-48h	14.8	9.3	10.4	11.5	2.9	93.9
	total (0-48h)	73.4	68.8	72.0	**71.4**	2.4	**96.3**
GI tract (tissue incl. contents)	stomach	0.008	0.009	0.013	0.010	0.003	3.09
	small intestine	0.033	0.036	0.028	0.033	0.004	3.74
	colon	0.345	0.052	0.122	0.173	0.153	16.84
	total GI	0.39	0.10	0.16	**0.22**	0.15	**9.72**
'Intrinsic'/ 'systemic'	whole blood	0.011	0.014	0.011	0.012	0.002	1.86
	liver	0.077	0.079	0.067	0.074	0.006	10.48
	remaining carcass	0.65	0.69	0.58	0.64	0.05	4.84
	total 'intrinsic'	0.73	0.79	0.66	**0.73**	0.06	**4.98**
skin/fur		0.43	0.46	0.40	**0.43**	0.03	**10.17**

Table 4: Radioactivity levels 48h after oral administration of ^{3}H-lignosulfonate to male rats (Main study)

(a) Radioactivity (in percent of given dose) in unchanged excreta, GI tract, blood and tissues 48h after oral administration of ^{3}H-lignosulfonate.

Male animals		M4	M5	M6	mean	SD
Urine	0-24h	1.09	1.19	1.67	1.31	0.31
	24-48h	1.12	1.17	1.45	1.25	0.18
	total (0-48h)	2.21	2.36	3.11	**2.56**	0.49
Cage wash		0.06	0.06	0.03	**0.05**	0.02
Faeces	0-24h	72.2	67.7	71.9	70.6	2.5
	24-48h	2.9	6.3	4.0	4.4	1.7
	total (0-48h)	75.1	74.0	75.9	**75.0**	1.0
GI tract (tissue incl. contents)	stomach	0.29	0.25	0.28	0.27	0.02
	small intestine	0.89	0.87	0.87	0.88	0.02
	colon	0.86	0.88	0.75	0.83	0.07
	total GI	2.05	2.00	1.90	**1.98**	0.07
'Intrinsic'/ 'systemic'	whole blood	0.57	0.61	0.51	0.56	0.05
	liver	0.79	0.74	0.73	0.76	0.03
	remaining carcass	13.95	13.72	11.98	12.88	0.87
	total 'intrinsic'	14.32	15.07	13.22	**14.20**	0.93
skin/fur		4.50	4.77	4.55	**4.61**	0.14
Recovery		98.3	98.2	98.7	**98.4**	0.3

(b) Radioactivity (in percent of given dose) in excreta, GI tract, blood and tissues after removal of water by drying or by separating low molecular weight fraction (urine).

Male animals		M4	M5	M6	mean	SD	% of 'wet'
Urine	0-24h	SEC on pooled			0.04		3.18
	24-48h	SEC on pooled			0.00		0.00
	total (0-48h)				**0.04**		**1.63**
Faeces	0-24h	68.2	64.9	67.8	67.0	1.8	94.9
	24-48h	2.2	5.7	3.2	3.7	1.8	84.2
	total (0-48h)	70.4	70.6	71.0	**70.7**	0.3	**94.2**
GI tract (tissue incl. contents)	stomach	0.010	0.009	0.005	0.008	0.002	2.86
	small intestine	0.032	0.030	0.030	0.031	0.001	3.50
	colon	0.045	0.058	0.038	0.047	0.010	5.63
	total GI	0.09	0.10	0.07	**0.09**	0.01	**4.30**
'Intrinsic'/ 'systemic'	whole blood	0.010	0.010	0.009	0.010	0.001	1.71
	liver	0.083	0.073	0.079	0.079	0.005	10.39
	remaining carcass	0.68	0.69	0.66	0.68	0.01	5.25
	total 'intrinsic'	0.77	0.77	0.75	**0.76**	0.01	**5.38**
skin/fur		0.41	0.42	0.49	**0.44**	0.05	**9.56**

Table 5: Summary table of radioactivity levels before and after removal of tritiated water

Radioactivity in % of dose 48h after oral administration of ^{3}H-lignosulfonate to male rats (Pilot study). The corresponding table for the main study is shown in section 5.

	direct measurement (LSC)	after 'subtraction' or removal of water
Faeces 0-24h	*56.96*	*57.09*
Faeces 24-48h	*7.97*	*7.00*
Total Faeces	**64.93**	**64.09**
Stomach	*0.27*	*0.01*
Small Intestine	*1.50*	*0.07*
Colon	*0.31*	*0.04*
Total GI (incl. contents)	**2.08**	**0.12**
Skin/Fur	**3.50**	**nd**
Blood	*0.76*	*0.01*
Liver	*0.90*	*0.08*
Remaining carcass	*13.37*	*0.76*
Total 'intrinsic'	**15.03**	**0.85**
Urine 0-24h	*1.42*	*0.06*
Urine 24-48h	*1.40*	*0.05*
Total Urine	**2.81**	**0.11**
Recovery	88.35	
systemic radioactivity (absorbed / excreted)	**< 18.0%**	**< 1.0%**

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