Formation of Hemoglobin Adducts of Acrylamide after Its Ingestion in Rats Is Dependent on Age and Sex

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The effect of fiber and fat contents of food and of age and sex of animals on the formation of hemoglobin adducts of acrylamide (AA-Hb) in blood has been studied. The results suggest that the absorption of acrylamide (AA) present in food is not affected by the fiber or fat contents of food. However, AA-Hb resulting from the intake of an aqueous solution of AA is dependent on the age and sex of rats: AA-Hb levels were higher in females than in males (3.53- and 2.55-fold higher, respectively, for AA doses of 25 and 100 mg/kg) and in younger than in older rats (30.1% higher in 1.5 month old as compared to 14 month old rats). In males, AA-Hb levels found after the oral administration of AA in an aqueous solution were significantly lower than those found after dietary or intravenous administration. In conclusion, these results show the existence of significant differences in AA bioavailability from an aqueous solution depending on the sex and age of animals. If similar differences also occur in humans, they would be relevant to assess the exposure of different subpopulations to AA.

KEYWORDS: Acrylamide; food; bioavailability; sex; age

INTRODUCTION

In April 2002, the Swedish National Food Agency and Stockholm University presented data that demonstrated the presence of acrylamide (AA), a probable human carcinogen, in regularly consumed fried and baked foods (*1, 2*). This was the result of a series of studies that began with the observation of a regular background exposition to AA (as measured by the levels of the hemoglobin adduct of AA, AA-Hb) in control persons without known exposure to AA (*3*). The fact that the average level of this adduct found in control persons, about 40 pmol/g of globin (*3–8*), could not be explained by the expected exposition to the then known sources of AA, as well as the lower background levels of this Hb adduct observed in wild animals (*2, 9*), suggested that the intake of cooked food might involve an exposure to AA. This hypothesis was later confirmed in rats fed a fried diet who showed an increase in AA-Hb adduct levels with respect to rats fed an unfried diet (*9*). The AA absorbed is very short-lived in blood, and the measurement of AA-Hb, formed as a result of a reaction with the amino group of N-terminal valine, has been extensively used as a biomarker of chronic exposure to AA (*3–5, 10, 11*); because these adducts accumulate over the life span of the erythrocyte, during chronic exposure, the adduct levels reflect the average exposure/internal

dose during the last month (*12*) (as the life span of erythrocytes is 120 and 60 days in human and rats, respectively).

A temperature-dependent formation of AA in human foodstuffs during cooking (at temperatures above 120 °C) was demonstrated in carbohydrate-rich foods, particularly potatoes, which showed very high AA levels (up to mg/kg) but could not be detected in raw or boiled food (*2*). AA may be generated from food components during heat treatment as a result of the Maillard reaction between asparagine and reducing sugars (*13, 14*).

Although AA is present and consumed in common foodstuffs, its relation with the prevalence of some cancers related to the diet is still unclear (*15*). However, the estimation of dietary intake of AA, on the basis of AA levels measured in food and food consumption statistics, reveals an average intake of 0.5 μg AA/kg body weight per day in adults (about 35 μg/day) (reviewed in *8*), and the World Health Organization (WHO) has estimated that a lifetime daily intake of 1 μg AA/kg body weight could result in a lifetime excess risk of approximately 0.7 cases per 1000 individuals (*16*). In addition, the report of the European large-scale HEATOX (Heat-Generated Food Toxicants: Identification Characterization and Risk Minimization) study concluded that, as compared with many related food carcinogens, the exposure of AA possesses a higher estimated risk to European consumers (*17*).

Thus, efforts to reduce both AA formation in food products and AA bioavailability to minimize human exposition to AA

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are of increasing interest (*18, 19*). In this sense, several studies have focused on the measures to reduce AA formation in food products (*20*) and to characterize the amount of AA in different foods (*19*); however, there are few studies regarding the parameters than can affect AA bioavailability (*21–24*). Up to now, the risk estimation to AA exposure from food sources has been based on the AA levels measured in food and on food consumption statistics; however, differences in AA bioavailability in different subpopulations have not been ruled out and, potentially, may be of interest. Here, the effects of food composition—particularly the fiber and fat content of food—and the age and sex of animals in AA bioavailability after oral ingestion in rats have been studied.

MATERIALS AND METHODS

Animals and Experimental Design. Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). The animal protocol followed in these studies was reviewed and approved by the bioethical committee of our university, and guidelines for the use and care of laboratory animals of the university were followed.

Study 1. Effect of Dietary Fiber and Fat Amounts in Food on AA Bioavailability. Three month old female Wistar rats (weighing 244 ± 7 g) were fed for 7 days with standard (1.5% fiber and 10% fat), high-fiber (12% fiber and 9.1% fat), or high-fat (1.2% fiber and 29% fat) cookies fortified or not with AA to a total amount of either 25 or 100 mg/kg of body weight; animals were also provided free access to standard chow diet ($n = 6$ in each group). The amount of cookies offered to animals was 10.5 g per day (this amount was previously checked to be finished by the animals during the same day). Blood was collected by animal decapitation at the end of the treatment.

Study 2. Effect of Sex on AA Bioavailability. Adult male (weighing 575 ± 20 g) and female (weighing 327 ± 11 g) Wistar rats placed on a standard diet were treated with two different single oral doses of AA (25 or 100 mg/kg of body weight) and sacrificed by decapitation 24 h afterward for blood collection ($n = 6$ in each group). AA was dissolved in a total volume of 200 μL of water and given to animals using a pipet.

To assess differences between sex depending on the route of administration, adult male (weighing 588 ± 21 g) and female (weighing 322 ± 9 g) Wistar rats placed on a standard diet were exposed to a single dose of AA (25 mg/kg of body weight) using different routes of administration ($n = 6$ in each group). (a) Oral administration in an aqueous solution: AA was prepared dissolved in water to a final volume of 200 μL and given to animals using a pipet. (b) Dietary administration: Cookies fortified with AA were administered during the first hour after the beginning of the light cycle after overnight fasting. (c) Intravenous administration: AA was dissolved in saline and administered by intravenous injection into the saphenous vein after ether anesthetization (the volume of injection was 1 mL/kg). In all cases, animals were sacrificed by decapitation 24 h after AA administration, and blood was collected.

Study 3. Effect of Age on AA Bioavailability. Different aged female Wistar rats, 1.5, 3, and 14 months old (weighing 148 ± 4, 250 ± 6, and 331 ± 6 g, respectively) ($n = 6$ in each group), were given a single oral dose of AA (100 mg/kg of body weight) using a pipet. Blood samples were collected 24 h afterward by animal decapitation.

Blood Sampling and Isolation of Globin. Globin was isolated according to refs *25* and *26* with slight modifications. Briefly, blood was collected in heparinized tubes. Erythrocytes were isolated by centrifugation at 1000*g* for 10 min and washed three times with saline. Hemolysis was achieved by addition of 2.5 volumes of Milli-Q water and subsequent freezing at -20 °C. The lysed cell suspension was centrifuged at 5000*g* for 30 min, and then, for the isolation of globin, 1 volume of the supernatant was added to 5 volumes of 50 mM hydrochloric acid (Panreac, Barcelona, Spain) in 2-propanol (Panreac). After centrifugation at 2000*g* for 10 min, ethyl acetate (Panreac) was added to the supernatant, and the solution was kept at 4 °C for at least 2 h. After centrifugation at 2000*g* for 10 min, the precipitated globin was washed two times with ethyl acetate and once with *n*-hexane (Panreac) and finally dried overnight in a desiccator.

Table 1. Retention Times and Detected Mass of Pentafluorphenylthiohydantion Derivates of Analytes[a]

analyte	retention time (min)	detected masses (*m/z*)
N-2-ethoxyethylvaline	20.3	308, 363
N-2-carbamoylethylvaline	28.4	363, 378, 395

[a] Quantifier ions are underlined.

Derivatization of Globin Samples. Adduct monitoring was conducted according to refs *25* and *26*, with slight modifications. Briefly, 50 mg of globin was dissolved in 1.5 mL of formamide (Sigma, Madrid, Spain). Fifty microliters of NaOH (1 M), 10.03 μL of the internal standard (*N*-2-ethoxyethlvaline-alanine-anilide, 0.033 mg/mL) (Bachem Biochemica, Heidelberg, Germany), and 10 μL of pentafluorophenyl-isotiocyanate (PFPITC) (Sigma) were added. The samples were rotated overnight at room temperature and finally heated at 45 °C for 90 min in a water bath with constant agitation. The resulting pentafluorophenylthiohydantoin (PFPTH) derivates of the adducts at the N-terminal valine of the globin chain were extracted three times with diethylether $(2 + 2 + 1 \text{ mL})$, and the combined ether extracts were evaporated to dryness under a stream of N_2. The residue was then dissolved in 1 mL of toluene (Panreac) and washed twice with Milli-Q water (2 mL), twice with freshly prepared 0.1 M Na_2CO_3 (3 mL), and again with Milli-Q water (2 mL). Finally, the toluene phase was carefully evaporated to dryness under N_2 at 45 °C and redissolved in toluene (50 μL). One microliter was then analyzed by gas chromatography/mass spectrometry (GC/MS).

A calibration standard was prepared by mixing pooled globin from nontreated rats with a solution of the dipeptide standard (*N*-2-carbamoylethylvaline-leucine-anilide, Bachem Biochemica) in the range of 0–80 pmol.

GC/MS Analysis. Analysis was carried out on a Shimadzu QP-5000 GC/MS System equipped with a Shimadzu Autoinjector AOC-17 autosampler and a split/splitless injector operating in splitless mode. The inlet purge off time was 1 min.

Chromatography separation was performed using a DB-17 HT capillary column (Agilent Technologies, Las Rozas, Spain). Helium 5.0 was used as the carrier gas at a constant flow of 1.25 mL/min. The initial temperature of 90 °C was held for 1 min and then raised at a rate of 25 °C/min to 120 °C. It was then raised at a rate of 5 °C/min to 240 and finally raised 25 °C/min to 280 °C, remaining at this temperature for 20 min. For mass spectrometric detection of the derivates, electron impact (EI) mode was used. EI mass spectra of the PFPTH derivates were obtained at an energy level of 70 eV, and the electron multiplier voltage was 2600 V ($+600$ rel.). The transfer line temperature was maintained at 300 °C. The retention times and detected masses for the PFPTH derivates of adducts under the described conditions are summarized in **Table 1**. Quantification is based on a comparison of peak areas of the major fragments, indicated in **Table 1**. The reproducibility and the linearity of the method were previously checked.

Statistical Analysis. All data are expressed as the mean $\pm$ standard errors of the mean (SEM). Two-way analysis of variance (ANOVA) was used to determine the significance of two different factors (dose and cookie type, sex and dose, and sex and route of administration) on the AA-Hb adduct levels. One-way ANOVA followed by least significant difference posthoc comparison was used to assess statistical differences between the groups. Single differences between male and female rats were assessed by Student's *t* test. The threshold of significance was defined at $p < 0.05$.

RESULTS

Study 1. Effect of Dietary Fiber and Fat Amounts of Food on AA Bioavailability. Different types of cookies (standard, high fiber, or high fat) fortified or not with AA to a total dose of either 25 or 100 mg/kg of body weight were offered to female rats for 7 days. AA-Hb was not detected in the blood of animals fed with cookies without fortification, while levels were detectable in animals fed with the different types of cookies

Table 2. AA-Hb Detected in Blood of 3 Month Old Female Wistar Rats Fed for 7 Days with Standard (1.5% Fiber and 10% Fat), High-Fiber (12% Fiber and 9.1% Fat), or High-Fat (1.2% Fiber and 29% Fat) Cookies Fortified or Not with AA (25 and 100 mg/kg of Body Weight)[a]

doses	cookie type	AA-Hb levels (nmol/g Hb)
nonfortified	standard	ND
	high fiber	ND
	high fat	ND
25 mg AA/kg body weight	standard	25.9 ± 2.47
	high fiber	27.8 ± 3.95
	high fat	26.3 ± 2.27
100 mg AA/kg body weight	standard	38.4 ± 2.50
	high fiber	41.3 ± 1.99
	high fat	38.9 ± 4.32 D

[a] Results are expressed as the mean ± SEM of six animals per group. ND, not detected. D, effect of doses ($p < 0.05$, two-way ANOVA).
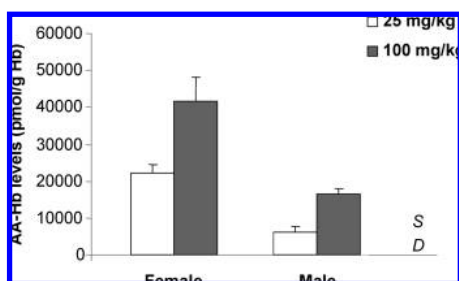

Figure 1. AA-Hb levels detected in blood of adult male and female Wistar rats 24 h after a single oral administration of two different doses of AA (25 or 100 mg/kg of body weight). Results are expressed as the mean ± SEM of six animals per group. *S*, effect of sex; and *D*, effect of dose ($p < 0.05$, two-way ANOVA).

fortified with AA. No differences were found depending on the type of cookies for each of the dose assayed (**Table 2**). As also seen in **Table 2**, AA-Hb levels after the intake of dietary AA were dependent on the dose of AA intake ($p < 0.05$, two-way ANOVA), although the formation of AA-Hb relative to the dose of AA intake (AA-Hb/dose) was higher with the lower dose of AA ($p < 0.05$, two-way ANOVA).

Study 2. Effect of Sex on AA Bioavailability. Male and female rats were given a single oral dose of AA dissolved in water (25 or 100 mg/kg body weight) using a pipet. For both doses of AA tested, female rats presented significantly higher levels of AA-Hb than male animals (effect of sex $p < 0.05$, two-way ANOVA) (**Figure 1**). In both sexes, AA-Hb levels were dose-dependent (effect of dose $p < 0.05$, two-way ANOVA), but the difference between both sexes was stressed when applying the low dose of AA (3.53- and 2.55-fold higher in females than in males, respectively, for the AA doses of 25 and 100 mg/kg), according to the previous observation of a relative higher AA-Hb formation with lower doses of AA intake (**Table 2**).

To ascertain whether differences in AA-Hb levels between sexes after AA intake were dependent on the route of AA administration, AA-Hb levels after intravenous AA injection and after the intake of cookies fortified with AA, in comparison with AA-Hb levels after oral administration of AA dissolved in water, were determined. In this study, the dose of AA assayed was 25 mg/kg. As expected from the previous study (**Figure 1**), AA-Hb levels after oral administration of AA dissolved in water were higher in females as compared with males. However, no differences between sexes were observed after the dietary administration of AA (cookies fortified with AA) or after intravenous injection of AA (**Figure 2**). Moreover, in females, no differences were observed in the levels of AA-Hb between
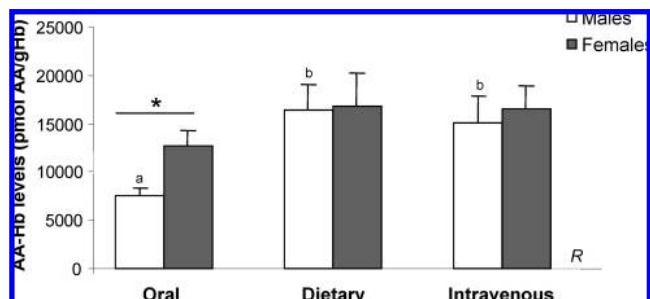

Figure 2. AA-Hb levels detected in blood of adult male and female Wistar rats 24 h after the administration of a single dose of AA (25 mg/kg of body weight) using different routes. (a) Oral administration: AA was dissolved in water to a final volume of 200 μL and given to animals using a pipet. (b) Dietary administration: cookies fortified with AA were administered during the first hour after the beginning of the light cycle after overnight fasting. (c) Intravenous administration: AA was dissolved in saline and administered by intravenous injection into the saphenous vein after ether anesthetization (the volume of injection was 1 mL/kg). Results are expressed as the mean ± SEM of six animals per group. *R*, effect of route of administration ($p < 0.05$, two-way ANOVA); a ≠ b ($p < 0.05$, one-way ANOVA); and *, male vs female ($p < 0.05$, Student's *t* test).
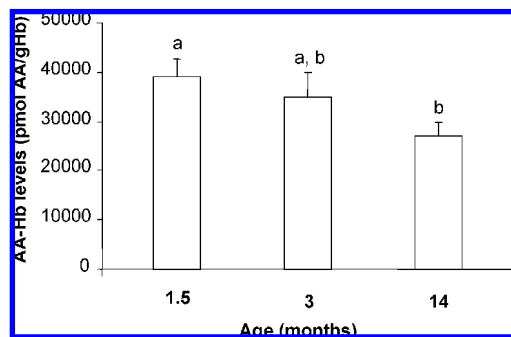

Figure 3. AA-Hb levels of 1.5, 3, and 14 month old female Wistar rats 24 h after the administration of a single oral dose of AA (100 mg/kg of body weight). Results are expressed as the mean ± SEM of six animals per group. a ≠ b ($p < 0.05$, one-way ANOVA).

the different routes of administration (orally in an aqueous solution, in cookies, and intravenous injection), whereas in males, AA-Hb levels found after the oral administration of AA dissolved in water were significantly lower than those levels found after dietary or intravenous administration.

Study 3. Effect of Age on AA Bioavailability. Results showed that AA absorption after the administration of a single oral dose of AA (100 mg/kg body weigh) dissolved in water was higher in young rats and decreased with age ($p < 0.05$, one-way ANOVA) (**Figure 3**). AA-Hb levels in 1.5 month old rats were 30.1% higher as compared to those found in 14 month old rats.

DISCUSSION

Since the description of the presence of AA in fried/owned food, different studies have focused on the study of AA formation as well as on the different conditions that may affect AA levels in foods with the aim of reducing AA formation—regarding the raw material and/or the processing of the food (reviewed in ref *20*). However, little is known with respect to AA availability in humans and animals.

In vitro studies using CaCo-2 cells have demonstrated that AA monomers are rapidly absorbed by a passive transport

mechanism (*27, 28*). However, there are only few studies on some aspects of AA bioavailability performed in animals (*21–23*) and even fewer in humans (*24*), and most of them have suggested an extensive absorption of AA ingested, although these studies have not compared AA absorption from different food matrices. Here, AA absorption from food with different composition and the effect of age and sex of animals on the bioavailability of AA in rats have been studied.

It is well-known that dietary fiber modifies the absorption of some nutrients and chemicals present in food and accelerates the gut transit time (*29*), and these properties have been associated with its beneficial physiological effect. In the present study, whether the dietary fiber content of food can affect the absorption of AA was checked. AA-Hb levels in rats fed with high-fiber cookies (12% fiber) or with normal-fiber cookies (1.5% fiber) fortified with AA for 7 days to a total amount of either 25 or 100 mg of AA/kg body weight have been determined. It was found that AA-Hb levels did not differ within groups, pointing out that, in our model, the fiber content of food did not affect AA bioavailability. In the same way, the fat content of food did not seem to affect AA availability either: AA-Hb levels in rats were similar after the ingestion of high-fat cookies (29% fat) or of normal-fat cookies (10% fat) fortified with AA (both 25 and 100 mg/kg of body weight). Thus, in the conditions used in this study, the food composition (regarding the fiber and fat content) does not seem to interfere with AA bioavailability.

In humans, it has been previously shown that most of the AA ingested with a controlled food is absorbed (*24*). Moreover, another study performed in swine showed that AA-Hb levels were strongly correlated to the administered dose of AA and were the same after the ingestion of AA given with food or by drinking water, suggesting that the absorption of AA from a food matrix is comparable to that of the compound dissolved in water (*21*). However, other studies have reported a lower absorption of AA by oral administration from a food matrix in comparison with aqueous gavage in mice (*22*) and rats (*23*), thus showing differences depending on the animal species, as well as the experimental protocol, including the type of food used as a source of AA; although to the best of our knowledge, a true comparison between AA absorption from different sources of food has not been made in any of these studies.

Regarding the effect of sex, the main finding of this study is that female rats showed a higher absorption capacity of AA from an aqueous solution than male rats. In fact, 24 h after an oral dose of AA, female rats had AA-Hb levels that were significantly higher than in males. At the lowest dose of AA assayed (25 mg/kg body weight), females showed levels of AA-Hb 3.53-fold higher than males, and with the highest dose of AA (100 mg/kg body weight), the difference in AA-Hb levels between male and female rats was maintained but to a lesser extent (2.55-fold higher in females). This indicates differences in the absorption of AA between sexes and that this difference is more evident with low doses of AA, while at higher doses, the differences become less important.

To confirm that the differences in AA-Hb levels between male and female rats could be attributed to differences in the absorption of AA and to rule out differences in AA-Hb formation, different routes of administration, aqueous solution, fortified cookies, and intravenous injection, were checked. Interestingly, he differences in AA-Hb levels between sexes were only observed after oral AA administration in aqueous solution; AA-Hb levels were similar in male and female rats after the dietary or intravenous AA dosing. The absence of sex differences after intravenous AA administration supports the fact that the differences in AA-Hb levels after oral administration of AA can be attributed to differences in the absorption of AA between both sexes and not apparently to differences in AA-Hb formation due to a different metabolism of AA to glycidamide, the epoxy metabolite of AA, or to different excretion of AA. These results also showed that, in male rats, AA-Hb formation is dependent on the way or route of AA administration: AA-Hb levels were higher (and similar to female rats) after the administration of AA in the diet or by intravenous injection but were lower when AA was administered dissolved in water. Thus, the lower capacity of AA absorption in male rats as compared with females only becomes apparent when AA is administered dissolved in water. This could be due probably to the faster intestinal transit of water as compared to solid food. Thus, if male rats have a less efficient or a slower AA absorption as compared to female rats, in the case of a faster intestinal transit, this difference would become appreciable.

Sex differences in the mean serum time—concentration profile for the absorption of AA after a gavage dose of AA have been previously reported (*23*). These authors studied levels of AA and its epoxide metabolite, glycidamide, in serum and tissues of male and female F344 rats following gavage acute exposure to AA and found significant sex effects in kinetic parameters affecting AA elimination and AA absorption/distribution, with the net result being that the area under the curve for AA and GA was several fold higher in females (*23*). Sex differences of this magnitude have not been described in mice after gavage administration of AA (*22*). Doerge et al. (*23*) also found that dietary AA administration displayed a similar serum time—concentration profile in male and female rats. However, unlike our results, these authors found significant, sizable reductions in AA bioavailability on going from an aqueous gavage to a dietary matrix, particularly in female rats, thus showing that diet can decrease the absolute AA availability relative to that seen after gavage with an aqueous solution. These differences between our results and those of Doerge et al. (*23*) can be tentatively attributed to differences in the route of AA administration (gavage or administration with a pipet), in the matrix of food fortified with AA, in the experimental protocol, as well as in the doses of AA used, higher in our study. Even considering these discrepancies, both studies agree in that AA bioavailability is significantly higher in females than in males, particularly when AA is supplied acutely and in aqueous solution.

Whether these sex-dependent differences in AA absorption in rats can be extended to humans is not known. We are not aware of any published study in humans showing an accurate measure of the correlation between a fixed amount of AA in the diet and the resulting Hb adduct levels; however, it must be pointed out that in a screening of AA-Hb levels from blood donors of the Malmö Diet and Cancer Cohort (HEATOX project), differences between nonsmoking men and women regarding the correlation between the AA-Hb levels and the estimated exposure to dietary AA were found; while in men there was a correlation between the levels of AA-Hb and the estimated dietary exposure to AA, such a correlation was not found in women (*12, 30*). In these studies, the levels of AA-Hb between men and women were similar, while the calculated AA intake from all foods was lower in women than in men (*12, 30*). It should be pointed out that the amount of AA ingested was calculated based on a questionnaire regarding dietary habits and not with a controlled dosing schedule of AA.

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However, although not conclusive, the result of this study does not disagree with a higher AA absorption capacity in women as compared with males. Further studies are needed to check whether in humans there are differences in the biovailability of AA depending on sex.

Differences between adults and children concerning the intake of food containing AA have been reported: A higher intake of AA has been estimated in younger age groups (particularly children and adolescents due to a higher consumption of chips, French fries, snacks, etc.), reaching up to 3.4 μg/kg body weight daily (*8, 31, 32*), as compared with 0.5 μg/kg body weight daily in adults (*8, 31–33*). Here, we have shown that after a single oral dose of AA, younger rats (1.5 month old) displayed higher AA-Hb adduct levels than older rats (14 months old). It is not known whether this difference is due to differences in the absorption of AA in the gastrointestinal tract. Differences in erythrocyte or hemoglobin concentration values between younger and older rats could also help explain the different levels of AA-Hb. In fact, younger rats have higher erythrocyte counts and hemoglobin concentrations than adult animals (*34*), although this difference does not seem to be able to totally explain the different AA-Hb levels found between different aged rats after an oral dose of AA. If these results can be extrapolated to humans, this finding stresses the importance of controlling the AA-rich foods in early life, because a higher exposure to AA due to a higher intake of AA-rich foods could even be enhanced due to a higher capacity of AA absorption in this population group.

In conclusion, our results confirm in rats that AA is bioavailable from aqueous solution and from AA-fortified food and show important differences between sex and age of animals in the bioavailability of AA from aqueous solution: higher in females and in younger rats. If extrapolated to humans, these factors should be considered to assess exposition levels to this compound and estimate their risks, together with differences in the AA content of food and the theoretical food ingestion of different subpopulations.

ACKNOWLEDGMENT

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