Bioavailability of cadmium from linseed and cocoa

A LOUS follow-up project

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Contents

Foreword

“The List of Undesirable Substances (LOUS) was established by the Danish Environmental Protection Agency (EPA) as a guide for enterprises. It addresses chemical substances of concern, based on their hazardous properties and the volumes used in Denmark. The latest version of LOUS from 2009 includes 40 chemical substances and groups of substances [DEPA 2010].

During the period 2012-2015, all substances listed on LOUS will be surveyed and further need for risk management measures will be evaluated. In certain cases, implementation projects will be launched to achieve the goals laid down in the strategies for these substances.

The present project ”Bioavailability of cadmium from linseed and cocoa” was initiated as a LOUS follow-up project by the Danish EPA. The objective of this study was to investigate the bioavailability of cadmium in selected food items. The investigation was conducted as an oral feeding study in rats in combination with *in-vitro* studies simulating the conditions in the stomach of both rats and humans. The aim of the study was to provide data which can be used to further qualify the estimated exposure of the population to cadmium via food.

The project was carried out from June2013 till February 2014 by the National Food Institute at the Technical University of Denmark (DTU Food). Participants from DTU Food were senior advisor Max Hansen, senior scientist Jens Jørgen Sloth and scientist Rie Romme Rasmussen. The quality assurance was performed by Folmer Eriksen.”

Sammenfatning og konklusion

I dette projekt blev biotilgængeligheden af cadmium fra hele hørfrø, knust hørfrø, cacao og cadmiumklorid undersøgt i et fodringsforsøg med rotter og i et in vitro forsøg der skulle simulere forholdende i mavetarmkanalen hos henholdsvis rotter og mennesker.

I fodringsforsøget blev rotter inddelt i grupper, der blev doseret med cadmium fra forskellige kilder, henholdsvis hele hørfrø, knuste hørfrø, kakao og $CdCl_2$, samt en kontrolgruppe. Hørfrø eller kakao indgik med 10 % af foderets vægt og blev tilsat som erstatning for en kulhydratkilde. Dette blev gjort for at sikre koncentrationen af de øvrige nærringsstoffer i foderet forblev uændret. I cadmiumklorid gruppen blev cadmiumklorid dog iblandet foderet. Rotterne blev doseret i 3 uger og cadmium indholdet blev derefter målt i rotternes nyrer. Der blev set signifikante forskelle på indholdet af cadmium i nyrerne i de forskellige grupper. Forsøget viste at biotilgængeligheden af cadmium er nogenlunde lige stor fra de tre kilder. Der kan dog være en lidt større biotilgængelighed fra hele i forhold til knuste hørfrø og det ser ud til at biotilgængeligheden fra kakao er en smule lavere end fra hørfrø.

Der er forskel på mavetarmkanalen i rotter og mennesker. Det blev vurderet, at den væsentligste forskel i forbindelse med biotilgængeligheden af cadmium er, at pH er væsentligt lavere hos mennesker end hos rotter. Der blev derfor gennemført et in-vitro forsøg, hvor hørfrø og kakao blev ekstraheret med saltsyre i en koncentration der svarer til den der findes i maven hos rotter og mennesker. Forsøget viste, at den højere syrekoncentration i maven hos mennesker fører til en væsentlig større frigivelse af cadmium fra både hørfrø og kakao og dermed potentielt større biotilgængelighed.

Nærværende undersøgelse bekræfter den absorption af Cd fra fødevarer, der er fundet i andre studier (EFSA 2009a). Projektet kunne ikke bekræfte, at biotilgængeligheden af Cd hos rotter er lavere i hele hørfrø i forhold til knust hørfrø. En mulig forklaring kan være, at rotter, i modsætning til mennesker, tygger hørfrø og dermed øger muligheden for at Cd kan bringes i opløsning i mave-tarm kanalen. Den meget store forskel i frigivelse af Cd fra knust hørfrø i en simulation af menneskelig mavesaft sammenlignet med den rotte mavesaft indikerer, at Cd fra knust hørfrø er mere biotilgængeligt i mennesker end Cd fra hele hørfrø. Denne konklusion understøttes af resultatet i dyreforsøget, hvor det opløselige $CdCl_2$ synes at være den mest biotilgængelige. Disse resultater giver formentlig ikke Fødevarestyrelsen grund til at ændre rådgivningen af befolkningen omkring indtag af hele og knuste hørfrø. Et forsøg, hvor rotterne doseres med hele hørfrø via sonde kunne hjælpe med yderligere at afklare forskellen i biotilgængelighed mellem hele og knuste hørfrø. Biotilgængeligheden af Cd synes at være en smule lavere i kakao forhold til de andre matricer, men forskellene er ikke tilstrækkelige til, at det bør give anledning give særlige råd til befolkningen vedrørende indtag af kakao og chokolade.

Dette projekt har vist, hvordan en undersøgelse i forsøgsdyr kan bruges til at vurdere biotilgængeligheden af Cd hørfrø og kakao. Der er flere andre typer af fødevarer og fødevarekomponenter, som er væsentlige bidragydere Cd eksponering fra kosten. Det ville være yderst relevant, også at undersøge biotilgængeligheden af cadmium fra disse, på samme måde som biotilgængeligheden af cadmium i hørfrø og kakao blev undersøgt i denne undersøgelse.

Summary and conclusion

In this project, the bioavailability of cadmium from whole linseed, linseed, cocoa and cadmium chloride studied in a rat feeding study and in an in vitro assay simulating the conditions in the gastrointestinal tract in rats and humans, respectively.

In the feeding study rats were divided into groups, which were dosed with cadmium from various sources; whole linseed, milled linseed, cocoa and $CdCl_2$. Linseed or cocoa made up 10% of the feed in weight and was added as a replacement for carbohydrate source. This was done to ensure that the concentration of the other nutrients remained unchanged. In the $CdCl_2$ group cadmium chloride was mixed into the feed. The rats were dosed for 3 weeks and the cadmium content was measured in the rats' kidneys. Important differences in the level of cadmium in the kidneys were observed between the different groups. The experiment showed that the bioavailability of cadmium is much the same from the three sources. However, there may be a slightly greater bioavailability from whole linseed compared to crushed linseed and it seems that the bioavailability of cocoa is slightly lower than from linseed.

There are differences in the gastrointestinal tract in rats and humans. It was considered that the main difference in relation to the bioavailability of cadmium is that the pH is significantly lower in humans compared to rats. Therefore an in vitro study was carried out, where the linseed and cocoa was extracted with hydrochloric acid in concentrations equivalent to that found in the stomach of rats and humans. The experiment showed that the higher concentration of acid in the stomach of humans results in a substantially increased release of cadmium from both the linseed and the cocoa and thus a potentially increased bioavailability.

The present study confirms the absorption of Cd from food found in other studies reported by EFSA (EFSA 2009a). This project could not confirm that the bioavailability of Cd in rats is lower from whole linseed compared with crushed linseed. A possible explanation may be that the rats, unlike humans, are chewing linseed, thereby increasing the possibility of Cd can be brought into solution in the gastrointestinal tract. The very large difference in the release of Cd from crushed linseed in the simulation of human gastric juice compared to the rat gastric juice indicate that Cd from crushed linseed are more bioavailable in humans than Cd from whole linseed. This conclusion is supported by the results of the animal experiment, where the soluble $CdCl_2$ seems to be the most bioavailable. These results may not provide Danish Veterinary and Food Administration reason to change the advice to the public concerning the intake of whole or crushed linseed. A study in which rats are dosed with whole linseed by gavage probe could provide further information concerning the difference in bioavailability between whole or crushed linseed. The bioavailability of Cd appears to be slightly lower in cocoa compared to the other matrices, but the differences are not sufficient that it should give rise to specific advice to the public on the intake of cocoa and chocolate.

This project has shown how a study in experimental animals can be used to assess the bioavailability of Cd from linseed and cocoa. There are several other types of foods and food components that are major contributors to the Cd exposure from the diet. It would be highly relevant also to investigate the bioavailability of cadmium from these, in the same way as the bioavailability of cadmium in linseed and cocoa were investigated in this study.

1. Introduction

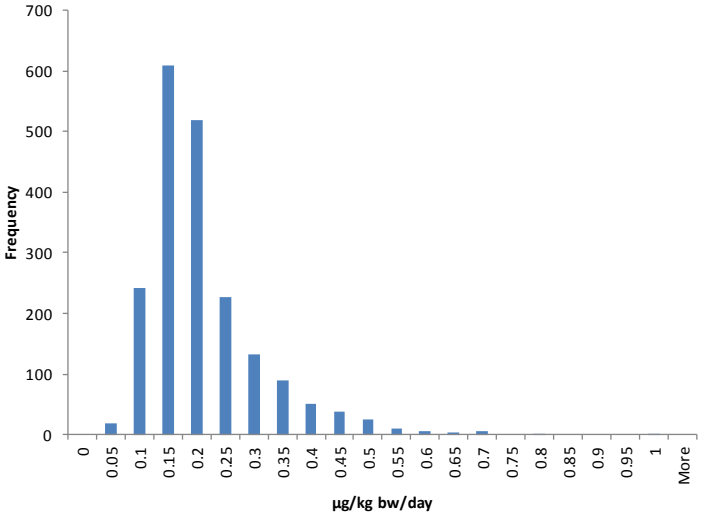
Cadmium (Cd) is a toxic element found as an environmental contaminant, both through natural occurrence and from industrial and agricultural sources. Food is the main source of cadmium exposure for the non-smoking general population. Besides foods, tobacco smoking and work place air have been identified as potential significant contributors to cadmium exposure.

Cadmium toxicity
Upon exposure cadmium is efficiently retained in the kidneys and liver in the human body, with a very long biological half-life ranging from 10 to 30 years. Cadmium is primarily toxic to the kidneys and may cause renal dysfunction. Cadmium can also cause bone demineralisation, either through direct bone damage or indirectly as a result of renal dysfunction. There is limited evidence for the carcinogenicity of cadmium following oral administration. In 2009 the CONTAM Panel of EFSA evaluated the dietary exposure to cadmium in the European population. Here a tolerable weekly exposure (TWI) value of 2.5 µg/kg bw/week was established, based on human studies on kidney effects (EFSA, 2009). This value was maintained in a statement from 2011 (EFSA, 2011) following a renewed evaluation due to a provisional tolerable monthly exposure (PTMI) of 25 µg/kg bw/month established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2010.

Absorption of cadmium
Absorption in rats and mice following oral administration of cadmium chloride varies from 0.2 to 3 % of the administered dose, depending on the dose and of the duration of the exposure. A refined diet high in fat and protein increases cadmium absorption, partially due to increased gastrointestinal passage time (EFSA 2009).

In humans, estimated daily intakes from the diet indicate that cadmium absorption from food is about 3-5 %. In 14 healthy adults, an average of 4.6 % of $CdCl_2$ administered in water taken with a meal was retained (McLellan et al., 1978). The influence of chemical complexation of cadmium on absorption was evaluated in seven volunteers who ingested brown crab meat (hepatopancreas) labelled with $^{109}CdCl_2$ by prior feeding of the crabs; whole-body counting ranged from 1.2 to 7.6 % with a mean of 2.7% (EFSA 2009).

Dietary exposure to cadmium
Recently, DTU Food issued a report on chemical contaminants in foodstuffs and results from the analysis of various types of foodstuffs during the period 2004-2011 were compiled and the dietary exposure to cadmium in the Danish population was estimated. The most important contributors to dietary exposure to cadmium in the Danish population are the food groups: cereals and cereal products (48.8%) and vegetables and vegetable products (34.3%) (DTU Food, 2013) but cocoa or chocolate was not included in this exposure assessment.
Figure X shows the dietary exposure to cadmium in the Danish population in µg/kg bw/day. The mean exposure at 0.18 µg/kg bw/day corresponds to 50% of the established TWI value at 0.36 µg/kg bw/day (=2.5 µg/kg bw/week) by EFSA (EFSA, 2009) and about 5% of the Danish population (primarily children) has an intake, which exceeds the TWI value.



		µg/kg bw/day
Mean		0.18
sd		0.10
N		1974
Percentiles	1	0.05
	5	0.08
	25	0.12
(median)	50	0.16
	75	0.22
	95	0.38
	99	0.52
Minimum		0.02
Maximum		0.97

FIGURE 1 DISTRIBUTION OF CADMIUM EXPOSURE IN µG/KG BW/DAY IN THE DANISH POPULATION (4-75 YEARS) (DTU FOOD, 2013).

These data indicate that the intake of cadmium from food in the Danish population is at a level where a health risk for high-consuming part of the Danish population cannot be excluded. However, considerations on the possible differences in bioavailability from the different types of food have not been taken into consideration.

Data from the Danish monitoring programme show that linseeds and cocoa are food commodities, which may contain elevated levels of cadmium. Linseed samples (N=13) had cadmium concentrations in the range 0.122-0.500 mg/kg (mean=0.391 mg/kg) and cocoa powder (N=18) contained in the range 0.026-1.91 mg/kg (mean=0.248 mg/kg). These commodities are usually only consumed in low to moderate amounts by the general population, but if consumed they can be significant contributors to the total dietary exposure to cadmium. Only little is known about the bioavailability of cadmium from specific food groups, including linseeds and cocoa.

The objective of this project is to investigate the bioavailability of cadmium from selected food sources to provide the Danish Veterinary and Food Administration with a tool to refine the consumer advises. The project was intended to contain the following parts:

1. In recent years several books and articles in journals and newspapers have suggested that it would be health beneficial to supplement the food with high quantities (up to 60 g/day) of crushed linseeds. It is assumed by the authors of these papers that the potential health beneficial unsaturated fatty acids are more bioavailable when the linseeds are crushed. The Danish Veterinary and Food Administration have advised the population not to eat high quantities of crushed linseed due to the potential increased bioavailability of cadmium. On the other hand the Danish Veterinary and Food Administration find it acceptable to eat whole linseed as it is assumed that these will pass the gastro intestinal tract almost unchanged although it has not been investigated whether the cadmium in whole linseed will be released during digesting. In the present project we have investigated the difference in bioavailability of cadmium from whole linseeds and crushed linseeds in rats and compared these values with the bioavailability of cadmium from the soluble cadmium salt $CdCl_2$. We have furthermore investigated whether the differences in the pH value in the stomach in rats and humans influence the bioavailability of cadmium differently in humans and rats.

2. In grain and grain products cadmium is bound mainly to the bran. The concentrations of cadmium in full grain products are therefore usually higher than the concentration in refined grain products. As much of the bran will pass the gastrointestinal tract without absorption, it is likely that cadmium bound to the bran will be less available than cadmium bound to the more digestible part of the food. It was initially a purpose of the project to investigate this in the rat experiment, but it was not considered possible due to relative low concentration of cadmium found in wheat flour (both full grain and refined grain) compared with the content in the cadmium in other part of the feed used in the experiment.

3. There is little knowledge on the bioavailability of cadmium from cocoa, which may contain relatively high concentrations of cadmium. The bioavailability of cadmium from cocoa is compared to the bioavailability of cadmium from linseed and the soluble cadmium salt $CdCl_2$. The potential differences in absorption between rats and humans due to differences in pH are investigated by *in vitro* experiments.

2. Study design and methods

Introduction
In this chapter the methods used in the project will be presented, as well as the study design and changes including the scientific arguments for the modifications in the project compared to the initial plan. All results are presented in the following chapter, but references to these are given in this chapter when necessary.

Animal experiments ethics and authorization
Animal experiments were carried out at the DTU Food (Mørkhøj, Denmark) facilities. Ethical approval was given by the Danish Animal Experiments Inspectorate. The authorization number given: 2012-15-2934-00089. The experiments were overseen by the National Food Institutes in-house Animal Welfare Committee for animal care and use.

Determination of cadmium concentration
Feed and kidneys: Subsamples (homogenized feed 0.2-0.4 g and whole single kidneys 0.6-1.5 g) were digested in high-pressure quartz vessels using microwaves (Multiwave 3000, Anton Paar, Austria) with 2 ml ultrapure water and 4 ml concentrated nitric acid (PlasmaPure, SCP Science, France). Prior to analysis the digests were further diluted to a volume of 40 ml with ultrapure water from a Millipore Element apparatus (Millipore, Milford, MA, USA).
Simulated gastric juices: The simulated gastric juice suspensions were first centrifuged (4700 rpm, 10°C, 15 min) followed by filtration with single use hydrophilic syringe filters (0.45 µm, Minisart, Sartorius, Göttingen, Germany) and prior to analysis aliquots (0.4 ml) were further diluted to a volume of 5 ml with ultrapure water.

Blood samples: Whole blood rat samples (200 µL) and reference material (Seronom L-2, Trace Elements Whole Blood (SEROAS, Billingstad, Norway) were diluted 25 times with an aqueous extract containing 1.2% 2-propanol (1.2%), 0.4 g/l (NH4)2EDTA, 0.4 g/l Triton X-100, 1% TMAH (Tetramethylammonium hydroxid) and 2 µg/l internal standard (rhodium). Cadmium and rhodium (Cd111 and Rh103) were measured by ICP-MS (Thermo Scientific iCAP Q, Thermo Fisher Scientific GmbH, Bremen, Germany) in standard mode with no further sample pre-treatment. External calibration standards (0; 0.05; 0.10; 0.25; 0.60 µg/l) matching the solvent (2-propanol, (NH4)2EDTA, Triton X-100) were applied for quantification. Empty sample tubes filled with the extraction mixture were subjected to the sample preparation procedure to correct for any possible contamination of Cd (reagents blanks).

The cadmium content was subsequently determined at m/z 111 by ICP-MS using an Agilent 7500ce instrument (Agilent Technologies, Waldbronn, Germany) equipped with a glass concentric nebuliser (Agilent) and a Scott-type double-pass water-cooled spray chamber (Agilent). Typical plasma conditions were 1,500 W RF power, 15 $lmin^{-1}$ plasma gas, 0.88 $lmin^{-1}$ carrier gas and 0.32 $lmin^{-1}$ makeup gas. Quantification was done by addition calibration with internal standardisation using ^{103}Rh as internal standard at 1 μgl^{-1} in all blanks, standards and samples. Standard stock solutions of cadmium and rhodium were obtained from SCP Science (Courtaboeuf, France).
The method is accredited according to ISO17025 by the Danish accreditation body DANAK.

Quality assurance parameters
The limit of detection, LOD, was calculated according to the three-sigma criterion at 0.6 µg/kg (kidneys) and 3 µg/kg (feed materials) from the standard deviation of the analytical blank values (N=17). The LODs were sufficiently low to detect the cadmium content in the present study with satisfactory precision. The trueness was verified from the analysis of the certified reference material BCR186 Pig Kidney (Institute of Reference Materials and Measurements (IRMM), Geel, Belgium) 2.83±0.17 mg/kg (N=6, mean ± 2sd), which results agreed well with the certified value (2.71±0.15 mg/kg).

Pilot study with Fischer 344 SPF rats
Initial calculation based on the concentration of cadmium in the wheat flour, whole linseed, crushed linseed and cocoa and on the expected absorption of cadmium in rats (0,2 - 3 %, EFSA, 2009) indicated that it would be possible to measure the blood concentration of cadmium with sufficient precision. The hypothesis was that the blood concentration in the rats would reach a steady state after a few weeks. The purpose of the pilot study was to investigate this further and if possible confirm the hypothesis. For the pilot study 6 male Fisher 344 SPF rats at an age of 6 weeks were acclimatised for one week. After acclimatisation they were dosed with feed containing cadmium chloride at concentration similar to the concentrations expected in cocoa (2,06 µg/kg) for 2 weeks. Blood samples were taken from the tissue under the tongue after 7 days of dosage, 11 days of dosage and at sacrifice at 14 days.

As basic feed for this study a semi synthetic feed from another study at the animal facilities at DTU was used (see table 1).

TABLE 1 FEED INGREDIENTS IN 1 KG OF FEED USED IN THE PILOT RAT STUDY

Potato protein (g)	100
Corn starch (g)	700
Fish meal (g)	80
ADEK-vit/oil (g)	50
Mineral mixture(g)	28
B-vitamin (g)	12
Cellulose (g)	30

The blood concentration of cadmium after 2 weeks was 0.05 ± 0.04 µg/l (mean ± sd) . This is an extremely low value considering the relative high concentration of cadmium in the feed. As this concentration was near the detection limit of the method it was considered unlikely that the analytical method had sufficient sensitivity to determine differences in the blood concentration of cadmium due to minor changes in the cadmium concentrations in the feed. Therefore it was decided not to use blood concentration as a marker for cadmium absorption in the main study.

All parts of the feed was analysed and a high cadmium concentrations in the potato protein of 132 µg/kg and fish meal 234 µg/kg were found (see table 3 in results section).

Main study
Due to the low concentration of cadmium in wheat flour (11 mg/kg) and whole grain wheat flour (27 mg/kg) it was decided not to include this in the main study. The study therefore included animals fed with either:

- control feed
- feed containing whole linseed
- feed containing crushed linseed
- Feed containing cocoa
- feed containing the water soluble $CdCl_2$.

Due to the smaller number of dosage groups it was decided to increase the number of animals in each dosage group from 6 to 8 and to increase the timespan for dosage from 2 to 3 weeks.

Based on the results from the pilot study it was decided to change the feed composition. In order to reduce the cadmium exposure from the feed ingredients, the protein sources (fish meal and potato protein) were replaced by caseinate with a low cadmium concentration (see table 1 in results for exact values). The dosed animals were given 10 % of the feed (determined by weight) as whole linseed, crushed linseed or cocoa. To achieve this and to make as few other changes in the feed composition as possible the substances replaced a similar amount of corn starch. Thereby the concentrations of all nutritional elements were the same in the different groups although the different feeds were not isocaloric.

Since it was not possible to measure the cadmium concentration in blood with sufficient precision in the pilot study, it was decided to use the kidney concentration of cadmium. As cadmium accumulates in the kidney it was expected that the concentration in this organ was higher compared with the blood concentration. The suitability of the analytical method to analyse cadmium in rat kidney was tested on two kidneys from rats at a similar age as the animals would be at sacrifice in the feeding study. There were no methodological problems and the cadmium concentration in these kidneys was much higher than the detection limit of the method. As the kidney concentration of cadmium reflect the lifetime exposure to cadmium it was necessary to be sure that the animals did not receive significant amounts of cadmium in feed before they were delivered to DTU. Therefore the standard altromin feed used at the facilities where the rats were breed was analysed. The cadmium content of this feed was high (60 – 70 µg/kg). It was therefore decided to buy 4 weaning mother animals with 10 male pups each and let these rats go directly from breast feed to the control feed. It was only possible to buy Wistar rats on these conditions. The consequences of changing from Fisher 344 to Wistar rats was expected to be a little higher variability of the results because the Wistar strain is more genetic heterogeneous compared to the Fisher 344 rats.

TABLE 2 FEED COMPOSITION FOR 1 KG OF FEED IN EACH OF THE GROUPS IN THE MAIN STUDY:

Feed	Control	Crushed linseed	Whole Linseed	Cocoa	Cd
Caseinate (g)	180	180	180	180	180
Potato starch (g)	220	220	220	220	220
Corn starch (g)	460	360	360	360	460
Crushed linseed	0	100	0	0	0
Whole Linseed	0	0	100	0	0
Cocoa	0	0	0	100	0
ADEK-vit/oil (g)	50	50	50	50	50
Mineral mixture(g)	28	28	28	28	0
Mineral mixture(g) added Cd	0	0	0	0	28
B-vitamin (g)	12	12	12	12	12
Cellulose (g)	50	50	50	50	50

Simulation of gastric juice in humans and rats

As cadmium in food is bound as inorganic cadmium the most important differences in the conditions in the human stomach and the rat stomach was considered to be the pH value of the gastric juice, which is pH 1- -2 in humans and about pH 4 in rats. To test whether this influenced the different food matrices differently an *in-vitro* experiment was set up, which simulated the human and rat stomach, respectively. Subsamples (approx. 10 gram) of the different food items stirred for 30, 60 or 120 minutes, respectively, in 100 ml hydrochloric acid 0.1 M, with initial pH of 1.5 and addition of 0.1 M NaCl and KCl. The samples were centrifuged at 800 g for 10 minutes and the supernatants were collected. The conditions in the rat stomach were simulated by adjusting the pH value to 4 using disodiumcarbonate and the samples were treated as described in the previous part.

3. Results and discussion

Cadmium in feed and feed ingredients

Table 3 shows the results from the analysis of cadmium in feed ingredients and the complete feeds. For corn and potato starch, vitamin mixtures, cellulose powder and caseinate the cadmium concentration was in all cases very low (up to 3 µg/kg). The mineral mixture used had a concentration of Cd at 197 µg/kg and the Cd probably originated from cadmium-containing impurities in some of the minerals used in the mixture. The cocoa powder (fairtrade organic raw cacao powder from The raw chocolate company) contained a high level of Cd at almost 1400 µg/kg. The whole linseeds (organic linseed from Biogan) contained a slightly higher Cd concentration (151 µg/kg) than the crushed linseeds (crushed linseed from naturdrogeriet) (123 µg/kg).

Four different subsamples of each of the complete feed samples were analysed in order to test the homogeneity of the feed. For the control, linseed feeds and cocoa feed satisfactory homogeneity was demonstrated with RSD values ≤12%. However, for the complete feed, which was added CdCl2, a high variability in the results for the four subsamples was observed (RSD=76%), indicating that the feed material was not homogeneous.

TABLE 3 CADMIUM CONCENTRATION IN FEED INGREDIENTS AND COMPLETE FEED

Feed/ingredient type	N	mean (µg/kg)	sd (µg/kg)	rsd (%)	range (µg/kg)		
Corn starch	1	< 3	-	-	-	-	-
ADEK vitamin mixture	1	< 3	-	-	-	-	-
BC Vitamin mixture	1	< 3	-	-	-	-	-
Cellulose powder	1	< 3	-	-	-	-	-
Potato protein	1	132					
Potato starch	1	< 3	-	-	-	-	-
Fish meal	1	234					
Caseinate	1	3	-	-	-	-	-
Mineral mixture	1	197	-	-	-	-	-
Cocoa powder	1	1379	-	-	-	-	-
Wheat flour	2	11	2	0.9	11	-	11
Wholemeal wheat flour	2	28	3	13	25	-	30
Linseeds, whole	2	123	2	1	122	-	124
Linseeds, crushed	2	151	1	1	150	-	152
Feed, pilot study	1	206					
Altromin whole feed	1	60-70					
Feed, control	4	6	1	12	6	-	7
Feed, crushed linseeds	4	22	1	6	21	-	24
Feed, whole linseeds	4	19	2	10	17	-	22
Feed, cocoa	4	164	3	2	161	-	169
Feed, $CdCl_2$	4	950	724	76	217	-	1795

Animals and feed intake in the main study

The feed intake was similar in all groups (see table 4). However, the weight gain was significantly higher in the groups given crushed and whole linseeds. That is probably caused by the high content of fat from the unsaturated oil in the linseed grains and The high weight gains in rats given whole linseeds indicate that the oil is bioavailable for the rats. The reason for that could be that rats, in contrast to humans, crush the whole linseeds by chewing when eating.

The high standard deviations in the feed intake are probably caused by uncertainties. The feed is a powder and some of it will be lost due to the behaviour of rat in the cage.

TABLE 4 FEED INTAKE, WEIGHT GAIN AND INTAKE OF CADMIUM IN THE MAIN STUDY.

	Control	Crushed linseed	Whole linseed	Cocoa	$CdCl_2$
Feed intake (g)	229 ± 87	195 ± 89	216 ± 83	204 ± 54	215 ± 55
Weight gain (g)	99 ± 17	136 ± 8	135 ± 11	114 ± 9	112 ± 17

Cadmium in rat kidney samples

Table 5 shows the concentration results of the analysis of cadmium in the rat kidneys. For all four groups receiving feed added linseeds, cocoa or $CdCl_2$ the concentration in the kidneys is significantly different from the control group (t-tests, 5 % level). The individual results for each rat and the mass of the kidney can be found in Annex X.

TABLE 5 CADMIUM CONCENTRATION IN RAT KIDNEYS OF EXPOSED RATS

Group	N	mean (µg/kg)	sd (µg/kg)	rsd (%)	range (µg/kg)		
Control	6	13	9	68	7	-	29
Crushed linseed	8	22	4	21	17	-	31
Whole linseed	8	28	5	19	20	-	37
Cocoa	8	156	55	35	81	-	257
$CdCl_2$	8	1379	254	18	958	-	1707

One of the results for the control group (animal no 2 at 29 µg/kg) could be identified as an outlying result by the Q-test (90% confidence level). When excluding this result the mean cadmium concentration in the control group is even lower, mean=9 µg/kg (rsd 35%).

Table 6 shows the cadmium content in the kidneys of the rats in each of the groups. These results are obtained by multiplication of the concentration with kidney weight and furthermore multiplication by 2 to get the cadmium content in both kidneys (assuming identical weight of the two kidneys).

TABLE 6 ABSOLUTE CADMIUM CONTENT IN RAT KIDNEYS OF EXPOSED RATS

	N	mean (µg)	sd (µg)	rsd (%)	range (µg)	
Control	6	24	12	49	13 -	46
Crushed linseed	8	36	7	21	25 -	49
Whole linseed	8	47	12	24	27 -	64
Cocoa	8	230	71	31	129 -	328
$CdCl_2$	8	2245	459	20	1622 -	2932

Again animal no 2 is an outlier (Q-test, 90% confidence level) and when excluded the mean content is 20 µg.

When taking the concentration of Cd in the feed into account and assuming that the amount of feed consumed by all groups is equal, a relative absorption can be estimated by division of amount of Cd in kidneys with the amount of Cd in the feed supplied throughout the feeding trial period (Table 7). The relative absorption was in the range of 0.9% to 2.0%. However, if discarding the outlying result for the control group (see text above) and using the corrected kidney mean at 9 µg/kg the factor for this group is 1.5 and similar to the factor for the other groups. The results indicate that differences in cadmium bioavailability exist from the different food items investigated in the present study. It is somewhat surprising that the bioavailability from crushed linseeds in the rat study seems to be lower than the bioavailability from the whole linseeds. This part of the study can probable not be extrapolated to humans and may be due to the crushing of the whole linseeds by chewing of the rats. In table 8 the release of Cd from the whole and crushed linseed indicate that the condition in the human stomach favour the release of cadmium from crushed linseed compared to the condition in the rat stomach and thereby increasing the bioavailability.

TABLE 7 PERCENT OF CD IN FEED DEPOSITED IN THE KIDNEY

Group	N	Percent of ingested Cd in kidneys (%)
Control	7	2,0
Crushed linseed	8	0.9
Whole linseed	8	1.5
Cocoa	8	0.7
CdCl2	8	4.6

Cadmium in simulated gastric juices

Table 8 shows the results from the analysis of cadmium in simulated gastric juices of humans and rats, respectively. The experiments were done on three different time lengths of 0.5, 1 and 2 hours, respectively. The length of the experiments did not influence the result, indicating that the bioaccessible fraction of the cadmium in the foodstuffs has been released to the liquid phase in less than half an hour and prolonging the time did not release more cadmium.

TABLE 8 CADMIUM CONCENTRATION IN SIMULATED HUMAN AND RAT GASTRIC JUICES.

		Simulated gastric juice	
Food type	Time (hours)	Human (µg/l)	Rat
Whole linseed	0.5	2,6	1,5
	1	2,5	1,7
	2	2,7	2,0
Crushed linseed	0.5	12	4,1
	1	11	4,4
	2	11	4,3
Cocoa	0.5	68	44
	1	68	45
	2	67	46

The results indicate that human gastric conditions (at pH 1.5) are more efficient to release cadmium from foodstuff compared to rat gastric conditions (at pH 4). The mean ratio between human/rat is 1.5 for both whole linseeds and cocoa, but even higher (2.6) for crushed linseeds. This factor needs to be taken into account when evaluating the data from the rat feeding trial and transferring these data to humans.
Furthermore, the data indicate that cadmium is more accessible from crushed linseeds compared to whole linseeds. When taking the concentration of cadmium in whole and crushed linseeds into account a factor describing the increased bioaccessibility of cadmium from crushed compared to whole linseeds can be calculated at 3.6 for humans and 2.0 for rats by the following approach:

$$Factor\left(\frac{crushed}{whole}\right) = \frac{C_{GJ.crushed}}{C_{GJ,whole}} \times \frac{C_{whole}}{C_{crushed}}$$, where

C_{GJ} = concentration in gastric juice
C = concentration in foodstuffs

The increased bioaccessibility in human gastric environment is most probably due to the lower pH (pH = 1.5) compared to the rat gastric environment with a higher pH value (pH = 4).
In the rat study the bioavailability was not significantly different between the rats exposed to whole linseeds and the rats exposed to crushed linseeds. This is probably because the rats crush the whole linseeds when chewing the feed and hence no difference was observed in the present study. In order to evaluate this, the linseeds should be force-fed to the rats (e.g. by gavage) in order not to let the animals chew on the linseeds prior to ingestion.

Perspective for risk managers

The present study confirms in general the expected level of absorption of cadmium from food in rats as well as humans from other studies (EFSA, 2009). The project could not confirm that the bioavailability of Cd is lower in whole linseed compared to crushed linseed in rats. The reason could be that the rats chew the linseed in contrast to humans. The very large difference in release of cadmium from crushed linseed in a simulation of human gastric juice compared with the rat gastric juice indicate that Cd from crushed linseed is more bioavailable than Cd from whole linseed. This conclusion is supported by the result in the animal experiment where the soluble $CdCl_2$ seems to be the most bioavailable. These results give no reason for the Danish Food and Veterinary Administration to change advises concerning whole and crushed linseed. A further experiment in rats where the rats are given the linseed by gavage could help clarified this issue further.

The present project has demonstrated how an animal study can be used to assess the bioavailability of Cd from two different Cd containing foodstuffs, linseeds and cocoa. There are several other types of foodstuffs that have elevated levels of Cd and also foodstuffs with lower level of Cd, but with higher consumption, which are significant contributors to dietary Cd exposure and hence it would be highly relevant to evaluate these food item similarly to the present study.
The bioavailability seems to be a little lower in cocoa compared to the other matrices but the differences are not sufficient to give special advices concerning cocoa and chocolate.

It would furthermore be very relevant to evaluate the bioavailability of other toxic elements, e.g. lead, mercury and inorganic arsenic, where there also is a lack of knowledge. Such studies would be helpful in the refinement of the risk assessment of dietary exposure to these compounds.

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Appendix 1: Results from the analysis of Cd in kidney and food

Animal no	Group	Analytical series	Kidney mass (g)	Cd result (µg/kg)	Cd in kidney (ng)
1	Control	2	1,472	7,7	22,7
2	Control	2	0,786	29,4	46,2
3	Control	3	1,130	8,4	19,0
4	Control	2	0,926	15,1	28,0
5	Control	Animal lost	-	-	-
6	Control	1	0,944	7,0	13,1
7	Control	Sample lost	-	-	-
8	Control	3	0,948	9,0	17,0
9	Crushed linseed	1	0,790	19,6	30,9
10	Crushed linseed	2	0,925	17,1	31,6
11	Crushed linseed	1	0,842	21,3	35,8
12	Crushed linseed	2	0,934	23,0	43,1
13	Crushed linseed	3	0,886	20,9	37,0
14	Crushed linseed	3	0,708	17,7	25,0
15	Crushed linseed	2	0,762	23,3	35,6
16	Crushed linseed	3	0,780	31,4	49,0
17	Whole linseed	3	0,857	27,0	46,3
18	Whole linseed	1	1,044	29,0	60,5
19	Whole linseed	1	0,768	33,2	51,0
20	Whole linseed	1	0,958	24,8	47,5
21	Whole linseed	1	0,865	36,9	63,8
22	Whole linseed	3	0,857	25,5	43,8
23	Whole linseed	1	0,792	25,2	40,0
24	Whole linseed	2	0,660	20,4	26,9
25	Cocoa	2	0,786	169	266
26	Cocoa	3	0,808	136	219
27	Cocoa	1	0,734	154	225
28*	Cocoa	1	0,399	81	129
29	Cocoa	2	0,813	202	328
30	Cocoa	2	0,769	113	174
31	Cocoa	3	0,664	135	179
32	Cocoa	3	0,626	257	322
33	$CdCl_2$	3	0,837	1436	2403
34	$CdCl_2$	1	0,731	1707	2495
35	$CdCl_2$	3	1,008	1454	2932
36	$CdCl_2$	3	0,803	1307	2099
37	$CdCl_2$	2	0,822	1605	2638
38	$CdCl_2$	2	0,707	1484	2099
39	$CdCl_2$	1	0,751	1080	1622
40	$CdCl_2$	2	0,872	958	1671

* only half of the kidney was available for analysis

Bioavailability of cadmium from linseed and cocoa

In Denmark and EU the exposure of cadmium from food is at a level that is relatively close to the Tolerable Daily Intake (TDI). This report describes an investigation of the bioavailability of cadmium in selected food items known to contain high levels of cadmium. The purpose was to provide data which can be used to further qualify the estimated exposure of the population to cadmium via food. The background for carrying out this investigation was the results from a survey of cadmium and cadmium compounds (Environmental Project no. 1471) conducted by the Danish EPA under the LOUS-review.

The investigation was conducted as a feeding study in rats in combination with in-vitro studies simulating the conditions in the stomach of both rats and humans. The results of the investigation do, however, not provide a basis for changing the current advice to the public neither regarding the intake of whole or crushed linseed nor the intake of cocoa and chocolate.

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