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Influence of pro- and antioxidants on the formation of mutagenic–carcinogenic heterocyclic amines in a model system

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The precursors of the heterocyclic amines (HAs), creatinine, glycine and glucose, were heated with the addition of different iron and copper compounds, hydroquinone, corn oil, β-carotene, tocopherols and synthetic antioxidants. The yield of HAs in the model system was affected by added pro- and antioxidants, while the species formed were not. Most of the antioxidants, as well as different forms of iron, were shown to increase the amount of 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline (MeIQx) formed in the model system. Copper had no effect on the formation of MeIQx. Hydroquinone had only a small increasing effect but, in combination with iron sulphate, an extreme increase in MeIQx, 2-amino-3-methylimidazo[4,5-*f*]quinoxaline (IQx) and 2-amino-3,7,8-trimethylimidazo[4,5-*f*]-quinoxaline (7,8-DiMeIQx) formation was observed. β-carotene and γ-tocopherol were shown to inhibit the synergistic effect of hydroquinone and iron sulphate, but had less effect in model mixtures containing none or only one of the pro-oxidants. Copyright © 1996 Published by Elsevier Science Ltd.

INTRODUCTION

The major food mutagens isolated to date from cooked meat and fish products are heterocyclic amines (HAs). All HAs tested have been shown to be multipotent carcinogens in rodent bioassays (for a review, see Ohgaki *et al.*, 1991) and 2-amino-3-methylimidazo[4,5-*f*]-quinoline (IQ) also in monkeys (Adamson *et al.*, 1990). Epidemiological studies have shown a relationship between the consumption of fried meat products and an elevated risk of colon and other cancers (Norell *et al.*, 1986; Schiffman & Felton, 1990; Steineck *et al.*, 1990; Willett *et al.*, 1990). The International Agency on Cancer Research (IARC) has classified several of the HAs as possible, and IQ as probable, human carcinogens, and recommend a reduced exposure to these compounds (IARC, 1993). However, in order to minimize exposure, a better understanding of their formation mechanisms is needed.

The mechanisms for the formation of HAs have not been clarified in detail, but model experiments have shown that these food mutagens are formed by heating the precursors creatin(in)e and amino acids with or without monosaccharides, as reviewed by Skog (1993). The Maillard reaction is involved, and after condensation of sugar and an amino acid, Amadori rearrangement followed by Strecker degradation takes place, and pyrazines, pyridines and aldehydes are formed (Mauron, 1981). Creatine, pyrazines or pyridines and aldehydes are assumed to condense to form IQ compounds (Jägerstad *et al.*, 1983). This hypothesis has been proven by heating creatin(in)e, glucose and amino acids in different model systems, resulting in the formation of IQ, 2-amino-3-methylimidazo[4,5-*f*]-quinoxaline (IQx), 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]-quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo-[4,5-*f*]quinoxaline (4,8-DiMeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Jägerstad *et al.*, 1991; Skog, 1993; Johansson & Jägerstad, 1993). Moreover, ^{14}C-labelled glucose was shown to be incorporated into MeIQx and DiMeIQx using an aqueous model system (Skog & Jägerstad, 1993). A remarkable correspondence between HAs formed in simple model experiments and those isolated from cooked meat products, confirms that model reactions are an important tool in providing basic information on the reaction conditions, precursors and inhibitors in the formation of HAs.

The yields of HAs in model systems are generally low, being 1–10 nmol/mmol reactant, which corresponds to low ppb levels in cooked meat products. However, the

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formation of HAs in model systems has earlier been reported to be influenced by, for example, lipids, iron and various antioxidants. In previous studies at our laboratory, the addition of oils and iron sulphate in a model system was found to significantly increase the formation of MeIQx (Johansson *et al.*, 1993). A higher lipid content in the model mixture also increased MeIQx formation. The reported effects of lipids on the formation of MeIQx might be explained by an enhanced formation of pyridines, pyrazines and Strecker aldehydes in the Maillard reaction (Arnoldi *et al.*, 1987, Arnoldi *et al.*, 1990; Buttery *et al.*, 1977; Kawamura, 1983; Watanabe & Sato, 1971*a,b*) or by the production of free radicals through thermic oxidation (Eriksson, 1987), or both. Free radical reactions have earlier been suggested to increase the formation of HAs (Barnes & Weisburger, 1984; Namiki & Hayashi, 1983; Yoshida & Mizusaki, 1985). Iron is known to act as a pro-oxidant in lipid oxidation and might potentiate the Maillard reaction. If radicals are involved in the formation of HAs, antioxidants might have inhibitory effects. Several studies have reported antioxidants to decrease the mutagenic activity of HAs (Wang *et al.*, 1982; Chen, 1988) and one study reported antioxidants to inhibit the formation of HAs during the frying of beef (Chen *et al.*, 1992).

The mechanisms by which HAs are formed require further attention to make a reduction in the daily human intake of HAs possible. The purpose of this study was to investigate the influence of various pro- and antioxidants on the formation of HAs in a model system. The precursors, creatinine, glycine and glucose, were heated with the addition of different iron and copper compounds, hydroquinone, tocopherols, β-carotene and synthetic antioxidants. The amount of HAs produced in the model system was quantified using HPLC.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were of HPLC or analytical grade. Water was taken from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All solvents, for example acetonitrile, methanol and dichloromethane, were purchased from Merck AG (Darmstadt, Germany). [^{14}C]MeIQx (10 mCi/mmol, 98% radiochemically pure according to NMR spectral analysis and TLC), IQ, 2-amino-1-methylimidazo[4,5-*f*]-quinoline (iso-IQ), IQx, MeIQ, MeIQx, 2-amino-3,4,7-trimethylimidazo[4,5-*f*]quinoxaline (4,7-DiMeIQx), 4,8-DiMeIQx, 2-amino-3,5,8-trimethylimidazo[4,5-*f*]quinoxaline (5,8-DiMeIQx), 2-amino-3,7,8-trimethylimidazo-[4,5-*f*]quinoxaline (7,8-DiMeIQx) and 2-amino-3,4,7,8-tetramethylimidazo[4,5-*f*]quinoxaline (4,7,8-TriMeIQx) were obtained from Toronto Research Chemicals (Downsview, Ontario, Canada). Iron(II) sulphate heptahydrate ($FeSO_4$), copper(I) chloride (CuCl) and butylated hydroxy-toluene (BHT) were obtained from Merck AG (Darmstadt, Germany). Anhydrous iron(III) chloride ($FeCl_3$) was obtained from Kebo (Stockholm, Sweden). Haemoglobin A ferrous, copper(II) sulphate pentahydrate ($CuSO_4$), hydroquinone (HQ), α- and γ-tocopherol, synthetic β-carotene, L-ascorbic acid, butylated hydroxy-anisole (BHA) and *n*-propyl gallate (PG) were purchased from Sigma (St Louis, MO, USA). Anhydrous copper(II) chloride ($CuCl_2$) and tert-butylhydroquinone (TBHQ) were obtained from Janssen Chimica (Geel, Belgium). Dimodan OT (distilled monoglyceride, made from edible, partially hydrogenated soy bean oil) was obtained from Grindsted (Denmark). Corn oil was purchased at a local shop. The materials used for PRS tandem extraction (Extrelut and BondElut, e.g. PRS and C_{18}) were obtained from Merck AG (Darmstadt, Germany) and Sorbent (Västra Frölunda, Sweden). Ecoscint A scintillation fluid was obtained from National Diagnostics (Somerville, NJ, USA).

Model system

The precursors—creatinine (0.9 mmol), glycine (0.9 mmol) and glucose (0.45 mmol)—were dissolved in Milli-Q water, to a total volume of 2.5 ml, before being heated in closed test-tubes at 180°C for 10 or 30 min, as described previously (Johansson *et al.*, 1993). Corn oil (0.5 or 1 g) was added to some mixtures. Five milligrammes of Dimodan OT were melted in the test-tubes before the addition of water and the precursors in order to establish an emulsion and dissolve the fat-soluble antioxidants.

The effect of different forms of iron and copper on the formation of MeIQx was studied through the addition of iron sulphate, iron chloride, copper sulphate, copper(I) and copper(II) chloride (0.01 and 0.09 mmol) or haemoglobin (0.005 and 0.02 g) to model mixtures containing corn oil (0.5 g) and the precursors mentioned above.

The effect of some antioxidants on the formation of MeIQx was studied in a model mixture containing creatinine, glycine, glucose and corn oil (1 g), and one of the following: TBHQ (100 and 1000 ppm), ascorbic acid (10, 100 and 1000 ppm), and α- or γ-tocopherol (10, 100, 200, 1000 and 10 000 ppm of the fat content).

Effects of pro- and antioxidants were further studied in a model mixture containing creatinine, glycine, glucose, iron sulphate (0.09 mmol) and corn oil (0.5 g) and one of the following: HQ (1000 and 10 000 ppm), BHA, BHT or PG (10 000 ppm).

The synergistic effects of the pro-oxidants iron sulphate (0.09 mmol) and hydroquinone (1000 ppm), and the naturally occurring antioxidants γ-tocopherol and β-carotene (1, 10, 100, 1000 and 10 000 ppm) on the formation of HAs were studied in a model system containing creatinine, glycine and glucose.

Purification

The heated samples were spiked with 8.0 nCi [^{14}C]MeIQx serving as an internal standard (i.s.). The

samples were extracted according to the method of Gross (1990) with some minor modifications (Johansson *et al.*, 1993). After purification, the samples were dissolved in 100 μl methanol. Aliquots (50%) of the samples were used for the determination of [^{14}C]MeIQx (i.s.) recovery, using a liquid scintillation counter (1219 Rackbeta LKB Wallac, Sweden).

HPLC fractionation

The sample volume was reduced to dryness under nitrogen and finally dissolved in 125 μl of HPLC buffer A (see below). Aliquots (20 μl) of the sample were injected (Varian 9095 Autosampler) into a Varian 9010 liquid chromatograph with a photodiode array UV detector (Varian 9065, Polychrom), equipped with an ODS 80 column (ToyoSoda TSK gel TM, 250×4.6 mm i.d., 5 μm particle size, Varian, Stockholm, Sweden) and a precolumn (Supelguard LC-18-DB, 20×4.6 mm i.d.) and eluted with a mobile phase of 10 mM triethylamine in water adjusted with acetic acid to pH 3.2 (A) or pH 3.6 (B), and acetonitrile (C). A gradient of 5–15% C in A for 10 min, then 15–25% C in B for 10 min, and finally 25–55% C in B for 5 min were used. The flow rate was 1.0 ml/min and the effluent was monitored at 263 nm.

Identification and quantification of HAs using HPLC

The HAs produced in the model system were fractionated using HPLC, as described above. The identities of the HAs were established by comparing the retention times of the peaks with the retention times of synthetic compounds namely IQ, iso-IQ, IQx, MeIQ, MeIQx, 4,7-DiMeIQx, 4,8-DiMeIQx, 5,8-DiMeIQx, 7,8-DiMeIQx and 4,7,8-TriMeIQx, obtained under the same conditions. In addition, some samples were also spiked with synthetic compounds before injection. Photodiode array UV spectra of synthetic compounds run under the same conditions and library entries were used to establish the identities of the peaks.

The HAs were quantified by comparing the peak area of the HPLC chromatographed sample with the area of a known amount of standard. The amount was corrected for incomplete recovery of [^{14}C]MeIQx.

Statistics

The amounts of HAs formed in the model mixtures containing different pro- and antioxidants were statistically compared using one-way ANOVA followed by Duncan's test (significance level 0.05) using the software package SPSS for MS Windows 5.0 (SPSS Inc., Chicago, USA).

RESULTS AND DISCUSSION

HAs produced in the model system

Addition of pro- and antioxidants to the model system did not affect the types of HAs formed. The same HAs were formed in all heated model mixtures with and without the addition of fat and different pro- and antioxidants. This is in agreement with earlier studies (Johansson & Jägerstad, 1993; Johansson *et al.*, 1993) showing iron and different forms of fats to affect, exclusively the yield of HAs. A chromatogram from the HPLC analysis of a heated model mixture containing creatinine, glucose, glycine, hydroquinone (1000 ppm) and γ-tocopherol (10 ppm) is shown in Fig. 1. On-line-recorded UV spectra of the peaks are compared with those of synthetic HAs. The retention times and UV spectra corresponded to authentic IQx, MeIQx and 7,8-

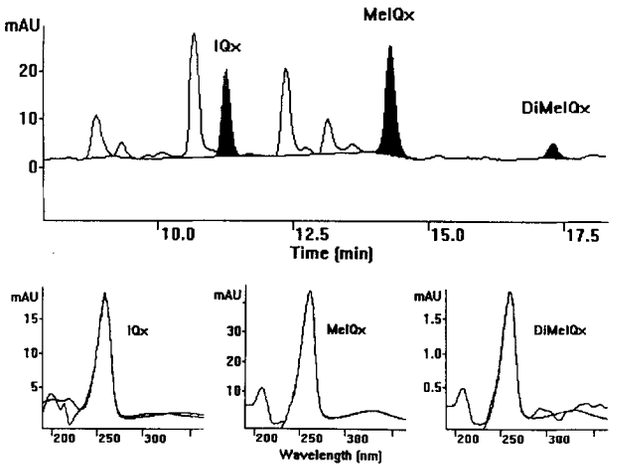

Fig. 1. Expanded region of a chromatogram from the HPLC analysis of a heated model mixture containing creatinine, glycine, glucose, hydroquinone (1000 ppm) and γ-tocopherol (10 ppm). The peaks corresponding to IQx, MeIQx and 7,8-DiMeIQx are indicated. On-line-recorded UV absorbance spectra are compared with those of synthetic IQx (lower), MeIQx and 7,8-DiMeIQx (upper).

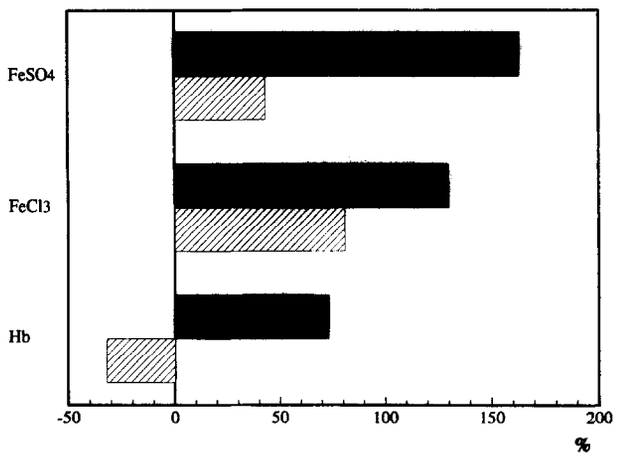

Fig. 2. The effect of iron sulphate (0.09 mmol), iron chloride (0.09 mmol) and haemoglobin (0.02 g) on the formation of MeIQx in a model mixture containing creatinine, glycine, glucose and corn oil (0.5 g) heated at 180°C for 10 and 30 min. Amounts are expressed as % of MeIQx content in a control sample without iron. The control samples contained 6.7 and 20.6 nmol MeIQx/mmol creatinine, respectively.

DiMeIQx. The formation of IQx and MeIQx in similar model mixtures has been established earlier using LC–MS (Johansson & Jägerstad, 1993; Johansson *et al.*, 1993). In contrast to earlier studies, 7,8-DiMeIQx and not 4,8-DiMeIQx was detected in the samples. However, 7,8-DiMeIQx has recently been identified using LC–MS in a similar glycine-containing model mixture (Johansson *et al.*, 1995*a*). The same study showed IQx, MeIQx and 7,8-DiMeIQx to be formed from creatinine, glucose and most amino acids, whereas 4,8-DiMeIQx was not as commonly formed. The formation of both 7,8- and 4,8-DiMeIQx in a creatine–glycine–glucose model system has been reported earlier by Skog & Jägerstad (1993).

Effects of iron and copper on the formation of HAs

Heating the precursors, creatinine, glycine, glucose and corn oil, at 180°C for 10 and 30 min produced 6.7 ± 2 and 20.6 ± 3.9 nmol MeIQx/mmol creatinine, respectively. The addition of iron(II) sulphate or iron(III) chloride (0.09 mmol) significantly increased the amounts of MeIQx formed by 163 and 130%, respectively, after heating for 10 min, and by 43 and 81%, respectively, after heating for 30 min, as shown in Fig. 2. Heating a mixture containing haemoglobin (0.02 g) for 10 min gave rise to a 73% higher MeIQx content than a control sample, while a similar mixture heated for 30 min contained 32% less MeIQx than the control sample. Lower concentrations of iron (0.01 mmol; 0.005 g) had similar effects on the MeIQx formation (data not shown). The addition of copper(II) sulphate, copper(I) or copper(II) chloride did not significantly affect the formation of MeIQx (data not shown).

The enhancing effect of iron, both as Fe^{2+} and Fe^{3+}, on the formation of MeIQx is in agreement with an earlier study showing iron sulphate to double the amount of MeIQx formed in a similar model system (Johansson & Jägerstad, 1993). Iron sulphate has also been reported to nearly double the mutagenic activity in a creatine phosphate–tryptophan model system (Taylor *et al.*, 1986). Moreover, iron, both as Fe^{2+} and Fe^{3+}, has been reported to enhance the formation of mutagenic activity in fried beefburgers by a factor of 2 (Barnes & Weisburger, 1984). Barnes & Weisburger (1984) also demonstrated the importance of iron on the formation of HAs during the cooking of meat, by reducing the mutagenic activity by 60 and 54% through the addition of EDTA and denaturing the haem protein, followed by extraction of haemoglobin, respectively. Both iron and copper ions, as ferrous sulphate and copper sulphate, were shown to catalyse the formation of pyrazines and pyridines when heated with oxidized oil and amino acids (Parihar *et al.*, 1981). The enhancing effect of iron on the formation of HAs can be explained by the formation of free radicals in the Maillard reaction, resulting in an increase in the formation of pyridines and pyrazines, which act as precursors for the IQ compounds.

We could not find any effect of copper on the formation of MeIQx in the model system. One explanation may be that concentrations of copper other than those we used are needed to obtain a pro-oxidative effect in the model system. Another explanation may be that copper ions, for some reason, do not produce free radicals under the conditions used in our model system, or if produced, the free radicals do not increase the formation of pyridines and pyrazines.

Effect of antioxidants on the formation of HAs

As shown in Figs 3 and 4, most antioxidants increased the formation of MeIQx in the model system. The highest amount of MeIQx was formed with the addition of 100 ppm TBHQ, being 220% higher than in a

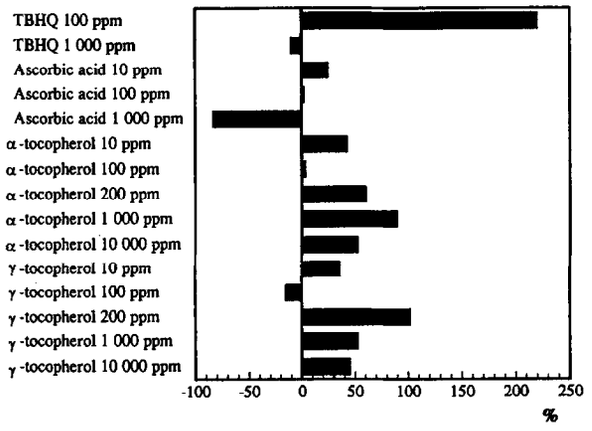

Fig. 3. The effect of different antioxidants on the formation of MeIQx in a model mixture containing creatinine, glycine, glucose and corn oil (1 g) heated at 180°C for 10 min. Amounts are expressed as percentage of MeIQx content in a control sample containing 11.2 nmol/mmol creatinine. Amounts of α- and γ-tocopherol are given in ppm of fat content.

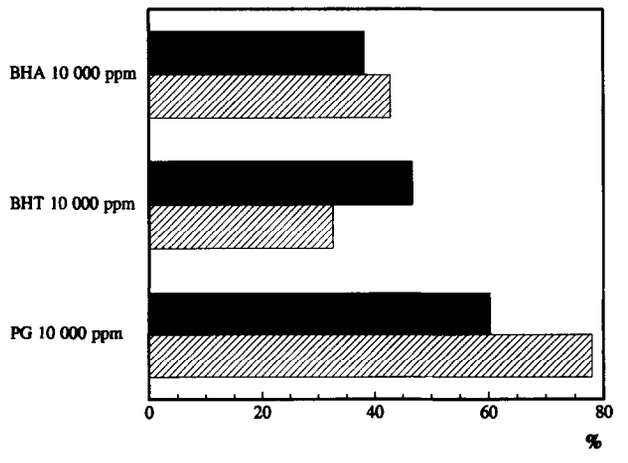

Fig. 4. The effect of synthetic antioxidants on the formation of MeIQx in a model mixture containing creatinine, glycine, glucose, iron sulphate (0.09 mmol) and corn oil (0.5 g) heated at 180°C for 10 and 30 min, respectively. Amounts are expressed as percentage of MeIQx content in a control sample. The control samples contained 17.6 and 29.5 nmol MeIQx/mmol creatinine, respectively.

control sample, containing 11.2±3.1 nmol MeIQx/mmol creatinine (Fig. 3). The addition of a higher amount of TBHQ (1000 ppm) had no effect. A high concentration of ascorbic acid (1000 ppm) reduced the MeIQx formation by 84%, while low concentrations (10 and 100 ppm) had no effect. The addition of α- or γ-tocopherol significantly increased the MeIQx formation at most concentrations. The synthetic antioxidants, BHA, BHT and PG, significantly increased the MeIQx formation in model mixtures containing iron sulphate (Fig. 4).

Antioxidants have previously been reported to decrease mutagenic activity. Wang *et al.* (1982) showed BHA to reduce mutagenic activity when added directly to beef extract, S9 and TA98. A reduction in mutagenic activity was also demonstrated by Chen (1988) and Chen *et al.* (1992) who reported BHA and PG to decrease the mutagenic activity of IQ, MeIQ and MeIQx, while the addition of BHT showed a concentration-dependent increase and decrease in the mutagenic activity of HAs. However, in these studies antioxidants were not shown to inhibit the formation of HAs but only their mutagenic activity *in vitro*. Barnes *et al.* (1983) showed BHA (50 mmol/100 g meat) to decrease the formation of IQ by 40% during the frying of beef. In contrast to the present study, Chen (1988) showed BHA, PG and TBHQ (100 ppm) to reduce the formation of HAs during the frying of beef. BHA was reported to reduce the amount of MeIQx formed by 56%, while PG and TBHQ gave 71 and 76% reductions, respectively. BHT was shown to increase the formation of DiMeIQx by 12%, while the amount of IQ and MeIQx decreased. However, the reported amounts of HAs in the fried beef are 100-fold higher than reported elsewhere making the results questionable. Chen (1988) also showed ascorbic acid and tocopherols to inhibit mutagen formation during the frying of beef. The reduction in HAs seen when adding reducing agents was explained by the scavenging of free radicals and oxygen, thus blocking the formation of IQ-like compounds.

Many antioxidants are known to exert both anti- and pro-oxidative effects depending on the concentration. Therefore, our results may simply be explained by the use of pro-oxidative amounts of the antioxidants in the model system. This should be kept in mind when adding antioxidants to meat products, as elevated amounts of HAs may be formed upon heating. Moreover, iron sulphate was present in the model mixtures together with BHA, BHT and PG, which might have influenced the results.

Synergistic effects of pro- and antioxidants on the formation of HAs

The addition of HQ in a creatinine–glycine–glucose model system did not increase the formation of MeIQx or IQx, while the amount of 7,8-DiMeIQx was increased by 150% (Fig. 5). However, as shown in Fig. 5, HQ (1000 ppm) combined with iron sulphate (0.09 mmol) gave rise to extreme increases in MeIQx, IQx and 7,8-DiMeIQx of 873, 703 and 1400%, respectively, compared with the amounts in a control sample containing neither HQ nor iron sulphate. The effect was less pronounced when fat was added. The addition of 1000 ppm HQ and iron sulphate (0.09 mmol) increased the amounts of MeIQx by 135 and 71%, in a mixture containing corn oil (0.5 g) after heating for 10 and 30 min, respectively,. The addition of a 10-fold higher amount of HQ increased the amount of MeIQx less (data not shown).

As shown in Fig. 6a–c, the addition of β-carotene or γ-tocopherol (100 ppm) to a mixture containing HQ and iron sulphate significantly decreased the formation of MeIQx, IQx and 7,8-DiMeIQx. The same results were obtained for all antioxidant concentrations tested (data not shown). The addition of β-carotene to control samples or mixtures containing HQ had no effect on MeIQx formation, while γ-tocopherol had a slightly inhibiting effect on MeIQx and IQx formation. β-carotene and γ-tocopherol had inhibiting effects on MeIQx and IQx formation in model mixtures containing iron sulphate. The content of 7,8-DiMeIQx was increased by both β-carotene and γ-tocopherol in a control sample and by β-carotene in an iron-sulphate-containing sample.

The most pronounced inhibiting effect of β-carotene and γ-tocopherol on the formation of MeIQx, IQx and 7,8-DiMeIQx was seen when added to mixtures containing a combination of the pro-oxidants iron sulphate and hydroquinone. This supports the free radical theory for the formation of HAs. HQ is known to be a radical when oxidized. Yoshida & Mizusaki (1985) heated amino acids with HQ at 250°C for 1 h, and some amino acids produced high mutagenic activity only in combination with HQ.

In a study recently performed at our laboratory, the amount of antioxidants, e.g. β-carotene and tocopherols, was shown to affect the formation of HAs during the frying of beefburgers using frying fats with different

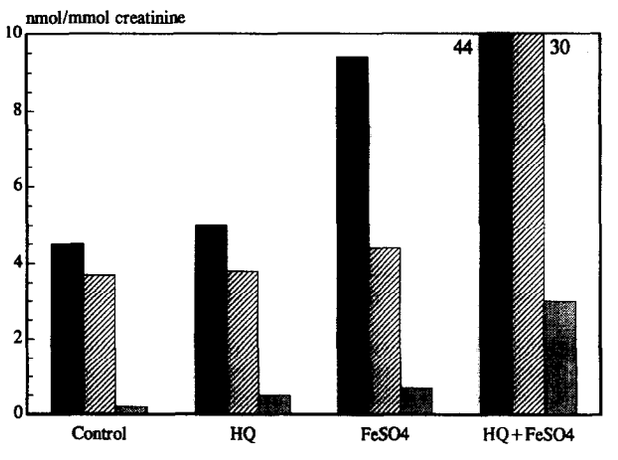

Fig. 5. The amounts (nmol/mmol creatinine) of MeIQx, IQx and 7,8-DiMeIQx formed in a model mixture containing creatinine, glycine, glucose and no pro-oxidant (control), HQ (1000 ppm), iron sulphate (0.9 mmol) or a combination of HQ and iron sulphate, heated at 180°C for 10 min.

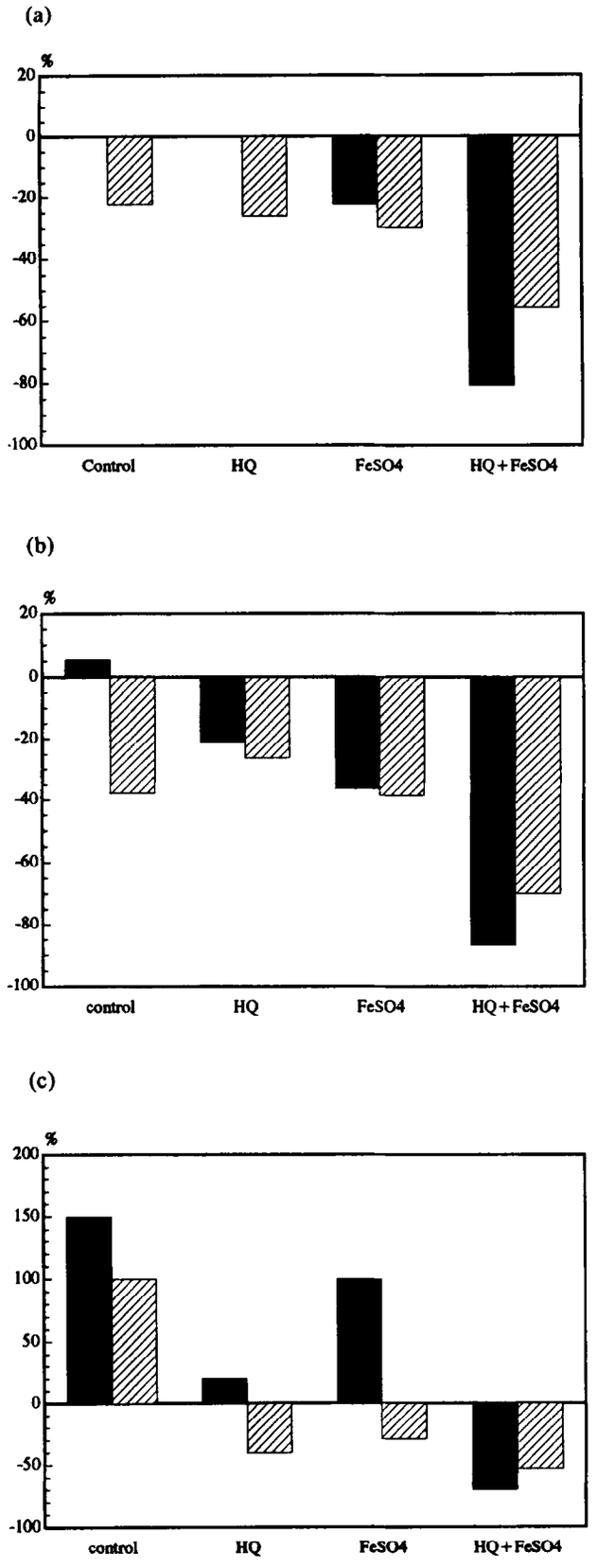

Fig. 6. The effect of β-carotene (100 ppm) and γ-tocopherol (100 ppm) on the formation of (a) MeIQx, (b) IQx and (c) 7,8-DiMeIQx in model mixtures containing creatinine, glycine, glucose and no pro-oxidant (control), HQ (1000 ppm), iron sulphate (0.09 mmol) or a combination of both HQ and iron sulphate, heated at 180°C for 10 min. Amounts are expressed as percentage of (a) MeIQx, (b) IQx and (c) 7,8-DiMeIQx content in a sample containing no antioxidant. The control samples contained 4.5, 3.7 and 0.2 nmol/mmol creatinine, respectively.

antioxidant levels (Johansson *et al.*, 1995*b*). Antioxidants were consumed during frying, and fats with an initial high antioxidant level gave rise to lower amounts of MeIQx and DiMeIQx in the beefburgers and the pan residues.

CONCLUSIONS

As shown in the present study, the formation of HAs can be affected by the addition of pro- and antioxidants, as well as by lipids, heating time and temperature, content of precursors, etc. The reaction mechanisms for the formation of HAs are complex and must be studied further in order to reduce the daily human exposure to these compounds.

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