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Research Article

Use of LC-MS for The Quantitative Determination of Advanced Glycation End-Products in Ultra-Processed Malaysian Foods: A Protocol Development Pilot Study

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ABSTRACT

Advanced glycation end-products (AGEs) are formed through non-enzymatic reactions between reducing sugars and proteins, accelerated by high-temperature food processing. Excessive dietary intake of AGEs has been linked to chronic diseases such as diabetes, cardiovascular disorders, and neurodegeneration. An AGE content database is lacking for Malaysian foods, unlike database developments in the United States and China. This pilot study aimed to establish a protocol for AGE quantification using liquid chromatography-mass spectrometry (LC-MS) in selected Malaysian foods. Eleven food samples, categorised by processing level according to the Nova classification, were analysed for two major AGE markers - Nε-carboxymethyllysine (CML) and Nε-carboxyethyllysine (CEL). Results showed significant variation in AGE content between samples, with the highest CEL levels found in deep-fried fish balls and steamed grouper fish, while pan-fried chicken sausages had the highest CML concentration; whereas levels in fresh fruits and minimally processed items like rolled oats were below detection. These findings suggest that food processing methods, particularly dry-heat techniques, significantly influence AGE formation. This developed protocol will be applied to a larger number of Malaysian foods to facilitate the development of an AGE food composition reference database for supporting dietary guidance in chronic disease prevention strategies.

Key words: Advanced glycation end-products (AGEs), CEL quantification, CML quantification, dietary AGEs, LC-MS analysis, processed foods

INTRODUCTION

Advanced glycation end-products (AGEs) are a heterogeneous group of stable compounds formed through non-enzymatic reactions between reducing sugars and free amino groups of proteins, lipids, or nucleic acids, primarily via the Maillard reaction (Luevano-Contreras & Chapman-Novakofski, 2010; Twarda-Clapa *et al*., 2022). This process progresses through the formation of Schiff bases, Amadori products, and eventually irreversible AGEs. AGEs arise endogenously as part of a normal physiological process in human metabolism; however, excessive accumulation is pathogenic, especially if associated with chronic diseases. AGEs readily bind to cell surface receptors, forming receptor for AGEs (RAGE) or form cross-links with proteins, altering their structure and function and contributing to oxidative stress, inflammation, and tissue damage (Uribarri *et al*., 2010; Byun *et al*., 2017). These mechanisms are implicated in the etiology of chronic conditions, including diabetes, cardiovascular disease, chronic kidney disease, neurodegenerative disorders, arthritis, and cancers (Byun *et al*., 2017; Prasad *et al*., 2017; Rungratanawanich *et al*., 2021).

AGEs may also accumulate in the body from exogenous sources such as tobacco smoke and thermally processed foods (Vadakedath & Kandi, 2018; Inan-Eroglu *et al*., 2020). High-temperature cooking methods such as frying, grilling, and roasting, especially in protein- and fat-rich foods, promote AGE formation, further influenced by factors such as low moisture, high pH, and prolonged heating (Uribarri *et al*., 2010; Mastrocola *et al*., 2021; Rungratanawanich *et al*., 2021; Li *et al*., 2022). Among the many AGEs identified - over 40 to date (Tian *et al*., 2023) - Nε-carboxymethyllysine (CML) is the most widely studied and serves as a proxy marker in both biological and food analyses (Luevano-Contreras & Chapman-Novakofski, 2010).

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Despite the growing body of research on the health impacts of dietary AGEs, there remains a significant gap in local data, particularly within the Malaysian context. Some international studies have quantified AGE content in various foods and beverages, but this data is limited to countries such as the United States (Hull *et al*., 2012) and China (Chen & Scott Smith, 2015). Malaysia lacks a comprehensive food composition database to provide AGE values. This absence limits the ability to assess dietary exposure to AGEs for the Malaysian population, hindering targeted public health strategies and dietary interventions.

Given the lack of a Malaysian food database on the content of dietary AGEs, we initiated a pilot study to establish a protocol for dietary AGE analyses. For this pilot study, we focused on ultra-processed foods (UPFs) such as instant noodles and snacks, which are potentially rich sources of dietary AGEs as they undergo high-temperature processing. The selection of foods followed the Nova food classification according to their level of processing, from not processed or minimally processed to highly processed (Petrus *et al*., 2021). For this protocol, we chose LC-MS instrumentation for AGE determination as it has been successfully used by others (Sharma *et al*., 2015; Corica *et al*., 2022; Zhang *et al*., 2024).

MATERIALS AND METHODS

Food sample selection

Food consumption statistics from the National Health and Morbidity Survey 2017 (Institute for Public Health, 2017) and the Malaysian Adult Nutrition Survey 2014 (MANS) (Institute for Public Health, 2014) were referenced to identify frequently consumed foods that could be classified according to the Nova food classification system (Petrus *et al*., 2021). Eleven food items were purchased from local grocery stores to represent varying processing levels, and these were commonly consumed in Malaysia. Accordingly, these were:

Highly processed foods: Cornflakes, granola bars, chicken sausages, fried fish balls, strawberry jam, and canned pineapple.

Minimally processed foods: Corn, rolled oats, grouper fish, strawberry, and pineapple.

Sample preparation

The protocol was primarily adapted from Zhang *et al*. (2024). All food items were freeze-dried at -40°C for 72 hr before grinding to a powder, excepting dry items such as corn flakes and granola, which were ground directly without freeze-drying. Ground samples were stored at -20°C until further analysis. About 2 g of powdered sample was mixed with 3 mL of a chloroform-methanol mixture (2:1, v/v) and centrifuged at 7000 rpm for 10 min at 4°C to isolate proteins. After careful removal of the supernatant, the precipitate was dried under a stream of nitrogen. A mixture of 1 mL sodium borohydride (1.0 M in 0.1 M sodium borohydride) and 2 mL borate buffer (0.2 M, pH 9.2) was added to the precipitate, vortexed, and incubated for 3 hr at 25°C to prevent neo-formation of AGEs from fructoselysine during acid hydrolysis. Next, the sample was hydrolysed using 3 mL of 6 M hydrochloric acid for 24 hr at 110°C. After hydrolysis, 500 µL of the hydrolysate was concentrated using a concentrator (Eppendorf Concentrator plus, Germany) at 45°C for 2 hr, reconstituted in 500 µL LC-MS grade water and filtered through a 0.22 µm membrane before LC-MS analysis. This procedure was carried out in triplicate for each sample. A stock solution containing all standards was prepared in LC-MS grade water at an initial concentration of 500 µg/mL. To create working standard solutions, the stock solution was diluted with LC-MS grade water to achieve a series of concentrations ranging from 50 to 500 µg/mL for CML (≥95%, Macklin Biochemical) and CEL (≥95%, Macklin Biochemical). All stock solutions were held at -20°C before use.

AGE determination

Each sample (n=3 for each selected food item) was subjected to LC-MS analysis using a Q Exactive™ Orbitrap LC-MS system with a 12-min run time and an ACQUITY UPLC® HSS T3 column (1.8 µm, 2.1 mm × 100 mm). The injection volume was 1 µL, flow rate 0.3 mL/min, and column temperature 40°C. The mobile phases were 5 mM nonafluoropentanoic acid (Thermo Fisher Scientific) in water (A) and acetonitrile (B), with a gradient from 5–100% B over 10 min and a 2-min isocratic hold. Mass spectra (MS) were acquired using heated-electrospray ionisation (HESI) in both positive and negative ion modes (m/z 100–1000). The resolution of the MS was fixed at 70,000 with a maximum injection time of 250ms for an MS full scan. Data-dependent MS2 acquisition was carried out on the top five most intense ions. The MS2 data was utilised at 17,000 resolutions, with a maximum injection time of 60ms. MS2 spectra were obtained using stepped normalised collision energy of 25,45, and 65 eV. The optimum HESI parameters were spray voltage, 4.0 kV (PI) and 3.5 kV (NI); auxiliary gas flow rate, 11L/min; sheath gas flow, 4 kV; capillary temperature, 350°C; S-lens radio frequency level, 60%. Xcalibur™ software version 4.3 (ThermoFisher Scientific, Waltham, Massachusetts, USA) was used to analyse the mass spectra.

Statistical analysis

Results are presented as mean ± SD, and statistical significance was assessed using one-way analysis of variance (ANOVA), followed by Duncan's multiple range test to compare means showing significant difference between sample types (P<0.05). All analyses were carried out using the SPSS software (IBM SPSS Statistics Version 25, SPSS Inc., Chicago, USA).

RESULTS

Quantification of CML and CEL in Food Samples by LC-MS

Table 1 shows results obtained from the LC-MS analysis with CML and CEL content of food samples presented as mean per serving size (mg/serving) and per 100 g of food (mg/100 g). CML and CEL content of food samples indicate significant variations. For CML, pan-fried chicken sausages exhibited the highest concentration at 0.031 mg/100g, followed by strawberry jam (0.030 mg/100g) and canned pineapple (0.007 mg/100g). Notably, CML was not detected in any of the remaining food

samples analysed. In terms of CEL content, deep-fried fish ball displayed the highest concentration at 86.114 mg/100g, followed by steamed grouper fish (59.519 mg/100g), pan-fried chicken sausages (36.548 mg/100g), corn flakes (11.631 mg/100g) and steamed corn (10.363 mg/100g). Remarkably, none of the remaining food samples examined contained CEL.

Table 1. CML and CEL Content in Food Samples Determined by LC-MS

Food samples	Cooking method	Serving size (g)	CML		CEL	
			mg/100g	mg/serving	mg/100g	mg/serving
Corn	Steamed	222.1	ND	ND	10.363	23.015
Corn Flakes	Extruded	45.0	ND	ND	11.631	5.234
Rolled Oats	Steamed	35.0	ND	ND	ND	ND
Granola Bar	Baked	45.0	ND	ND	ND	ND
Chicken Sausages	Pan-fried	34.0	0.031	0.011	36.548	12.426
Grouper Fish	Steamed	63.0	ND	ND	59.519	37.497
Fried Fish Ball	Deep-fried	63.0	ND	ND	86.114	54.252
Strawberry	Raw	47.0	ND	ND	ND	ND
Strawberry Jam	Boiled	18.0	0.030	0.005	ND	ND
Pineapple	Raw	130.0	ND	ND	ND	ND
Canned Pineapple	Simmered	140.0	0.007	0.010	ND	ND

#Values per food item are expressed as the mean of 3 samples. Abbreviation: ND=Not detected

Figures 1 to 4 present the chromatograms for the food samples with the highest and lowest CML and CEL concentrations, respectively.

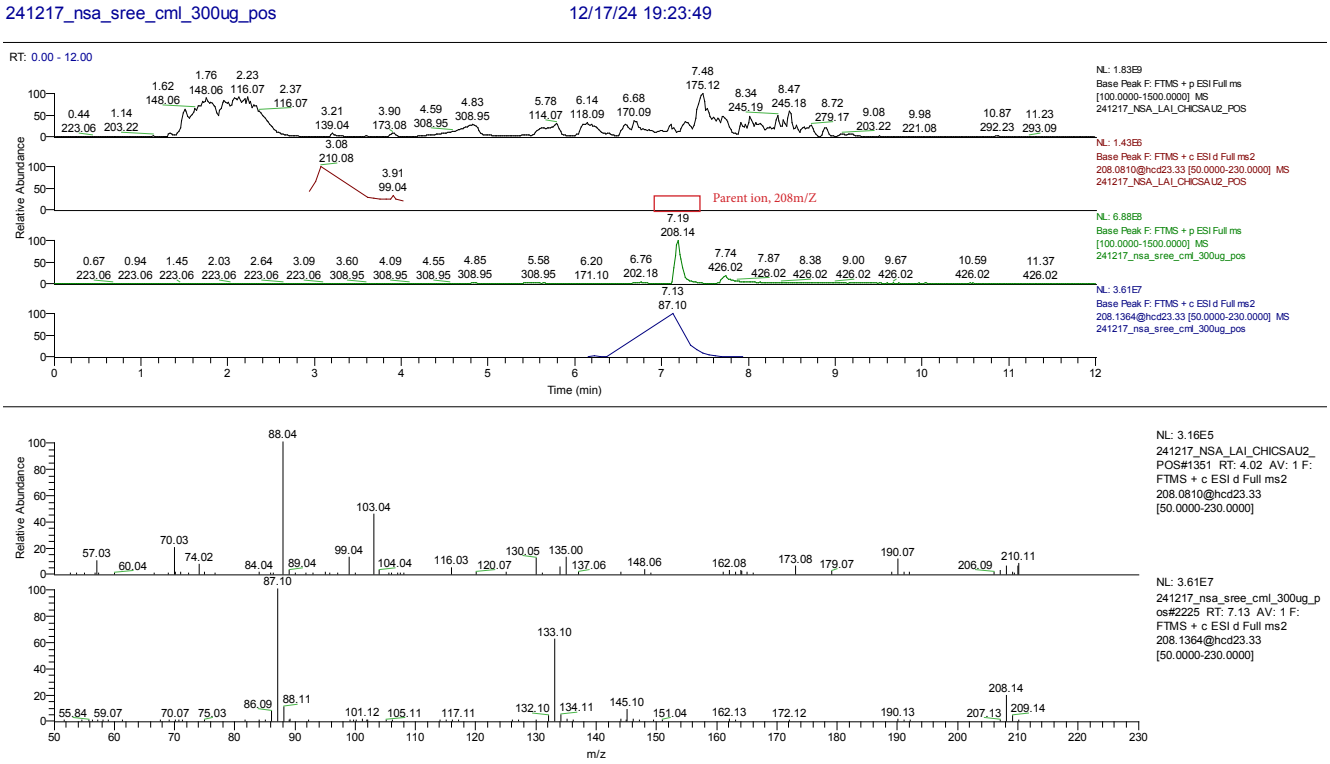

Fig. 1. Chromatogram for food samples with the highest CML content (chicken sausages).

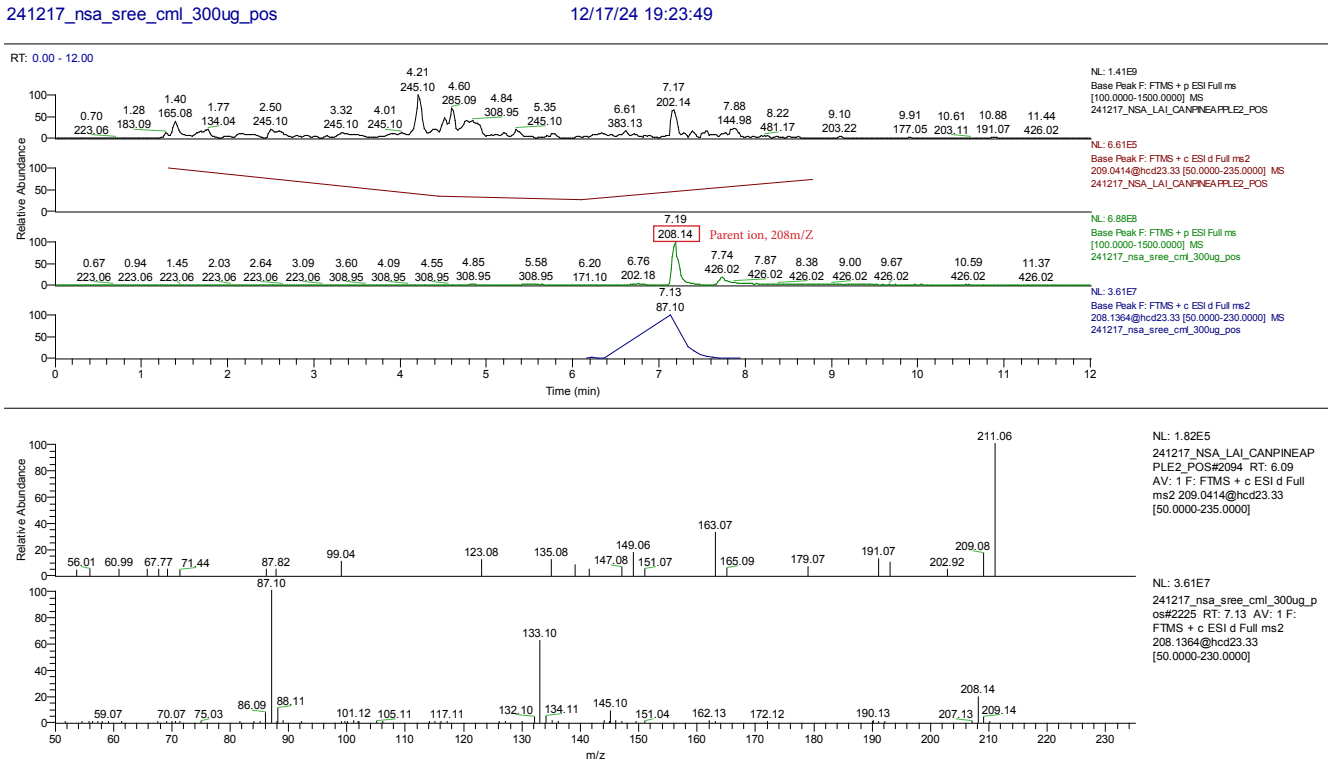

Fig. 2. Chromatogram for food samples with the lowest CML content (canned pineapples).

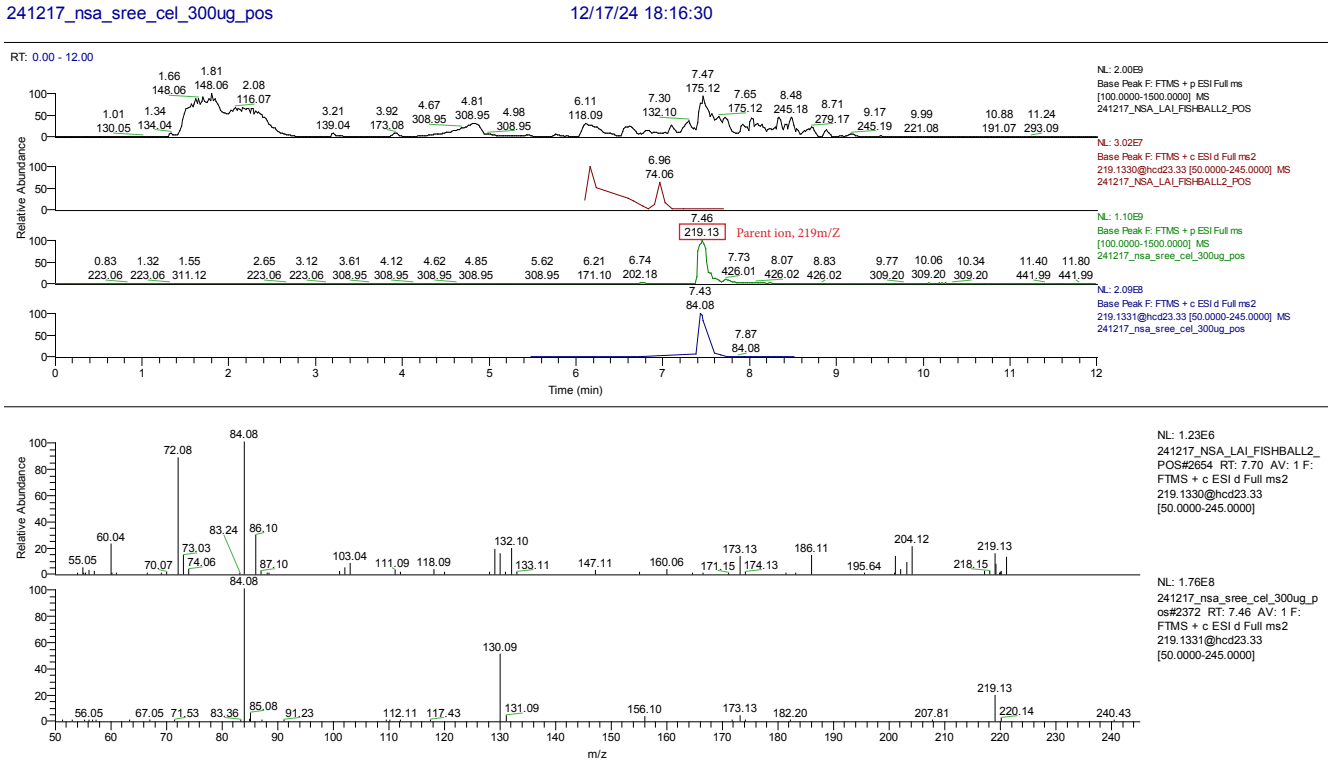

Fig. 3. Chromatogram for food samples with the highest CEL content (fried fish ball).

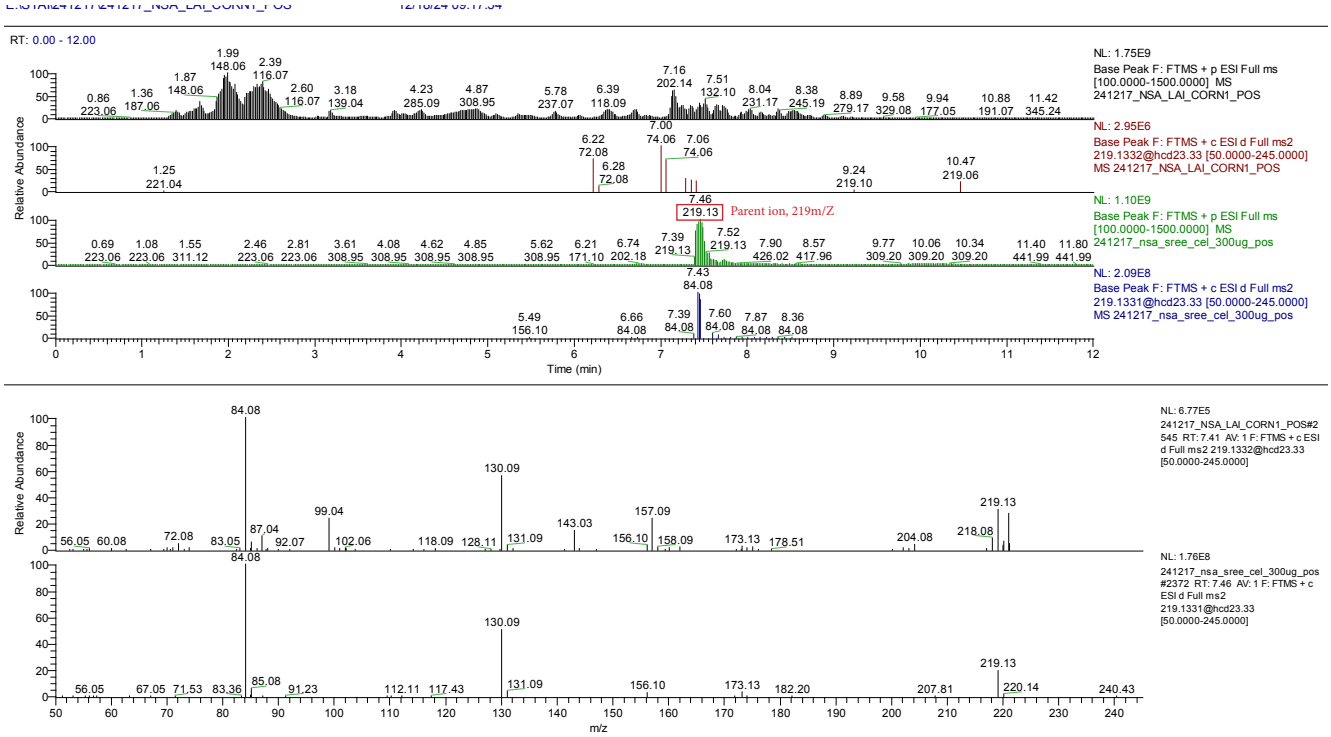

Fig. 4. Chromatogram for food samples with the lowest CEL content (corn).

DISCUSSION

This study utilised LC-MS for its sensitivity, speed, and lack of derivatisation requirements, enabling direct analysis of AGEs in food (Sharma *et al*., 2015; Corica *et al*., 2022; Zhang *et al*., 2024). Freeze-drying and fine grinding of samples improved extraction efficiency (Troise *et al*., 2015). Sodium borohydride was used to prevent artificial AGE formation during acid hydrolysis by stabilising Amadori products (Cheng *et al*., 2021; Niquet-Leridon & Tessier, 2011). CML and CEL were selected as markers due to their relevance in dietary intake (Snelson & Coughlan, 2019). Results expressed per 100 g of food allowed for better comparison with existing data (Zhou *et al*., 2015). AGE detection varied across foods, reflecting the influence of processing method and composition, with perhaps antioxidant content as an influencing factor (Sharma *et al*., 2015; Karadas & Yılmaz, 2021).

Steamed corn and corn flakes showed moderate CEL levels, while rolled oats and granola - both minimally or moderately processed - had no detectable AGEs, consistent with prior findings (Rasane *et al*., 2015; Scheijen *et al*., 2016; Zhang *et al*., 2024). Meat-based and processed foods generally showed higher AGE content, particularly in CEL, with deep-fried fish balls and steamed grouper fish recording the highest levels. This aligns with previous research linking dry-heat cooking, high fat and protein content, and intense thermal processing to elevated AGE formation (Uribarri *et al*., 2010; Inan-Eroglu *et al*., 2020).

In the USA, a comprehensive study (Uribarri *et al*., 2010) highlighted that processed foods, especially those subjected to grilling, roasting, or frying, had significantly higher AGE levels compared to foods prepared by steaming or boiling. Similarly, in China, the cooking methods and food types with the highest AGE content included grilled meats and fried foods, with low AGE levels found in boiled vegetables and rice (Zhang *et al*., 2024). These patterns underscore the global consensus that cooking methods involving high temperatures, especially dry-heat methods, promote significant AGE formation (Li *et al*., 2022; Zhang *et al*., 2024).

In this study, fresh fruits showed negligible AGEs, while processed variants like strawberry jam and canned pineapple had low but measurable levels, likely due to added sugars and heat (Patras *et al*., 2011; Çatak *et al*., 2022; Zhang *et al*., 2024). These findings are similar to those reported in Europe, where processed fruits like jams and fruit preserves have been shown to contain higher AGE levels due to the sugar content and the heat applied during their preparation (Scheijen *et al*., 2016).

Overall, dry heat promoted significantly more AGE formation than moist heat (Li *et al*., 2022), and protein was the most strongly correlated macronutrient, with increased temperature and processing time accelerating Maillard reactions and AGE precursor accumulation (Sharma *et al*., 2015; Liman *et al*., 2023; Zhang *et al*., 2024).

Since AGEs can become harmful when high levels build up in tissues and circulation, it is advised to limit the intake of foods high in AGEs, such as food subjected to intense heat treatment. However, the data obtained from this study only offers limited insights into the AGEs content in food available in Malaysia, which calls for an expanded food database development. Estimating the amount of dietary AGEs consumed daily can raise awareness and guide consumers in making healthier dietary choices and lowering the risk of chronic diseases by reducing the consumption of AGE-rich foods.

CONCLUSION

This pilot study was able to establish a protocol for AGE determination with LC-MS using 11 food samples, ranging from meat to fruit products, with varying processing levels and methods of heating. With these samples, variations in AGE content were found to support the hypothesis that foods with varying processing levels exhibit different CML and CEL content. This protocol will be adopted in a larger study of commonly consumed Malaysian foods for AGE content determination to facilitate the development of a food reference database for Malaysia.

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ETHICAL STATEMENT

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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