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Chinese chive and Mongolian leek suppress heterocyclic amine formation and enhance nutritional profile of roasted cod†

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Heterocyclic amines (HAs) are potent mutagens, which can form DNA adducts in various human tissues. There is increasing evidence that mutagenic HA formation and nutrition loss can occur concurrently in fish during vigorous heat treatment. Our study investigated the effects of five *Allium* spp. (garlic, onion, welsh onion, Chinese chive, and Mongolian leek) on reducing HA formation and improving nutritional quality of roasted cod (*Gadus morhua*). The results showed that cod patties pretreated with powders of the selected *Allium* spp. had significantly ($P < 0.05$) lower levels of HAs (82–92%, except garlic, 49%) than the control. The contents of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in the patties exhibited strong negative correlations with total antioxidant activity (−0.937), phenolic (−0.948), and lipophilic flavonoid (−0.933) contents, whereas the 2-amino-3,8 dimethylimidazo [4,5-*f*] quinoxaline (MeIQx) (made up only ~0.7–3% of total HAs) contents exhibited significant positive correlations with these antioxidant parameters. In terms of nutrient composition change, Chinese chive and Mongolian leek were the most effective in preventing oxidative degradation of proteins and unsaturated fatty acids in roasted cod patties, which was translated into significantly higher contents of soluble proteins, essential amino acids, and polyunsaturated fatty acids. This has been the first report on the strong HA-formation inhibitory effect of Chinese chive and Mongolian leek. The dual beneficial functionality of these two *Allium* spp. may be utilized to reduce the intake of hazardous by-products while enhancing the nutritional and antioxidant properties of roasted cod and probably other protein-rich heat-processed foods.

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1. Introduction

Cod (*Gadus morhua*) is one of the most popular seafood dishes worldwide. It has dense, flaky and white flesh with low fat content and a balanced protein profile. Some of its proteins could be a source of bioactive peptides that possess functional properties such as antioxidation and inhibition of angiotensin converting enzyme.[1,2] Meanwhile, these nutritional and potentially health promoting attributes could be lost during the process of heat treatment, which causes oxidative degradation of proteins, fatty acids (especially polyunsaturated fatty acids, PUFAs) and other nutrients.[3–7] Concurrently, heat-induced reactions among food components can also give rise to harmful compounds.[8] Heterocyclic amines (HAs) are one of the most important categories of these hazardous by-products in muscle-based foods including meat and fish.[9] More than two dozen HAs have been identified in thermally processed foods. Some of them, such as 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 2-amino-3,8 dimethylimidazo [4,5-*f*] quinoxaline (MeIQx) and 2-amino-3,4,8-trimethylimidazo[4,5-*f*] quinoxalin (4,8-DiMeIQx), are classified as possible human carcinogens by the International Agency for Research on Cancer.[10] Many reports indicate a dose–dependent relationship between HA intake and DNA adduct formation that may increase the risk of various cancers, especially liver, colon and prostate cancers.[11,12] Considering their wide occurrence in major dietary components, HAs represent a significant health risk in the long term.[12] Hence, it is of high priority to inhibit HA formation in order to attenuate the health risk due to dietary exposure, while preserving or improving the nutritional quality of the associated food products. HA contents in foods can be reduced by lowering heating temperature, reducing concentrations of HA precursors and/or using alternative heating methods known to generate less HAs.[13,14] The use of plant-based extracts and phytochemicals as additives or seasoning

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ingredients have also gained increasing attention and ample evidences suggest it to be one of the most promising approaches to attenuate HA-associated health risk.[15–17]

Members of the *Allium* genus are common culinary ingredients in many parts of the world. Garlic (*Allium sativum*) and onion (*Allium cepa*) have been the most extensively studied *Allium* species both as seasoning ingredients and as modulators of undesirable heat-induced reactions in foods. For example, they were reported to effectively lower the content of HAs in pan-fried pork patties and in deep-fried meatballs.[18,19] Garlic powders were also reported to inhibit acrylamide formation in a low-moisture chemical model and in baked bread.[20] Organosulfur compounds have been proposed to be major bioactive compounds in *Allium* plants.[21] Some organosulfur compounds, such as γ-glutamyl-*S*-allyl-cysteine peptide isolated from garlic, showed antiglycative activity in a bovine serum albumin-glucose model.[22] Meanwhile, accumulating evidence indicates that polyphenols, in particular flavonoids are another important class of bioactive constituents in *Allium* spp.[23–25] Lu and coworkers recently reported a significant negative correlation (coefficient (r) = −0.712 to − 0.853) between antioxidant capacity of spices (including garlic, onion, red chilli, paprika, ginger and black pepper powder) and total HA contents, which indicated that antioxidants in these spices played a significant role in inhibiting the formation of HAs during heating.[19] However, no further data was provided regarding the type of phytochemicals which were likely the principal inhibitors.

The distinctive flavor and aroma and rich phytochemical contents have been the major impetus for the increasing research interest in *Allium* spp. both as functional food ingredients and as modulators of sensory attributes in foods. Nonetheless, this genus remains underexplored, especially with regard to their potential for simultaneous improvement of nutrition profiles and mitigation of the formation of heat-induced toxicants in foods. The hypothesis of the present study was that pretreatment with powders of certain *Allium* spp. could decrease the content of mutagenic HAs and enhance the nutritional and antioxidant properties of roasted cod. Herein, the effects of powders of five *Allium* spp., garlic, onion, welsh onion (*Allium fistulosum*), Chinese chive (*Allium tuberosum*), and Mongolian leek (*Allium mongolicum Regel*) on the formation of major HAs (PhIP, MeIQx, and DiMeIQx), and on the changes in the profiles of major nutrients (proteins/amino acids, and fatty acids) in roasted cod patties were evaluated. Antioxidant activity, phenolic and flavonoid contents (total, lipophilic and hydrophilic) were also evaluated to better understand their relationships with the modulating effects of the selected *Allium* spp. on HA formation and nutrient composition changes.

2. Materials and methods

2.1 Chemicals and reagents

HA standards, PhIP, MeIQx, and 4,8-DiMeIQx were purchased from Toronto Research Chemicals (Toronto, Canada). Oasis MCX cartridges (500 mg) were from Waters Inc. (Milford, MA, USA). Amino acid standards, fatty acid methyl ester standards, Bond-Elut reservoirs, diatomaceous earth, Folin–Ciocalteu reagent, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-tripyridyl-*s*-triazine (TPTZ), and quercetin were from Sigma-Aldrich (St Louis, MO, USA). Mass spectrometry grade methanol, acetonitrile, ammonium acetate, and formic acid were purchased from Merck KGaA (Darmstadt, Germany). Amino acid test kit (MSLAB-45+AA) was from Beijing Mass Spectrometry Medical Research Co., Ltd. (Beijing, China). Aluminum chloride ($AlCl_3$), sodium carbonate and other general reagents were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2 Preparation of *Allium* powders

Five fresh *Allium* spp. (garlic, red onion, green welsh onion, Chinese chive, and Mongolian leek) were purchased in four batches (one in each season) from a local supermarket (Beijing, China) to minimize seasonal variation of the samples. After cleaning and drying (~4 h) to constant weight in an oven set at 95 °C, they were pulverized in a grinder (M 20, IKA, Germany). Powders of the four batches of each *Allium* spp. were mixed thoroughly and stored in a desiccator.

2.3 Roasted cod sample preparation

Cod fillets were obtained from a local supermarket (Beijing, China). Fillet samples (~120 g each; dimensions: 7.5 ± 0.5, 10.5 ± 1.0, and 2.0 ± 0.2 cm) were boned. For every batch of experiments, 100 g of minced cod was weighed for each treatment, which was mixed with 2 g (2%, w/w) of garlic, onion, welsh onion, Chinese chive, or Mongolian leek powders, respectively. The control group was not given any pretreatment. After marinating for an hour at 4 °C, the fillets were homogenized in a blender separately and four accurately weighed (30 ± 0.2 g) samples from each treatment were formed into patties with the aid of a Petri dish (diameter 6 cm, height 1.5 cm). The patties were roasted in a BAKLON-58M oven (Germany) at 225 °C for 10 min on each side. After roasting, each replicate was divided into two parts. One part was directly used for the analysis of HAs, and the other part was freeze-dried and stored at −80 °C until used for analysis of other endpoints.

2.4 UPLC-MS analysis of HAs in roasted cod patties

HA content of roasted cod patties was evaluated according to the literature with slight modifications.[26] Briefly, the patties from each treatment were homogenized in 150 mL of 1 M NaOH for 2 min to a dense paste. For each sample, 17.5 ± 0.1 g of the paste was mixed with 22.5 mL of 1 M NaOH in a beaker, and the mixture was sonicated for 20 min. The suspension was then mixed with diatomaceous earth and packed into empty reservoirs fitted with a bottom frit (20 μm). Eluate from the reservoir (200 mL ethyl acetate for each sample) was passed through an Oasis MCX cartridge, which was pre-conditioned sequentially with 6 mL of methanol, 6 mL of distilled water, and 6 mL of ethyl acetate. The cartridges were then dried under maximum vacuum for 5 min and washed with 6 mL of 0.1 N HCl, followed by 6 mL of methanol. The retained analytes were eluted with 6 mL of methanol–ammonia solution (19 : 1, v/v). The eluate was evaporated to dryness under nitrogen, and the residue was

re-dissolved in 300 μL of methanol for UPLC-MS analysis. Triplicate analyses were performed for each treatment.

Identification and quantification of HAs were performed on a UPLC equipped with a triple quadrupole mass spectrometer (Waters, Milford, MA, USA) and an electrospray ionization interface. Separation of the analytes was carried out on an ACQUITY UPLC BEH C18 reversed-phase column (1.7 μm particle size, 2.1 mm × 50 mm, Waters Inc.; USA) at 35 °C. Gradient elution with a binary mobile phase (solvent A: 0.1% formic acid and 5 mM ammonium acetate in water; solvent B: acetonitrile) was used: 0–0.1 min, 95% A, 5% B; 0.1–10 min, 70% A, 30% B; 10–14 min, 40% A, 60% B; 14–16 min, 20% A, 80% B; 16–18 min, 95% A, 5% B; 18–23 min, 95%A, 5% B. The flow rate was set as 0.3 mL min^{-1}, and the injection volume was 1 μL. Mass spectrometric detection was conducted in the multiple reaction monitoring (MRM) scan mode. The operating conditions were: capillary voltage 3.5 kV, ion source temperature 120 °C, and desolvation temperature 350 °C. Quantitative determination was performed using an external calibration curve. Correlation coefficients (r^2) for HA standard curves were 0.9995 for PhIP, 0.9996 for MeIQx, and 0.9999 for 4,8-DiMeIQx.

2.5 Preparation of hydrophilic and lipophilic extracts from roasted cod patties

Hydrophilic and lipophilic extracts of the samples were prepared for the determination of phenolic and flavonoid contents, and total antioxidant activity according to the method of Manful *et al.* with minor modifications.[5] Approximately 3 g of roasted cod samples were homogenized in 9 mL of sodium phosphate buffer (50 mM, pH 7.5). The homogenate was centrifuged (6000 rpm, 15 min) to obtain the supernatant as the hydrophilic extract. The residue was suspended in 9 mL of acetone/ethanol (50 : 50% v/v), vortexed, and incubated in darkness at room temperature for 30 min. The mixture was centrifuged to collect the supernatant as the lipophilic extract.

2.6 Analysis of antioxidant activity

Antioxidant activity was analyzed spectrophotometrically using the ferric reducing antioxidant power (FRAP) assay.[27] The FRAP reagent was freshly prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM ferric chloride hexahydrate in a ratio of 10 : 1 : 1 (v/v/v), and incubated at 37 °C for 60 min. Briefly, 100 μL of each standard or sample was reacted with 2.9 mL of the FRAP reagent in the dark for 30 min. Then absorbance of the samples was measured at 593 nm in a spectrophotometer (Tecan, Switzerland). Total antioxidant activity (TAA) values of the samples were the sum of their respective hydrophilic (HAA) and lipophilic (LAA) antioxidant activity values. Trolox (0–100 μM) was used as reference standard and antioxidant activities were expressed as μmol Trolox/g of cod.

2.7 Analysis of total phenolic contents

Total phenolic content (TPC) was determined by the Folin–Ciocalteau assay as described by Aryal *et al.* with minor modifications.[28] Diluted hydrophilic and lipophilic extract samples were each added with 1 mL of Folin–Ciocalteu reagent and 3 mL of 7.5% sodium carbonate solution. The resulting mixtures were incubated in darkness at room temperature for 2 h and absorbance was measured at 765 nm in a spectrophotometer (Tecan, Switzerland). TPC values were the sum of the respective hydrophilic (HPC) and lipophilic (LPC) values. Gallic acid (0–1 mg L^{-1}) was used to construct the standard calibration curve and phenolic contents were expressed as ng gallic acid/g cod.

2.8 Analysis of total flavonoid contents

Total flavonoid content (TFC) was measured by the method of Sharma *et al.*[27] One mL of extract or quercetin standard (0–1 mg mL^{-1}) solution was mixed with 1 mL of $AlCl_3$ (0.1 M), 1 mL of potassium phosphate monobasic (0.1 M, pH 5.5), and 7 mL of ultrapure water. The mixture was incubated for 30 min at room temperature followed by measurement of the absorbance at 415 nm against the blank in a spectrophotometer (Tecan, Switzerland). The data obtained were expressed as mg quercetin/g cod.

2.9 Total amino acid content analysis

Amino acid compositions of roasted cod patties were analyzed by LC-MS/MS (Shimadzu LC20AD, Kyoto, Japan and API 3200MD TRAP, Framingham, MA, USA). Approximately 0.1 g freeze-dried sample was hydrolyzed in 2 mL of 6 M hydrochloric acid solution at 110 °C for 24 h. The tube was pre-filled with nitrogen to prevent oxidation of amino acids. An aliquot (50 μL) of each sample was treated with sodium hydroxide solution to precipitate proteins. After centrifugation at 13 200 rpm at 4 °C for 4 min, 8 μL of the supernatant were incubated with 42 μL of labeling buffer and 20 μL of derivatization reagent at 50 °C for 15 min. The derivatized samples were chilled, centrifuged, and the supernatants analyzed by LC-MS/MS according to the method of Xu *et al.*[29]

Estimated protein efficiency ratio (ePER), an indicator of protein quality, was calculated using the following equation:

$$\text{ePER} = 0.468 + 0.454 \times \text{Leu} - 0.105 \times \text{Tyr} \quad (1)$$

where Leu is leucine content (μg mg^{-1} dw^{-1}) and Tyr is tyrosine content (μg mg^{-1} dw^{-1}).

Fischer ratio (FR) was also calculated to evaluate protein quality with the following equation:[30]

$$\text{FR} = (\text{Val} + \text{Leu} + \text{Ile})/(\text{Phe} + \text{Tyr}) \quad (2)$$

where Val, Leu, Ile, Phe, and Tyr represent the contents of valine, leucine, isoleucine, phenylalanine, and tyrosine, respectively.

2.10 Analysis of soluble proteins in roasted cod patties

Approximately 1 g of freeze-dried sample was homogenized in 10 mL of sodium phosphate buffer (50 mM, pH 7.5). The homogenate was mixed on a shaker at 4 °C for 4 h and then centrifuged (10 000 rpm, 10 min) to obtain the supernatant. Soluble protein content of the supernatant was determined

using the Pierce BCA Protein Assay Kit (Thermo Fisher, USA). Briefly, 20 μL of each albumin standards or supernatant was added to a microplate well containing 200 μL of the BCA reagent mixture. The mixtures were incubated in darkness at room temperature for 1 h and the absorbance was measured at 595 nm in a spectrophotometer (Tecan, Switzerland). Soluble protein contents were expressed as mg protein/g cod.

2.11 Total fatty acid analysis

Lipids were extracted from the freeze-dried samples according to the literature with minor modifications.[31] Approximately 0.1 g of sample was extracted with 2 mL of hexane at 50 °C on a shaker for 30 min, followed by derivatization with 3 mL of 0.4 M methanolic potassium hydroxide solution for 20 min. Fatty acid methyl esters (FAMEs) were extracted with 3 mL of hexane-water (2 : 1, v/v) and quantified with reference to calibration curves of FAMEs in a gas chromatography mass spectrometer system (7890B-5977A, Agilent Technologies Inc.; USA) equipped with a DB-23 column (0.25 μm film, 0.32 mm i.d. × 30 m length). GC-MS conditions were as follows: injection volume 1 μL; split injection; split ratio 5 : 1; carrier gas (helium) 3.0 mL min^{-1}; injection port temperature 250 °C; detector temperature 230 °C; oven temperature program: 50 °C held for 1 min, then increased at a rate of 25 °C min^{-1} to 175 °C and held for 1 min, followed by an increase to 230 °C at a rate of 4 °C min^{-1}.

After determining individual fatty acids, the contents of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were calculated.

2.12 Statistical analysis

Statistical analysis was performed using the SPSS statistical package (SPSS 20). Significant differences in treatment means were identified at a level of $P < 0.05$ by one-way analysis of variance (ANOVA) and the Duncan test. Pearson's correlations were used for factor analyses. Principal component analysis (PCA) was performed by using OriginPro 2019b.

3. Results and discussion

3.1 Effect of *Allium* powders on the formation of HAs in roasted cod patties

Heating of meat and fish can give rise to highly hazardous by-products, particularly HAs, through the Maillard reaction. Five *Allium* spp. in form of powders were used for pretreatment of cod samples respectively prior to heating, and the contents of three major HAs (PhIP, MeIQx and 4,8-DiMeIQx) which have been reported to account for most of the food-borne HA-associated mutagenic/carcinogenic activity were determined by LC-MS (ESI Fig. S1† and 1). In the control patties, the contents of PhIP, MeIQx and 4,8-DiMeIQx were 10.35 ± 0.95, 0.32 ± 0.07, and 0.07 ± 0.02 ng g^{-1}, respectively. Consistent with previous reports, PhIP was the most abundant and its content was much higher than that of MeIQx and 4,8-

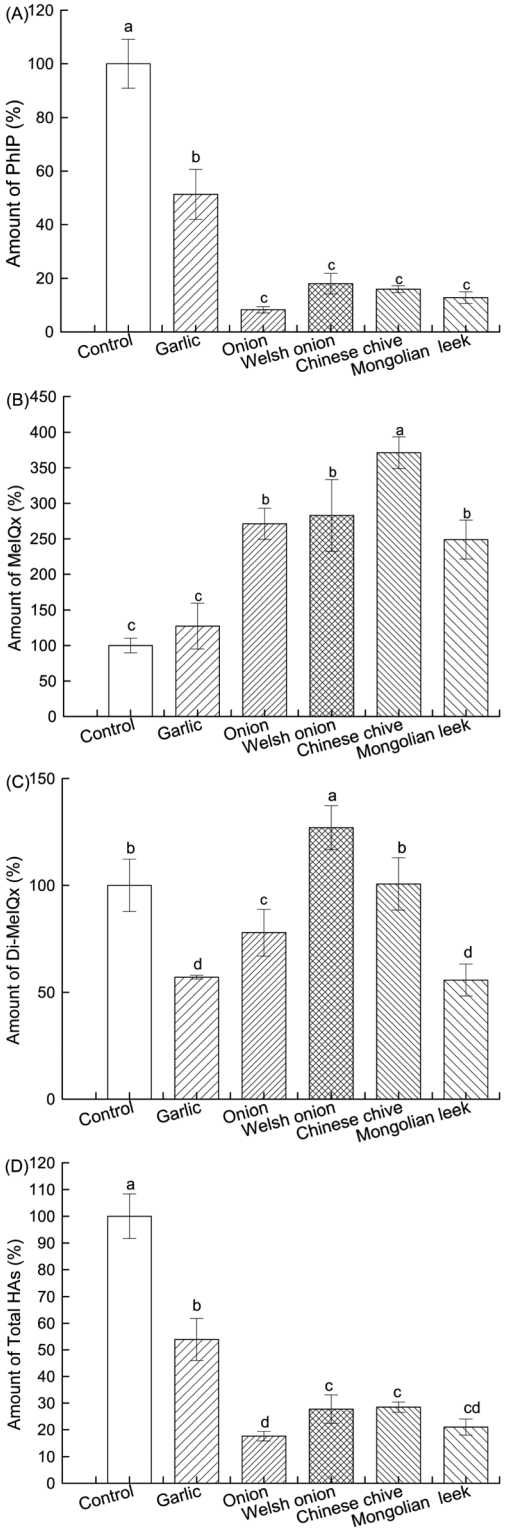

Fig. 1 Effect of *Allium* powders on the formation of PhIP (A), MeIQx (B), DiMeIQx (C), and total HAs (D) in cod patties roasted at 225 °C for 10 min on each side ($n = 3$). Values are expressed as mean ± standard deviation (SD) ($n = 3$). Bars denoted with different letters are significantly different ($P < 0.05$). PhIP: 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine, MeIQx: 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline, DiMeIQx: 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxalin.

DiMeIQx.[32] As shown in Fig. 1A, all *Allium* plants significantly inhibited the formation of PhIP in cod patties, which was in agreement with previous reports on effective inhibition of PhIP formation in fried beef patties by onion and garlic.[33,34] Except garlic, which reduced PhIP content by 49% relative to control, the inhibition rates of the other four ranged from 82% to 92%. In contrast, these four *Allium* plants resulted in higher MeIQx contents compared with control, while garlic did not significantly affect it (Fig. 1B). The totally different effects of onion on PhIP and on MeIQx formation was also reported in a previous study in fried chicken.[35] Another study using fried beef observed a dose–dependent enhancement in MeIQx formation by onion, and the authors attributed it to the introduction of sugar from onion into the meat during the marination and/or heating process.[36] For 4,8-DiMeIQx, similarly, no consistent inhibitory effect was observed (Fig. 1C); Mongolian leek, garlic and onion exhibited moderate inhibition ($P < 0.05$), while the others were either ineffective or mildly promoted its formation.

Based on the following two considerations: (1) the levels of MeIQx and 4,8-DiMeIQx only accounted for ~0.7–3% of total HAs in the roasted cod patties, and (2) the relative degrees of reduction in PhIP and in total HA contents by pretreatment with different *Allium* plants (Fig. 1A and D), Mongolian leek and onion represent the most promising candidates, followed by Chinese chive and welsh onion. This has been the first report on the potent HA-formation inhibitory effect of Chinese chive and Mongolian leek. In particular, 2% level of Mongolian leek-addition was able to reduce the content of total HAs by 80% relative to control.

3.2 Effect of *Allium* powders on antioxidant capacity, polyphenol and flavonoid contents, and their correlations with HA contents in roasted cod patties

Allium spp. are well-known phytochemical-rich culinary ingredients in many cuisines worldwide. Our data thus far showed that four out of the five selected *Allium* spp. could strongly reduce the contents of HAs in roasted cod patties by >70% relative to control. Considering ample evidence suggesting that antioxidant activity is a critical functional property of *Allium* spp.,[21,37–39] we next analyzed the antioxidant capacities and phenolic contents of the roasted cod samples pretreated with powders of the different *Allium* spp. As expected, *Allium*-pretreated roasted cod patties all had significantly ($P < 0.05$) higher TAA, LAA and HAA than the control (Fig. 2A). Consistent with the trend in their relative antioxidant activities, phenolic and flavonoid contents of the *Allium*-pretreated cod patties were all significantly ($P < 0.05$) higher than the control patties (Fig. 2B and C). *Allium* spp. are known to be good sources of phenolic and flavonoid compounds, such as quercetin, kaempferol, chlorogenic acid, gallic acid and others.[21,25,38,39] Consistent with literature data, the phenolic and flavonoid contents of *Allium*-pretreated patties were significantly higher than the control patties.[5] These data suggest that pretreatment of fish or meat with powders of certain *Allium* spp. prior to heating could indirectly improve our dietary intake of antioxidants.

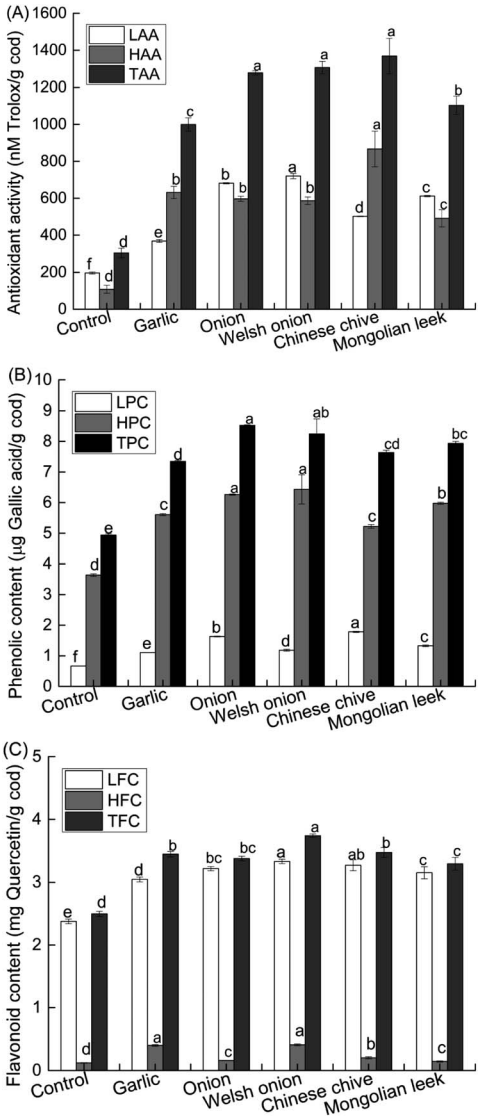

Fig. 2 Effect of *Allium* powders on the antioxidant activities (A), phenolic (B) and flavonoid (C) contents of roasted cod patties with or without pretreatment with *Allium* powders. Values are expressed as mean ± standard deviation (SD) ($n = 3$). Bars denoted with different letters are significantly different ($P < 0.05$). TAA = HAA + LAA, TPC = HPC + LPC, TFC = HFC + LFC. TAA: total antioxidant activity, HAA: hydrophilic antioxidant activity, LAA: lipophilic antioxidant activity; TPC: total phenolic content, HPC: hydrophilic phenolic content, LPC: lipophilic phenolic content; TFC: total flavonoid content, HFC: hydrophilic flavonoid content, LFC: lipophilic flavonoid content.

Next, we sought to understand the correlations between the antioxidant activity and phenolic and flavonoid contents of the roasted cod samples, as well as their correlations with the HA-formation inhibitory effects of the *Allium* spp. The phenolic and flavonoid contents (except for HFC) all had significant ($P < 0.001$) positive correlations with TAA of the roasted cod patties (Table 1), suggesting that *Allium* lipophilic flavonoids were key components of the antioxidant capacity of the roasted cod patties. In addition, it was found that the antioxidant activity, phenolic and flavonoid content parameters (except HFC) all had

Table 1 Pearson correlation analyses of antioxidant activities, phenolic and flavonoid contents in roasted cod patties with or without pretreatment with *Allium* powders[a]

Variable	TAA	LAA	HAA
TPC	0.929***	0.917***	0.733**
LPC	0.860***	0.641**	0.844***
HPC	0.841***	0.909***	0.599**
TFC	0.910***	0.779***	0.812***
LFC	0.965***	0.860***	0.834***
HFC	0.346	0.206	0.381
TAA	—	—	—
LAA	—	—	—
HAA	—	—	—

[a] TAA = HAA + LAA, TPC = HPC + LPC, TFC = HFC + LFC. TAA: total antioxidant activity, HAA: hydrophilic antioxidant activity, LAA: lipophilic antioxidant activity; TPC: total phenolic content, HPC: hydrophilic phenolic content, LPC: lipophilic phenolic content; TFC: total flavonoid content, HFC: hydrophilic flavonoid content, LFC: lipophilic flavonoid content. Significant at *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

strong negative correlations with the contents of PhIP in the roasted cod samples (Table 2). On the other hand, these parameters were significantly and positively correlated with MeIQx contents in the roasted cod patties. No significant correlation was found with 4,8-DiMeIQx. These data indicate an inhibitory and a promoting effect of *Allium* antioxidants on PhIP and MeIQx formation in roasted cod patties, respectively.

Thus far, no unified conclusion is available in the literature regarding the effect of antioxidants on PhIP and MeIQx/DiMeIQx formation. For instance, β-carotene and γ-tocopherol were reported to counteract the enhancing effect of some prooxidants such as iron compounds and hydroquinone on MeIQx/DiMeIQx formation in chemical models.[40] In another recent study, capsaicin from chilli pepper was found to be capable of inhibiting the formation of both PhIP and MeIQx-type HAs, whereas other chili pepper antioxidants promoted their formation in roasted beef patties.[41] Our previous study and other groups also found a lack of significant positive correlation between antioxidant activity and HA-formation inhibitory effect.[16,42] In particular, dietary polyphenols naringenin, quercetin-3-*O*-glucoside and quercetin significantly inhibited PhIP formation, hesperidin and rutin had no significant effect, while carnosic acid and rosmarinic acid enhanced it. These inconsistent findings could be attributed to the following factors: (1) heat-induced degradation fragments of some polyphenols may take part in the formation pathways of HAs;[42,43] (2) some polyphenols may function as prooxidants under the experimental conditions;[40] and (3) mechanism other than antioxidant/radical scavenging plays a dominant role in the effect of some polyphenols on HA formation.[44–46] Polyphenols are important dietary antioxidants that have been amply demonstrated to help attenuate the risk of many chronic diseases.[47] Apparently, the incorporation of plant extracts or purified polyphenols to our daily cuisine could be a convenient and economic way of obtaining the purported health benefits. However, literature data together with the data from the present study highlight a need to use these phytochemical-rich natural agents in heat-processed muscle-based foods with caution since they could potentially increase the contents of some hazardous chemicals including HAs.

Table 2 Pearson correlation analyses among antioxidant activities, phenolic contents, flavonoid contents and HA contents of roasted cod patties with or without pretreatment with *Allium* powders[a]

Variable	PhIP	MeIQx	Di-MeIQx	Total HAs
TAA	−0.937***	0.818***	0.054	−0.927***
LAA	−0.910***	0.695**	0.128	−0.910***
HAA	−0.750***	0.735**	−0.017	−0.733**
TPC	−0.948***	0.642**	−0.111	−0.959***
LPC	−0.850***	0.847***	−0.078	−0.830***
HPC	−0.867***	0.484*	−0.11	−0.889***
TFC	−0.816***	0.605**	0.079	−0.819***
LFC	−0.933***	0.737***	0.021	−0.932***
HFC	−0.128	−0.026	0.200	−0.140

[a] TAA = HAA + LAA, TPC = HPC + LPC, TFC = HFC + LFC. TAA: total antioxidant activity, HAA: hydrophilic antioxidant activity, LAA: lipophilic antioxidant activity; TPC: total phenolic content, HPC: hydrophilic phenolic content, LPC: lipophilic phenolic content; TFC: total flavonoid content, HPC: hydrophilic flavonoid content, LFC: lipophilic flavonoid content. Significant at *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

3.3 Effect of *Allium* powders on total amino acid and soluble protein profiles of roasted cod patties

During roasting, proteins/amino acids may be degraded or consumed in different chemical reactions, including the formation of HAs.[5,9,48] Having established that phenolic antioxidants from *Allium* powders could effectively reduce the contents of HAs in roasted cod patties, we then proceeded to examine if this beneficial effect could be accompanied by a protective effect on deterioration of protein/amino acid quality. Table 3 shows the amino acid content, FR, ePER and soluble protein content of roasted cod patties pretreated respectively with powder of the five *Allium* spp. versus control. The content of essential amino acids (EAAs) and non-essential amino acids (NEAAs) ranged from 142.67 to 217.34 μg mg^{-1} and 203.54 to 260.53 μg mg^{-1} dry weight, respectively. The ratios of EAA to AA and EAA to NEAA in dietary proteins have been used to evaluate protein quality because of their important effects on protein utilization.[49] Roasted cod patties pretreated with Chinese chive powder exhibited the highest EAA content and EAA to AA and EAA to NEAA ratios. Notably, the content of lysine in the Chinese chive-pretreated samples was 2-3-fold higher than the other samples, suggesting a strong protective effect against degradation of this EAA. Proteins with higher FR values are likely to have better health benefits owing to their potential antioxidant property, especially free radical scavenging and *anti*-peroxidation activity of the associated peptides.[50] Mongolian leek-pretreated cod patties had significantly higher FR and ePER values than the control and other *Allium*-pretreated cod

Table 3 Amino acid and soluble protein contents (μg mg^{-1} dw^{-1}) of roasted cod patties with or without pretreatment with *Allium* powders ($n = 3$)[a]

	Control	Garlic	Onion	Welsh onion	Chinese chive	Mongolian leek
Essential amino acids						
Leucine	30.00 ± 0.68^{BC}	31.16 ± 0.56^{B}	28.97 ± 0.38^{BC}	28.85 ± 0.74^{C}	30.83 ± 0.97^{BC}	34.95 ± 2.43^{A}
Lysine	41.25 ± 1.829^{C}	49.71 ± 0.68^{B}	31.59 ± 1.82^{D}	33.00 ± 1.14^{D}	96.29 ± 9.35^{A}	52.86 ± 0.71^{B}
Isoleucine	18.05 ± 4.54^{B}	18.17 ± 0.58^{B}	16.02 ± 0.97^{D}	17.58 ± 0.97^{BC}	16.42 ± 0.85^{CD}	22.51 ± 0.45^{A}
Valine	19.43 ± 1.52^{B}	16.70 ± 0.44^{C}	18.17 ± 1.33^{BC}	18.17 ± 0.91^{BC}	17.87 ± 1.29^{BC}	24.77 ± 0.51^{A}
Threonine	18.64 ± 0.69^{C}	20.16 ± 0.17^{B}	17.92 ± 0.62^{C}	20.10 ± 0.59^{B}	20.07 ± 0.57^{B}	24.30 ± 1.04^{A}
Phenylalanine	18.86 ± 0.94^{B}	16.98 ± 0.88^{C}	16.61 ± 0.86^{C}	16.68 ± 0.17^{C}	19.20 ± 0.33^{B}	21.84 ± 0.32^{A}
Methionine	14.44 ± 1.41^{BC}	14.73 ± 0.64^{B}	13.12 ± 0.64^{CD}	12.39 ± 0.21^{D}	16.67 ± 0.57^{A}	15.32 ± 0.65^{AB}
Tryptophan	ND	ND	ND	ND	ND	ND
Total essential amino acids (EAA)	160.67 ± 3.19^{C}	167.62 ± 2.88^{C}	142.67 ± 3.99^{D}	146.78 ± 2.88^{D}	217.34 ± 11.81^{A}	196.55 ± 3.24^{B}
Non-essential amino acids						
Tyrosine	10.18 ± 0.61^{D}	10.41 ± 0.60^{CD}	13.17 ± 0.80^{A}	11.80 ± 0.55^{B}	11.58 ± 0.83^{BC}	9.14 ± 0.86^{D}
Cysteine	4.62 ± 0.07^{A}	4.62 ± 0.16^{A}	3.07 ± 0.21^{D}	3.56 ± 0.01^{C}	2.96 ± 0.04^{D}	3.95 ± 0.01^{B}
Histidine	8.92 ± 0.02^{BC}	9.60 ± 0.85^{B}	7.63 ± 0.26^{D}	8.30 ± 0.20^{CD}	8.47 ± 0.10^{C}	10.75 ± 0.17^{A}
Glutamic acid	57.92 ± 1.51^{CD}	60.79 ± 0.75^{B}	56.79 ± 0.18^{D}	62.15 ± 0.32^{B}	58.87 ± 1.57^{C}	79.45 ± 0.21^{A}
Aspartic acid	30.74 ± 1.02^{CD}	31.74 ± 0.64^{BC}	28.31 ± 0.66^{E}	33.28 ± 0.69^{B}	28.96 ± 1.65^{DE}	39.40 ± 2.01^{A}
Arginine	33.12 ± 0.45^{A}	14.57 ± 0.33^{E}	25.77 ± 0.43^{B}	19.51 ± 1.06^{D}	22.93 ± 0.85^{C}	24.95 ± 0.64^{B}
Alanine	24.06 ± 0.51^{CD}	26.89 ± 0.58^{B}	22.80 ± 1.16^{CD}	25.11 ± 1.40^{C}	24.19 ± 1.10^{D}	31.22 ± 0.65^{A}
Glycine	22.56 ± 1.21^{A}	18.35 ± 0.33^{C}	14.91 ± 0.87^{D}	18.84 ± 0.65^{C}	16.03 ± 0.14^{D}	20.89 ± 1.48^{B}
Serine	19.68 ± 1.04^{BC}	21.34 ± 0.49^{B}	18.46 ± 1.76^{C}	20.38 ± 0.86^{B}	21.22 ± 0.44^{B}	24.04 ± 0.85^{A}
Proline	13.48 ± 0.28^{BC}	14.59 ± 1.06^{B}	12.63 ± 0.85^{C}	13.43 ± 0.57^{BC}	14.17 ± 0.43^{BC}	16.75 ± 1.23^{A}
Total non-essential amino acids (NEAA)	225.41 ± 3.24^{B}	212.90 ± 4.14^{C}	203.54 ± 5.01^{D}	216.34 ± 3.73^{C}	209.38 ± 3.00^{CD}	260.53 ± 4.85^{A}
Total amino acids (AA)	386.08 ± 2.78^{C}	380.51 ± 3.78^{C}	346.21 ± 8.91^{E}	363.12 ± 5.99^{D}	426.72 ± 10.34^{B}	457.08 ± 7.89^{A}
EAA/AA ratio	0.42 ± 0.01^{CD}	0.44 ± 0.008^{B}	0.41 ± 0.001^{D}	0.40 ± 0.0009^{D}	0.51 ± 0.02^{A}	0.43 ± 0.001^{BC}
EAA/NEAA ratio	0.71 ± 0.02^{CD}	0.79 ± 0.02^{B}	0.70 ± 0.01^{CD}	0.68 ± 0.002^{D}	1.04 ± 0.07^{A}	0.75 ± 0.01^{BC}
FR	2.33 ± 0.14^{B}	2.41 ± 0.02^{B}	2.12 ± 0.06^{C}	2.27 ± 0.05^{BC}	2.12 ± 0.09^{C}	2.66 ± 0.16^{A}
eFER	13.02 ± 0.24^{BC}	13.52 ± 0.30^{B}	12.24 ± 0.14^{C}	12.33 ± 0.35^{C}	13.25 ± 0.40^{BC}	15.37 ± 1.15^{A}
Soluble protein	166.05 ± 3.25^{D}	260.63 ± 2.60^{B}	258.17 ± 5.12^{B}	233.96 ± 2.90^{C}	263.01 ± 1.36^{B}	292.53 ± 3.63^{A}

[a] Values are expressed as mean ± standard deviation (SD) ($n = 3$). $^{A-E}$ Values in the same row with different letters are significantly different ($P < 0.05$). ND: not detected. FR: Fischer ratio, ePER: estimated protein efficiency ratio.

samples likely due to its high intrinsic content of branched chain AAs.[51] The soluble protein contents of the *Allium*-pretreated cod samples were 40–75% higher than control. These data together provide strong evidence that pretreatment with powder of *Allium* spp. could effectively preserve protein/amino acid quality in roasted cod likely by interrupting their participation in heat-induced reactions *via* the action of the *Allium* phenolic antioxidants.

3.4 Effect of *Allium* powders on fatty acid profiles of roasted cod patties

The profiles of fatty acids (FAs) in roasted cod patties were evaluated by GC-MS (Table 4). Significant differences ($P < 0.05$) in SFA, MUFA and PUFA contents were observed among samples pretreated with *Allium* powders and control. In agreement with the literature, unsaturated FAs (MUFAs and PUFAs) represented 65–73% of the total FA contents with oleicacid (C18:1n9c) and docosahexaenoic acid (DHA C22:6n3) being the dominant MUFA and PUFA, respectively. Although PUFAs are highly valued health promoting nutrients in cod,[52] they are susceptible to oxidative damage during thermal processes. Some of their peroxidation products, especially radicals may promote the formation of IQ- and MeIQx-type HAs.[9,53] A corollary to this proposition is that the antioxidants introduced from the *Allium* powder into the cod patties could function to prevent these heat-induced lipid peroxidation reactions. Surprisingly, PUFA contents in garlic- and onion-pretreated samples were significantly lower than that in control patties. This might be due to the prooxidative effect of some of the constituents in garlic and onion under the experimental conditions,[54,55] which caused oxidative degradation of PUFAs in the patties. The significantly higher contents of PUFAs in Mongolian leek, Chinese chive and Welsh onion pretreated samples were likely due to their much higher (~10-fold) contents of α-linolenic acid (C18:3n3),[56] an intrinsic property of these three *Allium* spp. that may be conveniently harnessed to increase dietary intake of PUFAs. These data tend to suggest that the phenolic antioxidants in the *Allium* spp. did not effectively prevent lipid peroxidation in the roasted cod patties at this condition, which could partly explain the lack of inhibition of MeIQx and 4,8-DiMeIQx formation. Meanwhile, the significantly higher contents of soluble proteins and amino acids in the *Allium* pretreated roasted cod patties (Table 3) together with their significant

Table 4 Fatty acid profiles (μg g^{-1} dw^{-1}) of roasted cod patties with or without pretreatment with *Allium* powders ($n = 3$)[a]

	Control	Garlic	Onion	Welsh onion	Chinese chive	Mongolian leek
C8:0	0.09 ± 0.03^{B}	0.11 ± 0.01^{B}	0.12 ± 0.02^{B}	0.16 ± 0.03^{A}	0.18 ± 0.01^{A}	0.17 ± 0.02^{A}
C10:0	0.17 ± 0.05^{BC}	0.14 ± 0.02^{C}	0.12 ± 0.01^{C}	0.20 ± 0.03^{AB}	0.23 ± 0.01^{A}	0.21 ± 0.02^{AB}
C12:0	1.05 ± 0.03^{CD}	1.20 ± 0.02^{BC}	0.88 ± 0.06^{D}	1.58 ± 0.24^{A}	1.44 ± 0.86^{AB}	1.55 ± 0.23^{A}
C14:0	55.92 ± 0.84^{BC}	53.22 ± 1.98^{D}	54.33 ± 0.80^{CD}	60.97 ± 1.85^{A}	58.01 ± 0.16^{B}	60.49 ± 0.21^{A}
C15:0	8.31 ± 0.01^{B}	8.08 ± 0.47^{B}	8.56 ± 0.19^{B}	9.91 ± 0.37^{A}	9.51 ± 0.45^{A}	9.66 ± 0.19^{A}
C16:0	493.96 ± 12.91^{B}	532.68 ± 38.95^{B}	505.29 ± 15.41^{B}	611.77 ± 17.90^{A}	646.34 ± 53.22^{A}	613.21 ± 15.65^{A}
C17:0	40.04 ± 0.65^{A}	38.39 ± 0.46^{B}	29.62 ± 0.25^{E}	31.71 ± 1.46^{D}	38.52 ± 0.31^{B}	37.04 ± 0.20^{C}
C18:0	581.84 ± 11.88^{A}	557.60 ± 7.60^{B}	497.62 ± 6.12^{D}	530.70 ± 23.13^{C}	544.88 ± 2.33^{BC}	534.50 ± 2.79^{C}
C20:0	2.07 ± 0.15^{E}	5.33 ± 0.35^{C}	3.29 ± 0.37^{D}	10.78 ± 0.01^{A}	10.17 ± 0.48^{AB}	10.26 ± 0.02^{B}
C22:0	35.25 ± 0.14^{D}	40.49 ± 0.41^{C}	39.65 ± 0.23^{C}	48.66 ± 1.57^{B}	50.73 ± 0.42^{A}	48.26 ± 1.43^{B}
C23:0	0.61 ± 0.11^{D}	1.88 ± 0.13^{C}	1.00 ± 0.03^{D}	4.20 ± 0.58^{A}	3.30 ± 0.04^{B}	3.69 ± 0.40^{AB}
C24:0	2.60 ± 0.14^{E}	5.48 ± 0.53^{D}	5.25 ± 0.48^{D}	19.72 ± 2.77^{A}	9.86 ± 0.38^{C}	17.11 ± 2.09^{B}
SFA	1227.86 ± 13.39^{B}	1244.60 ± 47.72^{B}	1145.74 ± 10.91^{C}	1330.36 ± 11.15^{A}	1373.17 ± 56.44^{A}	1335.47 ± 18.94^{A}
C16:1	80.36 ± 1.65^{A}	69.09 ± 0.83^{C}	63.60 ± 0.82^{D}	63.60 ± 2.22^{D}	72.34 ± 0.38^{B}	68.76 ± 0.16^{C}
C18:1n9c	395.76 ± 8.25^{A}	377.80 ± 4.02^{A}	355.80 ± 3.33^{B}	350.66 ± 15.04^{B}	338.45 ± 18.10^{B}	347.57 ± 2.68^{B}
C20:1	208.14 ± 4.60^{A}	180.88 ± 11.45^{B}	166.74 ± 1.76^{C}	165.78 ± 7.64^{C}	186.87 ± 0.86^{B}	180.40 ± 0.79^{B}
C22:1n9	15.35 ± 1.58^{A}	13.06 ± 0.68^{A}	10.77 ± 1.38^{B}	10.50 ± 1.12^{B}	13.64 ± 0.71^{A}	13.18 ± 1.63^{A}
C24:1	24.87 ± 2.16^{A}	16.53 ± 0.75^{C}	20.78 ± 1.99^{B}	20.45 ± 0.06^{B}	20.04 ± 1.47^{B}	22.19 ± 0.17^{B}
MUFA	724.48 ± 14.13^{A}	657.37 ± 14.71^{B}	617.68 ± 6.18^{C}	611.00 ± 25.37^{C}	631.35 ± 18.28^{BC}	632.11 ± 4.18^{BC}
C18:3n3	25.12 ± 0.27^{E}	29.80 ± 0.54^{E}	68.49 ± 1.67^{D}	257.18 ± 8.00^{B}	352.50 ± 27.24^{A}	222.24 ± 5.11^{C}
C20:3n3	ND	ND	ND	ND	ND	ND
C20:5n3 (EPA)	379.70 ± 6.47^{A}	345.17 ± 21.98^{B}	315.31 ± 3.17^{C}	320.95 ± 12.84^{BC}	333.60 ± 16.62^{BC}	331.38 ± 0.99^{BC}
C22:6n3 (DHA)	1593.94 ± 48.13^{B}	1442.57 ± 76.41^{C}	1533.38 ± 51.51^{BC}	1569.90 ± 51.68^{B}	1499.61 ± 64.85^{BC}	1753.92 ± 46.46^{A}
PUFA n-3	1998.75 ± 52.16^{C}	1817.54 ± 67.54^{CD}	1917.18 ± 50.23^{CD}	2148.04 ± 47.54^{B}	2185.71 ± 105.27^{B}	2307.55 ± 52.48^{A}
C18:2n6c	220.53 ± 8.97^{B}	153.33 ± 9.16^{E}	110.51 ± 1.23^{F}	210.88 ± 4.68^{C}	250.66 ± 4.56^{A}	199.24 ± 0.38^{D}
C18:3n6	ND	ND	ND	ND	ND	ND
C20:3n6	ND	ND	ND	ND	ND	ND
C20:4n6	100.77 ± 1.13^{B}	95.84 ± 0.47^{C}	105.52 ± 0.60^{A}	107.00 ± 3.14^{A}	95.51 ± 0.08^{C}	94.62 ± 0.20^{C}
PUFA n-6	323.30 ± 10.10^{B}	249.18 ± 9.24^{D}	216.03 ± 1.82^{E}	317.88 ± 7.82^{B}	346.17 ± 4.55^{A}	293.86 ± 0.19^{C}
C20:2	9.77 ± 0.17^{B}	9.06 ± 0.90^{B}	9.35 ± 0.04^{B}	10.22 ± 0.34^{A}	10.25 ± 0.39^{A}	9.79 ± 0.003^{AB}
PUFA	2331.82 ± 59.14^{C}	2075.78 ± 66.54^{D}	2142.56 ± 48.52^{D}	2476.14 ± 40.10^{B}	2542.13 ± 110.14^{AB}	2611.20 ± 52.66^{A}
EPA + DHA	1973.63 ± 51.93^{B}	1787.74 ± 67.07^{C}	1848.69 ± 48.59^{C}	1890.85 ± 39.69^{BC}	1833.21 ± 81.44^{C}	2085.31 ± 47.42^{A}
FA	4284.15 ± 81.01^{B}	3977.75 ± 120.26^{C}	3905.98 ± 55.00^{C}	4417.50 ± 13.95^{AB}	4546.65 ± 180.50^{A}	4578.79 ± 74.08^{A}
PUFA n-3/FA	0.467 ± 0.005^{C}	0.457 ± 0.007^{C}	0.491 ± 0.006^{B}	0.486 ± 0.010^{B}	0.481 ± 0.005^{B}	0.504 ± 0.004^{A}

[a] Values are expressed as mean ± standard deviation (SD) ($n = 3$). $^{A-F}$Values in the same row with different letters are significantly different ($P < 0.05$). MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, SFA: saturated fatty acid, FA: fatty acid.

correlations with inhibitory effects on PhIP formation (Fig. 1) indicate the consumption of antioxidant capacity in the protection against protein/amino acid degradation and/or their involvement in the formation pathway of PhIP. Nonetheless, considering the fact that MeIQx and 4,8-DiMeIQx only accounted for <3% of the total HAs in the roasted cod patties, it could be concluded that phenolic antioxidants are principal constituents in the tested *Allium* spp. that may help mitigate dietary exposure to HAs.

3.5 Principal component analysis of the quality changes associated with different treatments

Principal component analysis (PCA) was applied to have a more comprehensive comparison of the cod patties subjected to different pretreatments. As shown in Fig. 3, the first two principal components accounted for 73.89% of the total changes of the samples (45.46% PC1 and 28.43% PC2). Fig. 3A shows the biplot of the scores and loadings located in the four quadrants of a confidence region (95%). Fig. 3B displays the score points in detail with PC1 and PC2 as loading factors. The control patties fall in the upper left quadrant and are distinctive from the other treatments in their higher HA and MUFA contents. Of note, HA content positively correlated with MUFAs ($R = 0.910$, $P < 0.001$), which suggests that higher MUFA levels might play a role in promoting the formation of HAs.[57] Further investigation is warranted to clarify the potential correlation between the composition/content of MUFAs and HA formation in cod. Garlic-pretreated patties occupied a cluster in the lower left quadrant in the PCA plot near the control patties, which suggests that it may be a less preferable candidate to the other four *Allium* spp. in terms of potential to significantly improve the quality of roasted cod patties. Chinese chive- and Mongolian leek-pretreated patties occupied a cluster in the upper right quadrant which corresponds to their overall better nutrient profiles (*i.e.* n-3 PUFAs and soluble protein) than the other treatment groups. Thus, PCA indicates that powders of Chinese chive and Mongolian leek are promising culinary ingredients for improving both the safety and nutritional quality of roasted cod.

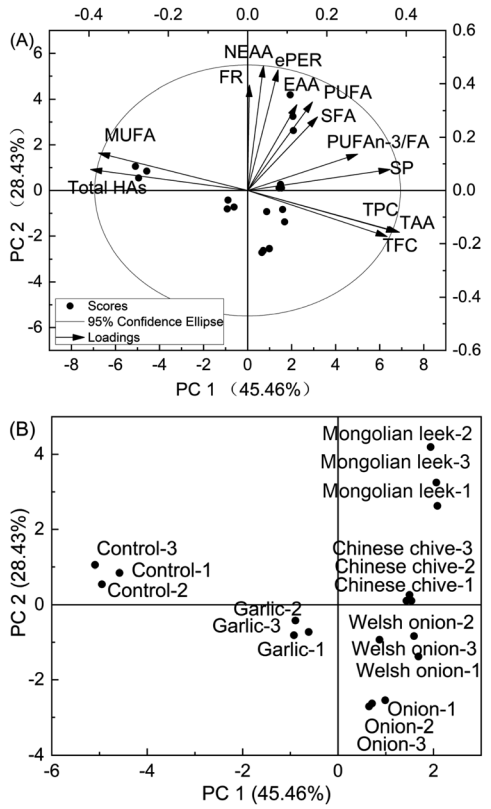

Fig. 3 The bioplot (A) and score plot (B) of principal component analysis (PCA) of HA contents, nutrient compositions, and antioxidant attributes of roasted cod patties.

4. Conclusion

In conclusion, the present study has demonstrated that pretreatment with powders of certain *Allium* spp. could lead to significantly lower HA contents and better nutritional profiles in roasted cod. This has been the first report on the strong HA-formation inhibitory effect of Chinese chive and Mongolian leek. It would be important to further characterize the principal phenolic component(s) and mechanism responsible for their differential effects on the formation of different types of HAs. The dual beneficial functionality of Chinese chive and Mongolian leek may be utilized to reduce the intake of hazardous by-products while enhance the nutritional and antioxidant properties of roasted cod and probably other protein-rich heat-processed foods.

Conflicts of interest

The authors declare no conflicts of interest

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