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In vitro digestion of selenium from selenium-enriched chicken*

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Abstract: Selenium (Se) is an essential trace element important for several biological functions, such as thyroid hormone metabolism, anti-aging, and antioxidant protection. Even though reports on Se species in different food products are increasing, the scientific knowledge on the bioaccessible amount of Se species from these products is not as well known. In this work, Se-enriched chicken meat was digested by an in vitro model using human gastrointestinal (GI) juices and different commercial enzymes. The aim of the study was to gain information on the bioaccessible amount of Se from meat after digestion by different relevant enzymes with the approach of replacing human liquids with commercial enzymes that could mimic the digestion in the human GI tract. The digestion with commercial enzymes such as pepsin, protease, and lipase resulted in highly variable extraction yield of Se in isotonic salt water (50–90 %). Se proteins were degraded into SeMet, while Se species with higher molecular mass were present in the extracts from human juices. The extraction yield using human juices was only about 70 % of Se from the meat, but Se in the extracts was normally distributed with a low standard deviation (4 %), indicating stability of the results from digested extract.

Keywords: bioaccessibility; enzymes; human juices; selenium.

INTRODUCTION

Scandinavia is a region in which levels of selenium (Se) in food are generally low, and since Se is essential for human health, strategies are currently being developed to increase the Se content of agricultural products [1,2]. Se is important for the activity of enzymes involved in antioxidant protection and thyroid hormone metabolism. Potent anti-tumor effects have been attributed to species-specific Se compounds; for example, low-molecular-mass (LMM) Se species, *Se*-methyl-selenocysteine, and its γ-glutamyl derivative, which have been identified in a number of edible plants of the Allium and Brassica genera [3]. Information on different Se species is important inasmuch as total food Se concentrations is an inadequate measure for evaluating the effect of increased Se levels in food on human health.

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In 2010, Fairweather-Tait et al. [4] stated that the forms in which Se is present in the foods should be focused on, but cautioned that methods devised to maximize extraction of Se may change the physico-chemical form of Se in the food matrix. Most studies on the identification, characterization, structural changes, and estimation of the bioaccessibility of Se from foodstuffs are based on in vitro digestion with commercially available enzymes of nonhuman origin, such as pepsin, different proteolytic enzymes, and bile salts [5–8]. Basically, proteolytic enzymes are used to digest proteins to amino acids and peptides prior to the identification and quantification of the Se species present [9–11]. Products analyzed include inorganic Se, organic molecules of selenomethionine (SeMet), and selenocysteine (SeCys) [9]. Optimally, foods ought to process using human gastrointestinal (GI) digestion to explore the forms of Se that occur naturally in foods. However, in vitro simulation models are easier to perform and more ethical. Without adequate knowledge of the Se speciation and possible changes during extractions, false conclusions may be drawn when assessing Se requirements for optimal health [3]. Thus, speciation studies to identify the chemical forms in which Se is present in food and changes that occur during digestion and uptake to tissues are needed [12]. Numerous reports have been published on the presence of organic Se compounds from Se-enriched products, but information on the distribution of Se species in products before and after human ingestion is scarce.

A promising approach to obtain information on Se accessibility to humans is to use human GI juices in controlled experiments [13,14]. As a naturally occurring liquid, human GI juices are easy to use, and should give precise and realistic information on degradation of foodstuffs. According to Hur et al. [15], in vitro methods that use complex enzymes (e.g., human juices) have the advantage of being more reproducible and realistic than those using single enzymes. Using a single purified enzyme rather than a complex biological mixture has, however, the advantage of facilitating standardization of the in vitro digestion models [16]. Several factors may influence the results of in vitro digestion models, such as sample characteristics, enzyme activity, ionic composition, applied mechanical stresses, and digestion transit time [15]. In addition, the results of in vitro digestion models often differ from those reported using in vivo models because of the difficulties in accurately simulating the complex physiochemical conditions and the physiological events occurring in the GI tract [15]. It is important, for example, that in vitro models measuring digestibility use physiologically relevant levels of enzyme concentrations.

In the present study, the accessibility of Se from chicken meat was estimated using physiologically relevant enzyme activities of human GI juices and different activities of commercial enzymes. The aim of the study was

- to gain information on the extraction efficiency of Se from chicken meat;
- to compare the type and amount of Se molecules (speciation, chromatographic profiles) after digestion using human GI juices and commercial enzymes, respectively; and
- to measure the influence of transit time and enzyme activity on structural changes of Se species present in the extracts after digestion.

MATERIALS AND METHODS

Reagents and solutions

Analytical reagent grade chemicals and commercial enzymes such as pepsin, lipase, and protease were purchased from Sigma-Aldrich unless stated otherwise. Sodium chloride was purchased from Heigar (Oslo, Norway), hydrochloric acid, sodium hydroxide, and ammonium acetate were purchased from Merck (Darmstadt, Germany), and MQ (Millipore) water (18 MΩcm) was used.

Se biofortification of chicken breast muscle

The animal feeding procedure to reach a total Se concentration of 1.9 ± 0.17 mg Se kg^{-1} in the chicken breast muscle is presented elsewhere [7]. In brief, five male chickens (*Ross 308,* Samvirkekylling, Norway) were fed Se-enriched wheat (1.2 mg Se Kg^{-1}) for 33 days at the Animal Production Experimental Centre (SHF) at the Norwegian University of Life Sciences (UMB, Ås, Norway). Chickens were randomly placed in separate pens, with free access to food and water. The breast muscles were vacuum-packed and stored frozen (–20 °C) prior to analysis.

The experimental research on animals followed internationally recognized guidelines. All animals were cared for according to laws and regulations controlling experiments with live animals in Norway (The Animal Protection Act of 20 December 1974, and the Animal Protection Ordinance Concerning Experiments with Animals of 15 January 1996); according to the rules given by Norwegian Animal Research Authority.

HUMAN JUICES AND COMMERCIAL ENZYMES

Human enzymes were obtained by collecting human gastric juice (HGJ) and human duodenal juice (HDJ) according to Holm et al. [17]. Aspirates were collected on ice and frozen at –80 °C. The HGJ and HDJ used were obtained from a pooled batch of 18 healthy individuals. Written consent forms were obtained from all donors, and the study was conducted by Østfold Trust Hospital, Norway (ØTH) and approved by the Ethical Committee of Norway.

Commercial enzymes (pepsin, lipase, and protease) were used to mimic the digestion in the GI tract. In the model mimicking the stomach, 4220 units mg^{-1} pepsin (from porcine gastric mucosa, PP6887) was used. In the model mimicking the duodenal, 263 units g^{-1} lipase (from *Aspergillus niger*, 62301) and 3500 units g^{-1} protease XIV (from *Streptomyces griseus*, P5147) were used.

Extraction procedure

Chicken breast muscles from five chickens fed Se-enriched wheat were freeze-dried, pulverized, and homogenized using a ball mill. To obtain information on the degradation and bioaccessibility of Se to humans, two GI digestion procedures were used, one with model enzymes consisting of commercially available enzymes and one with real human juices. At the time of the experiment, the pepsin activity in the pooled HGJ (pH 2.0) was 12.2 U ml^{-1} min^{-1} assayed with hemoglobin as substrate, according to Sanchez-Chaing et al. [18]. Total proteolytic activity in the pooled HDJ (pH 7.0) was 12.9 U ml^{-1} min^{-1} assayed with casein as substrate, according to Krogdahl and Holm [19]. The α-amylase activity in HDJ was 36 500 unit l^{-1} and the bile salt was 4.5 mMl^{-1} as analyzed at ØTH. All values and contents of the HGJ and HDJ juices are in detailed described by Eriksen et al. [20]. Physiologically relevant activity levels of HGJ and HDJ from the pooled batches were calculated to give the enzyme activities per gram of substrate (sample) listed in Table 1. Protein concentrations in the chicken breast muscles were calculated by multiplying the nitrogen concentration (Kjeldahl) with a factor of 6.25, giving a protein content of 14.6 %.The enzyme activity mimicking the digestion in the stomach was set to an average activity of 16 units g^{-1} chicken sample. Low protease activity in the duodenal juice was set to 16 units g^{-1} chicken sample and high to 55 units g^{-1} chicken sample.

The commercial enzyme pepsin was made up to a solution of 420 units ml^{-1} with isotonic salt solution (0.9 %) and an enzyme activity of 16 units g^{-1} chicken muscle was used. Lipase and protease XIV were made up with isotonic salt solution to a solution with lipase activity 0.5 units ml^{-1} and protease activity of 10.5 units ml^{-1}. Protease XIV activity was added equal to the lowest and highest activity of HDJ, respectively, and mg of protease XIV used for identification purposes [7,9,10] (Table 1).

One ml of 0.9 % NaCl was added to 0.1 g (DM) of chicken breast muscle and mixed for 30 s using a ball mill. After 10 min, the pH was adjusted to 2.5 with 1 M HCl, HGJ or commercial pepsin were added, and the samples were digested for 2 h at 37 °C according to J. L. Kaye [21]. The pH was then adjusted to 7.5 with 1 M NaOH, and HDJ or commercial protease XIV plus lipase were added, giving the activity levels presented in Table 1. The samples were digested at 37 ± 0.5 °C for 6, 17, or 24 h in the dark using an incubator cupboard, on a Roto-Shake (Genie) at 37 °C, which should simulate the contractions in the GI tract. All the digested samples were centrifuged at 10 000 × g for 10 min at 4 °C and the supernatants were then freeze-dried. The freeze-dried material was dissolved in 2 ml MQ water; i.e., 1 ml for Se chromatographic analysis and 1 ml for total Se analysis.

Table 1 Activity of human juices (HGJ and HDJ) and commercial enzymes (pepsin, protease XIV, and lipase) added per grams of chicken muscle.

Sample	HGJ units g^{-1}	HDJ units g^{-1}	Pepsin units g^{-1}	Protease XIV units g^{-1}	Lipase units g^{-1}
1–5	15	16	16	16	3
6–10	15	31	16	56	5
11–15	15	55	16	161	56

Analysis

Total Se concentrations in muscle and extracts were based on measurement of ^{82}Se by inductively couple plasma-mass spectrometer (ICP-MS) (Perkin Elmer Elan 6000) with Cinnabar spray chamber (20 ml Cyclonic), after microwave (UltraCLAVE, Milestone) decomposition of 0.1 g (DM) breast muscle or 1 ml extract in 1.25 ml HNO_3 (ultrapure) at 250 °C for 120 min. All samples were diluted to 25 ml with 2 % ethanol. Standard reference material (SRM) National Institute of Standard and technology (NIST) wheat flour (1567A 1.1 ± 0.4 µg Se g^{-1}) and Bovine liver (1577B 0.73 ± 0.06 µg Se g^{-1}) were used for quality control. Tellurium and indium were used as internal standards to control loss of analyte during the decomposition process. Isobaric interference of krypton on Se-82 was adjusted for. The use of ethanol increases the ionization of the sample, increasing the count statistics of Se-82 (27).

Chromatographic separations were performed using a Model 1100 HPLC pump (Agilent, Wilmington, DE, USA) as the delivery system. Injections were done by using a Rheodyne valve with a 100-µl loop. The exit of the column was directly coupled to a Meinhard nebulizer (Glass Expansion, Romainmotier, Switzerland) of an ICP-MS equipped with a collision cell (Agilent 7500c, Yokogawa Analytical Systems, Tokyo, Japan) by means of polyetheretherketone (PEEK) tubing. Chromatographic columns and conditions used in the experiment are listed in Table 2.

Table 2 Conditions for size exclusion and reversed-phase chromatography.

Size exclusion column	Superdex 75
Buffer solution	100 mM ammonium acetate, pH 7.5
Elution program	Isocratic
Flow	0.7 ml min–1
Sample volume	100 ml
Aqua column	Atlantis T3
Eluent	A: 0.1 % HFBA in water B: 0.1 % HFBA in methanol
Flow	1 ml min–1
Eluation program	0–5 min: 5 % B, 5–35 min: 5–40 % B; 35–40 min: 40 % B, 40–42 min: 40–5 % B, 42–52 min: 5 % B
Sample volume	100 ml

Statistical analysis

Statistical analysis was conducted using Minitab 15 (Minitab Inc.). Normality was tested using the Anderson-Darling test ($p < 0.01$). The significance of the differences between the two methods was analyzed by the Mann–Whitney procedure, since the results from commercial enzymes were not normally distributed.

RESULTS AND DISCUSSION

The results showed that bioaccessible Se accounted for 65 ± 10 and 62 ± 5 % of total Se concentration after digestion using commercial enzymes and human juices, respectively (Fig. 1). No differences in the extraction yields were obtained between the two extraction procedures ($p = 0.4$). In the experiments, physiologically relevant activities of commercial enzymes and human juices were applied. Interestingly, increasing the enzyme activity in both model systems from 16 to 55 units g^{-1} did not increase the overall Se bioaccessibility from the chicken muscle ($p > 0.05$), nor did prolonged digestion time. The results indicate that the main generation of LMM Se components occurs within the first 6 h of the digestion period and that the overall human digestion procedure does not reveal more than 70 % of the Se from the chicken muscle (Fig. 1). The extraction yields are somewhat lower than reported by others, however, in most studies results on bioavailability or bioaccessibility of Se using commercial enzymes refer to mg of enzyme or % w/v, without taking into account the activity of the enzymes [8]. Reyes et al. [22] simulated bioaccessibilty with a mixture of enzymes, resulting in a bioaccessible fraction of 89 % of Se from selenized yeast, while others found highly variable extract yields (50–100 %) using similar approaches [23]. Clinical trials, however, showed an apparent Se absorption of 83 ± 4 % from shrimp and 90 % from yeast to humans [24,25], and these results are somewhat higher than the bioaccessible yield of Se obtained from chicken muscles in the present experiment. In the present work, enzymes were added to isotonic salt solutions with low buffer capacity. Cabanero et al. [26] compared extraction of Se with enzymes in water or Tris-HCl solution and found that enzymes had a lower extraction capacity in water than in Tris-HCl buffer. Earlier experiments with Tris-HCl buffer and 20 mg protease gave an extraction yield of 91 ± 5 % of Se from the chicken muscle.

The large standard deviation in the Se molecule extraction yields using the commercial enzymes reflects that the reproducibility and precision of the procedures in water are low (Figs. 1 and 2). For human juices increased digestion time and higher enzyme activity lowered the variation of extracted Se molecules, whereas no such trend for the commercial enzymes was found. This result is probably due to the lack of buffer capacity and complexity of the human juices. Results for Se in human extracts were

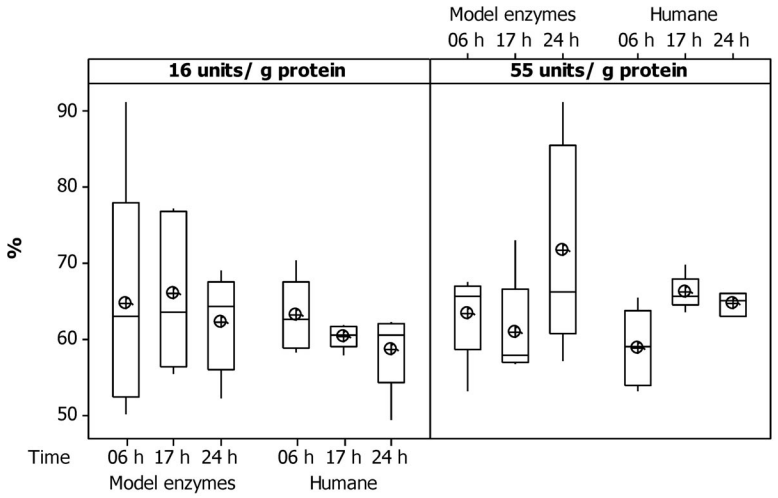
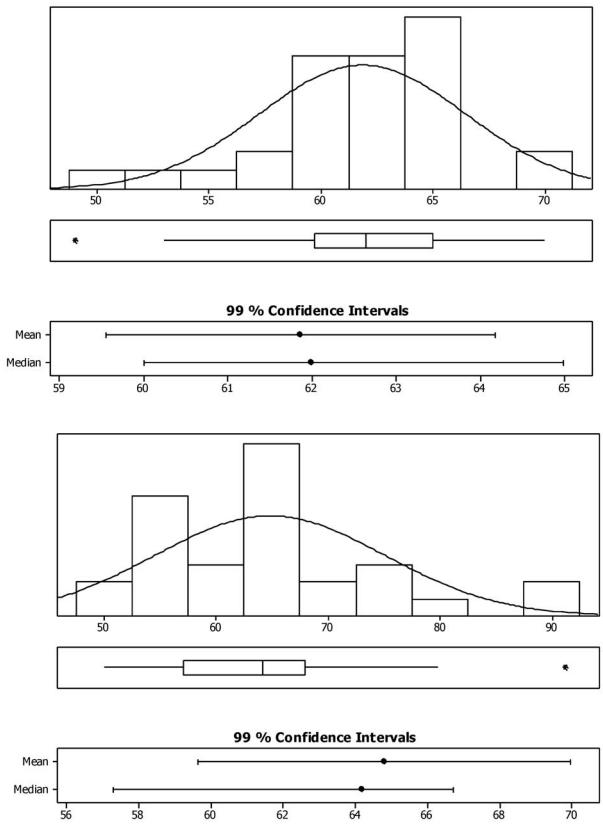


Fig. 1 Relative fraction (%) of Se extracted from Se-enriched chicken breast muscle with low enzyme activity (16 units g^{-1}) and high enzyme activity (55 units g^{-1}) with human juices and commercial enzymes, respectively. Horizontal line is the median, the crossed circle the mean, the top and the bottom of the box show the 75th and 25th quartiles. The top and the bottom of the whiskers show the maximum and minimum values.



Anderson-Darling P-value	Human juices 0.15
Mean	61.9
StDev	4.6
Variance	21.2
Skewness	-0.76
Kurtosis	1.2
N	30

Anderson-Darling P-value	Commercial enzymes 0.020
Mean	64.8
StDev	10.3
Variance	105.5
Skewness	1.1
Kurtosis	1.2
N	30

Fig. 2 Normality distribution of Se in extracts using with human juices and commercial enzymes, respectively, to mimic the digestion of Se-enriched chicken ($p < 0.01$). The *P*-value for normality was set to 0.1.

normally distributed (p = 0.15), whereas results from commercial enzyme extractions were not (p = 0.020) (Fig. 2). This is in accordance with the assumption that a mixture of complex enzymes increases the reproducibility and the precision compared to the addition of single enzymes [15]. Increasing the activities of the commercial enzymes to concentrations used for identification and quantification did not significantly change the extraction yield, but the mean value increased from 64 to 71 (±11) % with the highest enzyme activity (results not shown). Cabanero et al. [26] compared different sample/enzyme ratios and found that the bioaccessible fraction of Se was highly dependent on this ratio. This is in accord with the results in this experiment; the higher the enzyme activity of commercial enzymes, the higher the bioaccessible fraction of Se. The large variation in extract efficiencies from commercial enzymes may be due to a pre-steady state of the enzyme-substrate reactions. Many proteases and inhibitors exist in the human juices with a wide range of substrate specificities and utilizing a variety of catalytic mechanisms, so extraction method with human juices seems to have progressed to a steady state, resulting in more reproducible data.

Size exclusion chromatography (SEC) was used to gain information on the size distribution of Se molecules in the different digestions, and the distribution pattern of Se molecules depended on the digesting agents. In the human juices digestion procedure, the extracts contained more high-molecular-mass (HMM) Se molecules compared to LMM Se molecules obtained with commercial enzymes (Fig. 3). The presence of HMM indicates that the human juices digestion is less efficient in breaking down the Se proteins to LMM Se molecules than the use of commercial enzymes. The results are in accordance with Eriksen et al. [14] who compared GI digestion of caprine whey proteins with human juices vs. commercial enzymes (porcine and proteases), finding different degradation profiles of the proteins and the peptides. This indicates that accurate mimicking of the digestion in the GI tract with commercial enzymes is difficult since the results differ from results obtained when digests are made with human juices. By increasing the enzymatic activity from 16 units g^{-1} protein to 55 units g^{-1} protein after 6 h total digestion, a small shift from HMM Se molecules toward LMM Se molecules occurred (Fig. 3). Even though the human juices extraction yield did not increase with time, a shift toward LMM with increasing enzymatic activity showed that SEC speciation is needed to identify the size distribution of Se molecules of relevance for estimating bioaccessibility of Se in Se-enriched food products. Experiments using intestinal cell membranes are also needed to investigate differences in the cross-membrane transport of Se molecules after digestion using different enzymes. In extracts from commercial enzymes, Se proteins were broken down to LMM molecules, and the retention time of the peak matched the retention time of SeMet (Fig. 3). No information is available on the concentration of SeMet and SeCys in the SEC chromatograms, and the human bioavailability cannot be estimated based on these results. From these data, it is evident that larger molecules than SeMet are present in the human digest. These may be di- or tri-peptides which contain SeMet. These results are in accordance with Hinojosa et al. [22] where SeMet was the major component identified after GI extraction, but only 41 % was present as free SeMet, the remaining SeMet seemed to be associated to Se-containing peptides. Other authors have reported important transformations of Se species in in vitro studies such as the transformation of γ-glu-Se-MeSeCyst to Se-MeSECyst, formation of organic species such as MeSeCys, $SeCys_2$, and degradation of SeMet to inorganic Se [8].

Since SEC is a relatively imprecise method, an attempt to identify Se peptides or other Se species associated with macromolecules from the different digests was made using reversed-phase chromatography. The only peak identified was SeMet with four times higher intensity using commercial enzymes compared to human juices (Fig. 4). This difference may be due to interactions with the column material or retention by the guard column of the HMM Se species in the human extracts. Low-speed centrifugation and electrospray ionization (ESI) together with standard addition in the chromatographic separations could possibly provide information on HMM Se species in the future, although problems with the analysis of unknown species at low Se concentrations may be a problem using ESI.

Mimicking the human GI tract is difficult due to the complexity of the digestive system. However, the in vitro digestion with human juices having physiologically relevant enzyme activities plus other

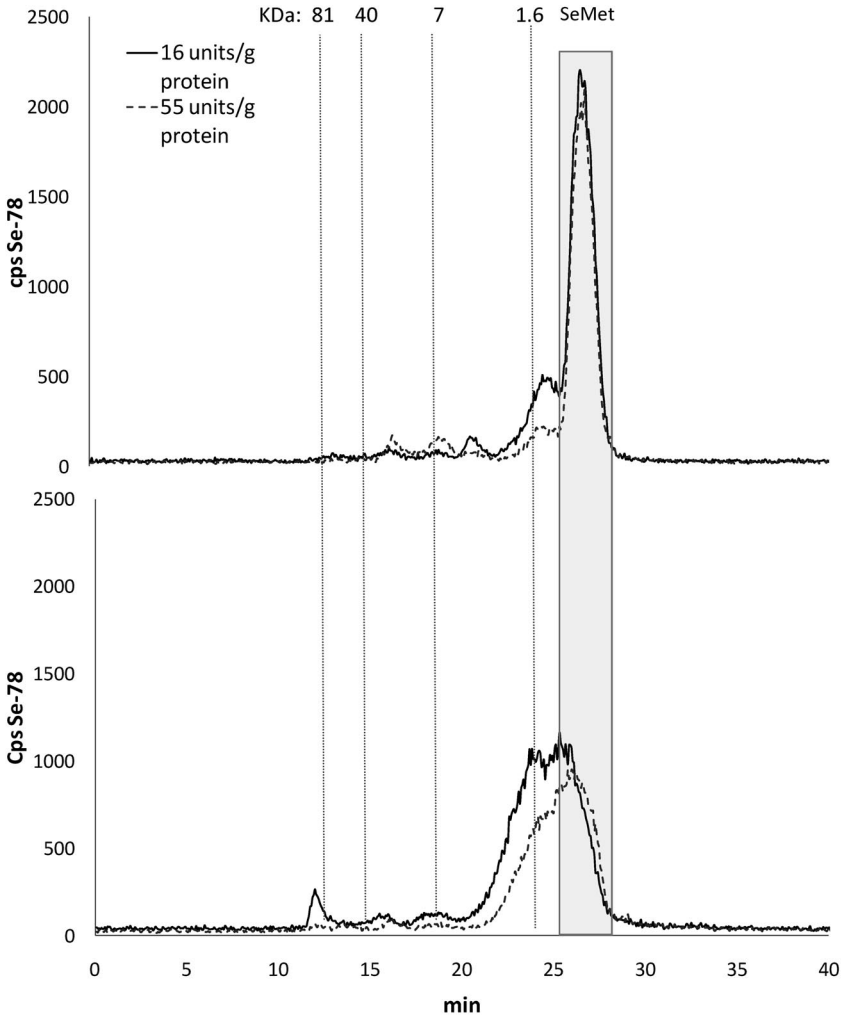


Fig. 3 Size exclusion chromatograms of Se from digests of Se-enriched chicken breast muscle with (a) model enzymes and (b) human juices. The breast muscles were extracted with low enzyme activity (16 units g^{-1}) and high enzyme activity (55 units g^{-1}) for 6 h. The retention time of SeMet is indicated with gray.

natural occurring components resulted in a lower extraction yield and a SEC Se profile with HMM molecules in contrast to results obtained when commercial enzymes were used. Digestion with commercial enzymes did not reflect the Se molecular mass profile obtained with human juices, and the extraction efficiency was highly variable in the present experiment. Sample preparation may also be a critical factor when it comes to reproducibility as results presented by other authors generally show higher reproducibility [8], though lower sample/enzyme ratio and buffer solutions may explain the difference. In vitro extraction efficiency with human juices gives an estimate on the bioaccessible amount of Se from the chicken breast muscle to humans. Extraction with commercial enzymes is not just highly variable, but due to the degradation of HMM Se species to SeMet, this model also overestimates the amount of free SeMet in human digestion.

Foodstuffs other than chicken meat with different Se species should be analyzed in the future, as SeMet may not be the most important species in relation to human health. Furthermore, analysis after 6 h does not give any information on the kinetics of the extraction process of Se from the meat or at

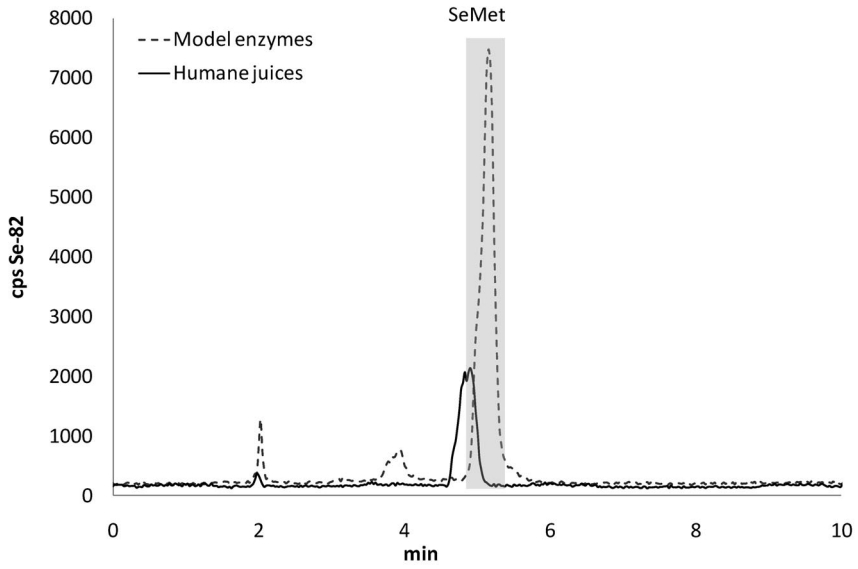


Fig. 4 Se profile of extracts of Se-enriched chicken breast muscle digested with model enzymes (pepsin, lipase, and protease XIV) and human juices after 17 h of digestion at 37 °C. Gray box shows the retention time of SeMet standard.

which point in time the reactions reaches a steady state. A time series investigation is needed to fully understand the degradation processes occurring in the GI tract.

CONCLUSIONS

The results indicate that care should be taken when commercial enzymes are used to mimic the digestibility and bioaccessibility of Se from different foodstuffs. One should always search for optimal conditions to mimic the degradation processes in man. The use of human juices is a good alternative, although the relationship between in vitro and in vivo needs to be investigated further in the future. It is important to take the results from the HJ into consideration when working with an international standardized protocol for in vitro approaches.

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