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RESEARCH

Effects of Nuts, Dried Fruits, Dried Seeds and Black Olives as Enrichment Ingredients on Acrylamide Concentrations in Sweet and Savoury Biscuits

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Abstract

This study investigated the effect of adding 10% almonds, pistachios, apricots, plums, sesame seeds, sunflower seeds, and black olives to sweet and savoury biscuits on acrylamide (AA) concentration. These ingredients, which are increasingly used to enrich bakery products, can increase the final AA content, because they contain its precursors and sometimes AA itself, due to the processing treatments they undergo. The AA and some quality characteristics of all ingredients and biscuit samples were analysed. The results showed that sweet biscuits with almonds, pistachios and apricots had AA concentrations exceeding the European benchmark level (350 µg/kg), while those with plums had a lower value (190.7 µg/kg). The enriched savoury biscuits had significantly higher AA concentrations than the control (198.9 µg/kg), up to + 163%. A 10% addition of enrichment ingredients to biscuits can lead to high AA concentrations, highlighting the need for further studies aimed at its mitigation in bakery products.

Keywords Acrylamide · Bakery products · Dried fruits · Nuts · Dried seeds · Black olives.

Introduction

Acrylamide (AA) is a heat-generated contaminant classified as probably carcinogenic to humans (Group 2 A) and is found in various food as a result of thermal processing and treatments (IARC, 1994, SNFA, 2002). The harmfulness of this substance is due to the fact that it is easily and rapidly absorbed through the skin, respiratory system, and digestive tract, leading to widespread distribution in the body and causing genotoxicity, germ cell toxicity and carcinogenic potential. In addition, AA can affect nerve endings and cause both central nervous system symptoms such as

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hallucinations and convulsions and peripheral nervous system symptoms such as tremors, weakness, ataxia and alteration of sensory perception. Most cases of AA intoxication involved peripheral neuropathy and carcinogenic effects caused by consumption of contaminated heat-processed food and occupational exposure. However, it must be taken into account that the dose, the exposure rate and the duration of contact have a significant influence on how severe the effects are (Yamamoto et al., 2023).

The formation of AA in food results mainly from Maillard reactions and is related both to the type of ingredients used in the recipe and to their interactions during the preparation process to which the various food are subjected. The main precursors of this toxic compound are reducing sugars (e.g. glucose and fructose) and free amino acids (e.g. asparagine) (Keramat et al., 2011; Mesías & Morales, 2015; Schouten et al., 2022b). Following the discovery of AA in food, the European Commission, Member States, researchers and the food industry have made joint efforts to investigate its occurrence and mechanisms to control it. As it is found in various types of everyday food, the risk not only concerns all consumers but also food processing companies. In 2017, the European Commission established mitigation measures and benchmark values to reduce the presence of AA in several food categories at particular risk through Regulation (EU) 2017/2158. An important AA risk food group mentioned in this regulation is bakery products, which include biscuits and crackers, consumed daily by different consumer groups. Over the years, numerous strategies have been studied to mitigate AA in bakery products, encompassing interventions across all stages of production from the use of the enzyme asparaginase to the control of the main baking conditions (Açar et al., 2012; Anese et al., 2016; Keramat et al., 2011; Kumar et al., 2014; Sarion et al., 2021; Schouten et al., 2022b). However, it has become evident that there is still insufficient data on the AA presence in certain food that are not listed in Regulation (EU) 2017/2158. These food, although not included in the regulation, may contain significant amounts of this toxic compound and/or contribute to its dietary exposure. To initiate a step towards addressing this concern, in 2019, the European Commission adopted Recommendation 2019/1888/EC, which focuses on AA monitoring in other food products. The new list of food includes numerous specialities in the different categories (e.g., rösti and potato croquettes, pancakes, croissants and coffee substitutes not based on chicory or cereals) as well as other products, including roasted nuts, roasted seeds, dried fruits and olives.

Several studies in the literature have shown that nuts (e.g. almonds, pistachios, hazelnuts, peanuts) and dried fruits, including apricots, plums, sultanas, dates, chestnuts, may contain amounts of AA (Amrein et al., 2005, 2007; Asadi et al., 2020; Bahrami et al., 2021; Constantin et al., 2018; De Paola et al., 2017; Liu et al., 2023; Nematollahi et al., 2020; Surma et al., 2018; Tepe et al., 2020; Zhang et al., 2011; Zhu, 2016). This is due to both their high content of precursors and the heat treatments to which these products are subjected (e.g. drying, blanching and roasting) to improve their sensory properties and stability during storage. In the case of roasted nuts, the considerably high roasting temperature (160-180 °C) and time (25-30 min) are the key factors for the formation of AA. The drying process of fruits, on the other hand, usually takes place at a rather low temperature (70-80 °C), but the duration of exposure to heat is long enough (24-36 h) to allow AA to form. In addition, the drying process (up to about 20% moisture) increases the sugar and asparagine concentration, which can promote the formation of AA (De Paola et al., 2017). According to Nematollahi et al. (2020), the AA content in 17 commercial roasted nuts, including peanuts, almonds, pistachios and hazelnuts, varied from 83.4 to 250.9 μg/kg. The highest mean value of AA was found in roasted almonds with a concentration of 176.9 μg/kg and the lowest in roasted hazelnuts with a concentration of 90.6 μg/kg (Nematollahi et al., 2020). The AA concentration in dried fruits ranged from < LOQ for dates, sultanas and cranberries, to 141 μg/kg for plums in the analysis of 28 commercial samples (Surma et al., 2018). The wide variability between different commercial nuts and dried fruit samples can be attributed to several factors, such as origin and variety, roasting time-temperature conditions and methods that could determine the amount of AA precursors and influence the AA formation and degradation rate.

Seeds and table olives are also subjected to some heat processes that can cause the formation of AA (AL-Ansi et al., 2019; Amrein et al., 2007; Berk et al., 2019; Boateng & Yang, 2021; Casado et al., 2010, 2014; Charoenprasert & Mitchell, 2014; Crawford & Wang, 2019; De Paola et al., 2017; Duedahl-Olesen et al., 2022; Fernández et al., 2022; Martín-Vertedor et al., 2021; Nematollahi et al., 2020; Pérez-Nevado et al., 2018; Salamatullah et al., 2021). AA concentrations of 33.4-171.8 μg/kg were found in commercial roasted seeds, including three types of commercial sunflower seeds, two types of pumpkin and watermelon seeds (Nematollahi et al., 2020). Concentrations of AA of 135-633 μg/kg were found in sesame seeds roasted for 10 min at 150, 180, 200 and 220 °C, indicating a significant influence of the adopted process conditions (Berk et al., 2019). The results of the analysis of AA in 31 table olives showed concentrations below the detection limit of up to 1100 μg/kg. The enormous variations in AA concentration can be ascribed to the effect of different processing and storage procedures in combination with the geographical origin of olive products (e.g. Californian style, Greek style, sterilisation, pasteurisation, with brine) on the formation of this toxic substance (Duedahl-Olesen et al., 2022).

All of the above products are commonly consumed as healthy snacks, appetisers or side dishes, but are also increasingly used as additional ingredients to enrich various sweet and savoury food due to their appealing health-promoting effects and pleasant taste or aroma (Asadi et al., 2020; Berk et al., 2020; Nematollahi et al., 2020; Žilić, 2016). Therefore, they could contribute to a high intake of AA in different ways and in different product categories; moreover, the current Reg. (EU) 2017/2158 is likely to be renewed soon. The ongoing discussion revolves around three key aspects: the review of the current benchmark levels, the establishment of new ones for certain foods mentioned in Rec. (EU) 2019/1888, and the introduction of maximum levels for certain food (e.g. processed cereal-based products for infants and young children) (European Commission, 2023; Hyslop, 2022).

The aim of this study was to investigate the effects of adding certain ingredients, namely peeled almonds, roasted pistachios, dried apricots and plums, sesame and sunflower seeds, and black olives with and without brine, as enrichment components in sweet and savoury biscuits on the concentration of AA. Although these selected ingredients are known to contain AA and its precursors, their contribution to the further formation of AA when added to a complex matrix, such as in bakery products, has not yet been investigated. It is important to recognise that some properties (e.g. water binding and particle size) and composition (e.g. free precursors and antioxidant content) of additional ingredients added to a bakery formulation can either promote or inhibit the formation of AA. It is known that the interaction between the different ingredients within the matrix can vary during the different stages of the process, especially during baking, which influences the behaviour of the chemical reactions and consequently the formation of this potentially harmful compound (Schouten et al., 2022b, 2023). In line with this research framework, in the present study, the biscuits were prepared on a laboratory scale by adding 10% of the enrichment ingredients to mimic commercially available recipes. All enrichment ingredients and biscuit samples were analysed for AA and some quality parameters such as moisture, water activity, weight loss, pH, colour and texture.

Materials and methods

Preparation of Sweet and Savoury Biscuits

The biscuit doughs were formulated according to a sweet and a savoury basic recipe. The four sweet biscuit types were enriched with peeled almonds, roasted pistachios, dried apricots and plums, while the four savoury biscuit types were enriched with sunflower seeds, sesame seeds, black olives without and with brine. The additional ingredients were added at 10%, a percentage chosen in line with ingredients used by bakery companies to fortify similar product categories.

The basic sweet recipe was prepared with wheat flour (250.0 g), sucrose (62.5 g), pasteurised eggs (62.5 g), sunflower oil (50 g) and raising agent (7.5 g); the basic savoury recipe was prepared with wheat flour (250 g), water (100 g), extra virgin olive oil (25 g), salt (5 g) and raising agent (5 g). All ingredients were procured at the local market (Cesena, FC, Italy), making sure that the different packages belonged to the same production batch.

To prepare the dough, flour, sugar and raising agent were placed in a kitchen mixer (Bimby Robot TM31, Vorwerk, Wuppertal, Germany) and mixed by adjusting the speed regulator to level 3 for 15 s; then the liquid ingredients (i.e. eggs, oil, water) were added and mixed on speed level 4 for 1.30 min. Subsequently, each additional ingredient was added to the basic dough and mixed for 1 min in reverse mode on speed level 3. Almonds and pistachios were previously crushed into grains; apricots, plums, and black olives were reduced into small pieces, while the seeds were left whole. The control sample was made with the basic recipe only. The doughs were then removed from the mixer and worked by hand for 1 min to obtain homogeneous mixtures, which had to rest in the refrigerator at about 4 °C for 20 min before moulding. After the resting period, the dough was rolled using a semi-industrial rolling machine (S-420 INOX, Gam International, Santarcangelo di Romagna, Italy), with the roll gap set at 3 mm. Finally, the biscuits were formed into a round shape using a plastic mould with a diameter of 8 cm (average weight of the raw biscuits = 19.8 g). The preparation of the dough was based on preliminary tests.

For each formulation, 6 biscuits (approx. 120 g uncooked dough) were baked in static mode in an electric oven preheated to 175 °C (Apollo PS1 Steamer, AEG-Elettrolux, Berlin, Germany). The optimal baking times for each biscuit type were selected on the basis of preliminary tests by monitoring of baking parameters evaluated and are given in Table 1 with the sample codes.

Two dough and two baking replicates were made for each sample (12 biscuits for each biscuit recipe, 96 biscuits in total).

Chemicals

AA (acrylamide, molecular weight 71.08 g/mol), 2,3,3-d_3-AA (AA-d_3) standard solution, hexane (molecular weight 86.18 g/mol), ethanol (molecular weight 46.07 g/mol), LC-MS acetonitrile (Sigma-Aldrich, St. Louis, MO,

Table 1 Sweet and savoury biscuit samples with corresponding baking times at 175 °C

Sample	Sample code	Baking time (min)
Sweet biscuits		
Control (C)	C_{SW}	14
With peeled almonds (AL)	AL_{SW}	9
With roasted pistachios (PI)	PI_{SW}	9
With dried apricots (AP)	AP_{SW}	13
With dried plums (PL)	PL_{SW}	13
Savoury biscuits		
Control (C)	C_{SA}	20
With sunflower seeds (SU)	SU_{SA}	20
With sesame seeds (SE)	SE_{SA}	20
With black olives without brine (ONB)	ONB_{SA}	23
With black olives in brine (OB)	OB_{SA}	23

USA), HPLC-grade formic acid (99%) (Merck, Darmstadt, Germany) and ultrapure water (Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). By dissolving 10 mg in 10 mL of water, the stock solution of AA was predisposed. All operative solutions by diluting appropriate volumes of the stock solutions with water to obtain various concentrations were daily prepared. For the quantification of AA, an internal standard (IS) named AA-d_3 was used. A specific aliquot of AA-d_3 stock solution (3500 ng/mL) was combined with the different concentrations of the standard solutions of AA. Sample clean-up was performed using two SPE (solid phase extraction) cartridges: Bond Elut-Accucat (3 mL, 200 mg, Agilent Technology, Santa Clara, CA, USA) and Oasis HLB (6 mL, 200 mg, Waters, Milford, MA, USA). Each sample was filtered using a Phenex™ RC syringeless filter (0.2 µm, 4 mm, Phenomenex, Castel Maggiore, BO, Italy) prior to instrument (ultra-high-performance liquid chromatography-tandem mass spectrometry, UHPLC-MS/MS) analysis. The syringeless filter was captiva PTFE (0.45 µm, 13 mm, Agilent Technology, Santa Clara, CA, USA).

Analytical Determinations

The additional ingredients used in the formulation of the biscuits were analysed for moisture, water activity (a_w), pH, colour and acrylamide (AA) content. Moisture, a_w, pH and colour characteristics were determined for all types of raw biscuits and in addition to these parameters, the baked ones were also analysed for weight loss, texture and AA concentration. The methods used for each analysis performed are described in the following paragraphs.

Moisture and Water Activity

The moisture (%) of ground enrichment ingredients, raw and baked biscuits was assessed at 105 °C until constant weight using the gravimetric method (laboratory stove, UF110, Memmert, Schwabach, Germany).

The a_w of ground enrichment ingredients, and biscuits was determined at 25 °C with a dew point hygrometer (AQUALAB, Metre 4 TE, Pullman, Washington, USA).

Six replicates of moisture and a_w were conducted for each sample.

Weight Loss

The weight loss (%) of the biscuits after baking was assessed with an electronic balance (KERN GAB-N, Balingen, Germany). Twelve biscuits were evaluated for each sample.

pH

The pH of enrichment ingredients, raw and baked biscuits was assessed using a pH meter (METTLER TOLEDO, FiveEasy F20-Std kit, USA). About 3 g of sample and 6 g of distilled water were mixed with a vortex for 30 s and the pH was measured in the liquid fraction. Six replicates were evaluated for each sample.

Colour and Visual Appearance

The surface colour of enrichment ingredients, raw and baked biscuits was determined with a spectrophotometer (45°/0° geometry, ColourFlex EZ, HunterLab, Sunset Hills Road Reston, Virginia, USA). The CIE L*a*b* standard scale was used for express the samples colour. Hue angle (h°) was calculated from the a*(green-red) and b* (blue-yellow) values using the equation reported by Schouten et al. (2022a). Six replicates were evaluated for each sample.

To analyse the colour appearance of both raw and baked biscuits a computer vision system (CVS) was used. The biscuits were positioned in a dark box against a black background ensuring and under controlled lighting conditions with four fluorescent lamps (Natural Daylight, 18 W/965, 6500 K, TL-D Deluxe, Philips, USA). The RGB (Red, Green, Blue) pictures of the biscuits were obtained using a vertically-oriented digital camera (D7000, Nikon, Japan) and a 105 mm lens (AF-S Micro Nikkor, Nikon, Japan).

Texture

Texture analysis of baked biscuits was carried out at room temperature using a texture analyzer TA-HDi500 (Stable Micro System, Surrey, UK) equipped with a three-points bending ring, a horizontal stainless-steel probe and a 25 kg load cell. In detail, the speeds were 1.00, 5.00, 10.00 mm/s for the test, the pre-test and post-test, respectively. The probe distance was 5 mm, while the spacing between the two beams was 20 mm. The recorded parameters included fracture hardness (N), determined by the maximum force values, and fracturability (1/mm), expressed as inverse of the distance (mm) from the origin to the point of biscuit breakage (Romani et al., 2012). Twelve replicates were evaluated for each sample.

Extraction, Purification and Quantification of Acrylamide

The AA quantification in enrichment ingredients and baked biscuits followed previous analytical methods by Schouten et al. (2022a) with slight modifications. In detail, the previous method was improved by freeze-drying and consequent sample concentration. Before taking the biscuit sample, 10 g from each replicate batch (=20 g) were mixed and thoroughly homogenised. For the extraction and purification, 5 g of sample (ingredient or mixed biscuits) were weighed into two 250 mL glass flasks and spiked with 0.5 mL of AA-d_3 solution (3500 ng/mL). Subsequently, 49.5 mL of water was added and the sample was shaken with a magnetic stirrer for 1 min. At room temperature, ultrasound extraction was performed for 1 h at a frequency of 40 kHz and 100% power (FALC, Treviglio, Italy). Then, the supernatant was filtered with filter paper and lipids were removed by liquid-liquid extraction with hexane (performed four times with different volumes: 10 + 10 + 10 + 5 mL). After defatting, the aqueous solution was freeze-dried until a constant weight was achieved. The resulting dried extract was dissolved in 5 mL of water, and the concentrated liquid extract was centrifuged and purified using two SPE cartridges. In the case of dried apricots and plums, before SPE, an aliquot (2 mL) of the concentrated liquid extract was treated with 2 mL of ethanol to allow the precipitation of interfering pectin.

The purification procedure with two SPE cartridges, i.e., Oasis HLB and Bond Elut-Accucat has been carried out following previous methodology (Schouten et al., 2021, 2022a). Finally, samples were filtered with a 0.2 µm filter and injected into UHPLC-MS/MS.

An Agilent 1290 Infinity Series and a Triple Quadrupole 6420 (Agilent Technology, Santa Clara, CA, USA) were employed for conducting the UHPLC-MS/MS quantification. The MS system was composed of an electrospray ionisation source (ESI) operating in positive polarity. The mobile phase was water (A) and acetonitrile (B), both containing formic acid (0.1%). The separation was achieved at a flow rate of 0.8 mL/min using a gradient elution mode. The composition of the mobile phase underwent a gradient elution with the following specifications: 0-2.5 min (isocratic condition), 85% B; 2.5-3.5 min, 85-70% B; 3.5-5.5 min (isocratic condition), 70% B; 5.5-6.5 min, 70-60% B; 6.5-10 min (isocratic condition), 60% B; 10-12 min, 60-85% B. A 2 µL injection volume was used while maintaining the drying gas temperature of the ionisation source at 350 °C with a gas flow rate of 12 L/min. The nebuliser pressure was set at 45 psi and the capillary at 4000 V. Multiple Reaction Monitoring (MRM) mode was performed for data acquisition with the most abundant transition used for quantification and the others for analyte confirmation. The limit of quantification (LOQ) and detection (LOD) were established at 10 and 3 times the signal-to-noise ratio, respectively. LOQ and LOD for AA were 5 and 1.5 µg/kg, respectively. The method exhibited comparable sensitivity to a previously developed and published method employed for biscuits (Schouten et al., 2022a). Two replicates of extraction and quantification were performed for each sample.

Statistical Analysis

One-way analysis of variance (ANOVA) and Tukey's post-hoc comparison test were applied for statistical analysis of the data in order to identify possible differences between the samples ($p \leq 0.05$, STATISTICA, Statsoft Inc., version 8.0, Tulsa, UK). The correlation between the average AA concentration in the final biscuits and the main physico-chemical properties of the biscuits was assessed using the Pearson correlation coefficient (r). A value of $0.60 \leq r \leq 1.00$ indicates a positive linear correlation, while $1.00 \leq r \leq 0.60$ indicates a negative linear correlation and $0.60 < r < 0.60$ indicates no correlation ($p \leq 0.05$, STATISTICA, Statsoft Inc., version 8.0, Tulsa, UK).

Results and Discussion

Main Quality Characteristics of Biscuits

Certain quality characteristics of the baked biscuit samples were analysed to gain a deeper understanding and insight into the physicochemical changes induced by the addition of additional ingredients in the product matrix. These changes have the potential to influence the chemical reactions responsible for the formation of AA and subsequently affect the final concentration in the biscuits. The main quality characteristics resulted for the enrichment ingredients and for the sweet and savoury biscuits are presented in Tables 2, 3 and 4, respectively.

In the sweet biscuits, weight loss was observed after baking for all samples, but in different ways depending on the type of additional ingredient used (Table 3). The control sample (C_{SW}) had the highest weight loss compared to the

Table 2 Moisture, water activity, pH and colour values of the additional ingredients used in the sweet and savoury biscuit formulations

Extra ingredient	Moisture (%)	Water activity (a_w)	pH	L* (lightness)	h° (hue angle)
Peeled almonds	4.4 ± 0.1^c	0.57 ± 0.00^c	6.0 ± 0.0^a	81.4 ± 0.3^a	86.1 ± 0.6^a
Roasted pistachios	1.8 ± 0.1^d	0.28 ± 0.01^d	6.0 ± 0.0^a	61.4 ± 0.6^b	83.8 ± 0.4^a
Dried apricots	25.3 ± 1.2^b	0.67 ± 0.00^b	5.0 ± 0.0^b	23.8 ± 0.5^c	48.5 ± 2.1^c
Dried plums	40.0 ± 0.3^a	0.83 ± 0.00^a	3.7 ± 0.0^c	7.3 ± 0.9^d	56.7 ± 1.6^b
Sunflower seeds	4.0 ± 0.1^B	0.57 ± 0.01^B	6.1 ± 0.0^B	58.4 ± 0.4^A	78.8 ± 0.0^A
Sesame seeds	4.7 ± 0.0^B	0.53 ± 0.00^B	6.0 ± 0.0^B	64.3 ± 0.2^A	76.9 ± 0.2^A
Black olives without brine	75.6 ± 1.4^A	0.98 ± 0.01^A	7.6 ± 0.2^A	19.0 ± 1.0^B	54.7 ± 0.5^C
Black olives in brine	77.2 ± 1.3^A	0.98 ± 0.00^A	7.5 ± 0.0^A	14.8 ± 0.2^B	61.6 ± 0.2^B

Different letters in the same column indicate significant differences between samples (Tukey's test, $p \leq 0.05$)

other samples, while the AP_{SW} and PL_{SW} samples had the lowest and similar weight loss. This result can be attributed to the lowest moisture reduction in these biscuit samples after baking, which could be due to the structure of the dried apricots and plums, which retain more water during baking and thus reduce evaporation. On the other hand, the samples C_{SW}, AL_{SW} and PI_{SW} had a more uniform structure, resulting in a higher dehydration rate during baking. Furthermore, the raw AP_{SW} and PL_{SW} doughs had the highest initial water content compared to all other samples. This result is due to the higher moisture content of the dried apricots and plums compared to the other added ingredients (Table 2).

All raw doughs had significantly similar a_w values regardless of the added ingredient, with an average of 0.86. The a_w data of the baked biscuits also decreased after baking and were statistically similar in C_{SW}, AL_{SW} and PI_{SW}, while the samples with dried apricots and plums reached significantly higher values. As a result of baking, an a_w reduction of –55.6% was observed in C_{SW} and in AL_{SW}, –66.7% in PI_{SW}, –12.5% in AP_{SW} and –22.2% in PL_{SW}. As for moisture, the AP_{SW} and PL_{SW} samples underwent the lowest reduction in a_w value after baking, due to the presence of dried apricots and plums, which had higher a_w values than the other ingredients (Table 2).

The previous results were confirmed by the texture data of the AP_{SW} and PL_{SW} biscuits, whose structure was characterised by lower hardness and fracturability values compared to the other samples, suggesting that these biscuits were softer and less crunchy than the others due to the presence of dried fruit. The biscuits with almonds and pistachios had a texture with a slightly higher fracturability compared to the control biscuits, indicating a more uneven structure due to the presence of nuts grains (Table 3).

As expected, the use of enrichment ingredients in the recipes resulted in a different colour of the raw and baked biscuit surfaces. The raw biscuit C_{SW} showed higher L* and h° values compared to some of the other enriched samples, indicating a bright and intense yellow colour. The raw biscuit PL_{SW}, on the other hand, had the lowest colour parameter values, indicating a darker and less yellow colour than all other samples. This result is due to the darkest colouration of the dried plums added in the recipe (Table 2). Baking led to an increase in L* and h° values in all samples. These colour changes are due to the migration of water through the surface and the change in the surface structure of the biscuits due to the increase in volume, which resulted in a greater reflection of light compared to the raw biscuits (Romani et al., 2012). The colour differences between raw and baked biscuits are also visible in the RGB images in Fig. 1**(a)**.

In terms of pH, all raw dough of the sweet samples had values around 7, which varied slightly depending on the type of additional ingredient used in the formulation. In particular, the PL_{SW} dough (6.9) had a slightly significantly lower pH than all the others. After baking, the pH values of almost all biscuit samples increased slightly, except for the sample with plum (PL_{SW}). The baked biscuits reached pH values of 7.5 for control sample, 7.4 for AL_{SW}, 7.6 for PI_{SW}, 7.3 for AP_{SW}, and 6.8 for PL_{SW}. This increase is due to the decrease in moisture during baking, which resulted in a higher concentration of salts and other alkaline compounds (Nasser & Hammood, 2020). The slight differences in pH between the samples can be attributed to the pH of the additional ingredients (Table 2).

For the savoury biscuits types, the control sample (C_{SA}) had a weight loss of 20.1% after baking, while the other ones had a significantly higher weight loss in the range of 21.4-24.6% (Table 4). Similar to the sweet biscuits, this result can be attributed to the higher moisture loss between raw and baked biscuits, which was –44.7% for C_{SA}, –62.9% for SU_{SA}, –59.4% for SE_{SA}, –50.0% for ONB_{SA} and –54.7% for OB_{SA}. The moisture contents of the raw doughs and the corresponding baked biscuits were related to the moisture content of the additional ingredients (Table 2).

The a_w values were similar in all doughs with an average of 0.96. After baking, the reductions in a_w were –13.4, –23.0, –27.9, –13.7 and –14.5% for C_{SA}, SU_{SA}, SE_{SA}, ONB_{SA} and OB_{SA} samples, respectively. Statistical analysis revealed that the baked C_{SA} sample had a significantly higher a_w compared to SU_{SA} and SE_{SA}. Conversely, OB_{SA} and ONB_{SA} showed a_w values significantly similar to the control (Table 4).

Table 3 Weight loss, moisture, water activity, texture (hardness, fracturability) and colour (lightness, hue angle) of raw and baked sweet biscuit samples (C_{SW} = sweet control; AL_{SW} = with peeled almonds; PI_{SW} = with roasted pistachios; AP_{SW} = with dried apricots; PL_{SW} = with dried plums)

Sample	Weight loss (%)		Moisture (%)		Water activity (a_w)		Hardness (N)		Fracturability (1/mm)		L* (lightness)		h° (hue angle)	
	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked
C_{SW}	-	14.1 ± 0.7^a	18.2 ± 0.1^c	5.3 ± 0.4^c	0.9 ± 0.0^a	0.4 ± 0.0^b	-	55.8 ± 12.6^a	-	2.4 ± 0.8^b	70.9 ± 0.4^a	82.3 ± 0.8^a	82.3 ± 0.3^a	85.7 ± 0.4^a
AL_{SW}	-	13.1 ± 0.7^b	17.1 ± 0.1^d	4.9 ± 0.5^c	0.9 ± 0.0^a	0.4 ± 0.1^b	-	51.4 ± 7.9^a	-	2.6 ± 1.1^{ab}	68.8 ± 0.2^a	80.7 ± 0.5^b	81.1 ± 0.6^a	85.6 ± 0.1^a
PI_{SW}	-	13.2 ± 0.6^b	16.8 ± 0.3^d	4.7 ± 0.3^c	0.9 ± 0.0^a	0.3 ± 0.0^b	-	53.8 ± 8.7^a	-	3.9 ± 1.4^a	60.1 ± 1.5^c	73.9 ± 0.6^c	82.1 ± 0.4^a	84.9 ± 0.5^b
AP_{SW}	-	9.0 ± 0.7^c	19.0 ± 0.4^b	11.1 ± 0.7^b	0.8 ± 0.0^a	0.7 ± 0.0^a	-	15.5 ± 3.9^b	-	0.9 ± 0.2^c	63.1 ± 1.6^b	73.3 ± 1.3^c	78.8 ± 1.9^b	80.2 ± 0.9^c
PL_{SW}	-	9.0 ± 0.5^c	20.3 ± 0.2^a	12.7 ± 0.1^a	0.9 ± 0.0^a	0.7 ± 0.0^a	-	15.7 ± 2.7^b	-	0.7 ± 0.1^c	42.2 ± 1.8^d	55.6 ± 1.8^d	65.5 ± 1.2^c	71.9 ± 1.0^d

Different letters in the same column indicate significant differences between samples (Tukey's test, $p\leq0.05$)

The SU_{SA} and SE_{SA} biscuits, which had the lowest final moisture content, had a texture characterised by higher values of hardness and fracturability compared to the other samples, probably due to the presence of dried seeds.

Similar to the sweet biscuits, the additional ingredients in the savoury ones reduced the L* and h° values of the doughs, especially the black olives (ONB_{SA} and OB_{SA}), compared to the control (C_{SA}) (Tables 2 and 4). In fact, the raw biscuit C_{SA} had the highest values of L* and h°, indicating a lighter and more yellow colouration than all other samples of raw and baked biscuits. Also in this case, the L* and h° values increased after baking for all samples, but not significantly for those formulated with black olives. From the RGB images acquired by CVS (Fig. 1**(b)**), it is possible to confirm the trend of the colour parameters and the differences between the samples.

Significant differences in pH were found in the additional ingredients used, but without finding significant differences between the seeds and the olives types (Table 2). The differences found had no particular effect on the values found in the doughs, which averaged to 7.4. The baked biscuits reached pH values of 7.5 for C_{SA}, 7.3 for SU_{SA}, 7.3 for SE_{SA}, 7.5 for ONB_{SA} and 7.4 for OB_{SA}. Compared to the corresponding raw samples a slight increase in pH after baking was observed only for C_{SA}, ONB_{SA} and OB_{SA}, but this was not significant.

Acrylamide Concentration of Biscuits

The concentrations of AA found in the sweet biscuits after baking and in the additional ingredients added are shown in Fig. 2. Specifically, the concentrations of AA in the biscuits were: 284.3 ± 11.4 μg/kg in C_{SW}, 819.8 ± 147.9 μg/kg in AL_{SW}, 516.2 ± 82.4 μg/kg in PI_{SW}, 694.6 ± 53.8 μg/kg in AP_{SW} and 190.7 ± 10.2 μg/kg in PL_{SW}. The control sample (C_{SW}) had similar AA concentrations to those found in a previous study (Schouten et al., 2022a).

The significantly higher AA content of AL_{SW} and PI_{SW} biscuits compared to the control may be attributed to the use of almonds and pistachios in the recipes, which had AA contents of 551.4 ± 85.2 and 92.8 ± 7.8 μg/kg, respectively. Almonds and pistachios contain the AA precursors and due to their drying and roasting processes to improve their sensory properties and shelf-life, these nuts also contain the same AA (Žilić, 2016). The commercial peeled almonds used contained the highest AA concentration because they were probably subjected to heat treatment for drying after peeling. Indeed, the most common industrial processing of peeled almonds involves a blanching phase in hot water or steam, mechanical removal of the tegument by abrasion and a final drying phase to remove the excess water (Chen & Pan, 2022; Shakerardakani & Bagherinasab, 2019). The

Table 4 Weight loss, moisture, water activity, texture (hardness, fracturability) and colour of raw and baked savoury biscuit samples (C_{SA} = savoury control; SU_{SA} = with sunflower seeds; SE_{SA} = with sesame seeds; ONB_{SA} = with black olives without brine; OB_{SA} = with black olives in brine)

Sample	Weight loss (%)		Moisture (%)		Water activity (a_w)		Hardness (N)		Fracturability (1/mm)		L* (lightness)		h° (hue angle)	
	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked	Raw	Baked
C_{SA}	-	20.1 ± 1.5^{d}	34.0 ± 0.4^{b}	18.8 ± 1.3^{a}	0.9 ± 0.0^{a}	0.8 ± 0.0^{a}	-	40.8 ± 9.4^{b}	-	0.2 ± 0.1^{c}	83.9 ± 0.4^{a}	85.8 ± 0.7^{a}	88.5 ± 0.4^{a}	90.8 ± 1.0^{a}
SU_{SA}	-	22.2 ± 1.3^{bc}	30.6 ± 0.3^{c}	11.3 ± 0.6^{b}	0.9 ± 0.1^{a}	0.7 ± 0.0^{b}	-	71.9 ± 6.6^{a}	-	0.4 ± 0.1^{a}	75.7 ± 1.8^{b}	82.0 ± 1.7^{b}	85.5 ± 0.5^{b}	88.9 ± 0.4^{b}
SE_{SA}	-	21.4 ± 0.9^{c}	30.8 ± 0.3^{c}	12.5 ± 0.3^{b}	0.9 ± 0.0^{a}	0.7 ± 0.0^{b}	-	75.4 ± 11.1^{a}	-	0.5 ± 0.1^{a}	76.4 ± 0.8^{b}	82.4 ± 0.8^{b}	87.7 ± 0.8^{a}	90.2 ± 0.4^{a}
ONB_{SA}	-	23.4 ± 0.9^{ab}	37.5 ± 0.2^{a}	18.8 ± 1.1^{a}	0.9 ± 0.0^{a}	0.8 ± 0.0^{a}	-	40.1 ± 8.2^{b}	-	0.3 ± 0.1^{b}	71.4 ± 0.4^{c}	71.5 ± 0.8^{c}	80.1 ± 0.4^{d}	81.5 ± 0.3^{d}
OB_{SA}	-	24.6 ± 1.8^{a}	37.8 ± 0.3^{a}	17.1 ± 0.2^{a}	0.9 ± 0.0^{a}	0.8 ± 0.0^{a}	-	46.7 ± 9.3^{b}	-	0.3 ± 0.1^{b}	68.9 ± 0.6^{d}	69.4 ± 2.3^{d}	82.3 ± 0.8^{c}	82.5 ± 1.0^{c}

Different letters in the same column indicate significant differences between samples (Tukey's test, $p\leq0.05$)

formation of AA after the thermal processes of almonds is determined by their high free asparagine content, which can reach 2760 mg/kg (Amrein et al., 2005). The lower AA content in commercial roasted pistachios compared to almonds and thus in PI_{SW} biscuits could be related to the lower asparagine concentration in this nut, which can be around 112 mg/kg (Hou et al., 2019). The amount of AA, found in commercial hot air, infrared and microwave roasted pistachios ranged from 57 to 851 µg/kg. Infrared roasted pistachios had the highest AA content, while microwave roasted pistachios yielded lower concentrations (Asadi et al., 2020). It has also been reported that roasting conditions (time, temperature, voltage and power etc.) can significantly affect the AA formation (Asadi et al., 2020), suggesting that the AA content in commercial nuts depends on the conditions used by the companies.

Comparing the concentrations of AA in the nuts with those in the corresponding biscuits, it appears that the pistachios, despite their significantly lower AA content, contributed to a greater increase in AA in the corresponding biscuit (+455%) than the almonds (+49%). This could probably be due to the presence of some free precursors in the pistachios or other compounds that may have reacted with other ingredients in the biscuit matrix during the baking process. Nevertheless, further studies are needed to confirm this hypothesis.

The AA level found in commercial dried apricots was of 59.7 ± 3.0 µg/kg, within the range of 13-100 µg/kg found by Surma and co-workers (2018) in their analysis of 6 different varieties of dried apricots. In dried fruits, AA forms as a result of the long drying processes that the fruits undergo to reduce the moisture content to about 20% (Amrein et al., 2007; Surma et al., 2018). In fact, dried plums may also contain AA after the drying process (Amrein et al., 2007; Constantin et al., 2018; De Paola et al., 2017; Surma et al., 2018; Žilić, 2016). Amrein et al. (2007) found concentrations in dried plums ranging from 730 to 1680 µg/kg AA, while a more recent study by Surma et al. (2018) measured lower average AA values of 43.9 µg/kg, similar to the concentrations found in the commercial dried plums used in the present study, namely 39.8 ± 1.2 µg/kg. These very wide AA concentration ranges in dried fruits can be attributed to the use of different varieties with different precursor concentrations as well as to the drying conditions applied. Comparing some properties of the commercial dried fruit ingredients used in sweet biscuit formulations (Table 2), it is observed that dried plums have a higher moisture content compared to apricots, indicating a lower degree of dryness and a lower pH (3.7), which could contribute to slowing down the Maillard reactions during the drying process.

The biscuits enriched with dried apricots (AP_{SW}) had similarly high AA values to the AL_{SW} sample, despite a

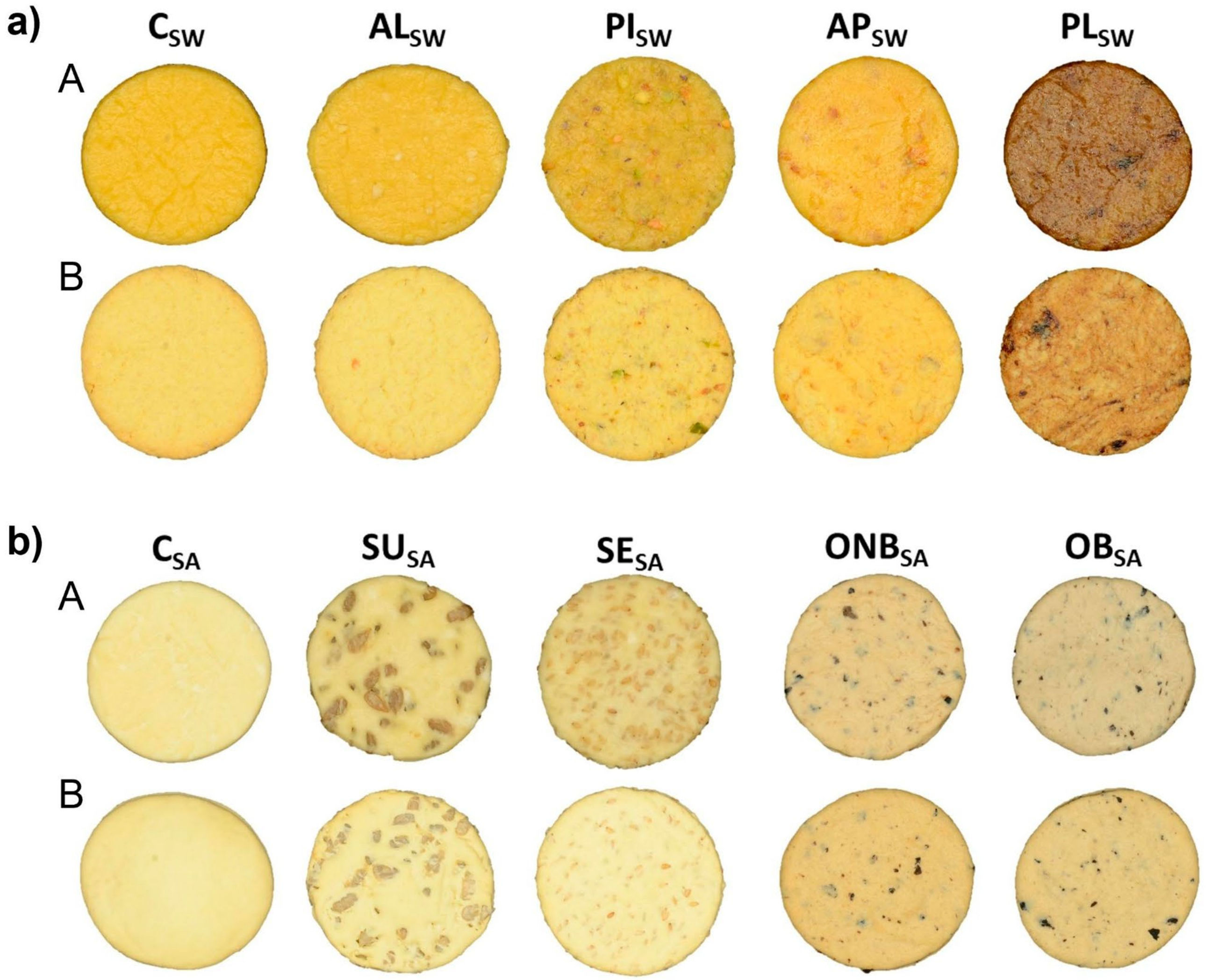

Fig. 1 Visual appearance of the raw (**A**) and baked (**B**) sweet (**a**) and savoury biscuits (**b**) (sweet biscuits: C_{SW} = sweet control; AL_{SW} = with peeled almonds; PI_{SW} = with roasted pistachios; AP_{SW} = with dried apricots; PL_{SW} = with dried plums; savoury biscuits: C_{SA} = savoury control; SU_{SA} = with sunflower seeds; SE_{SA} = with sesame seeds; ONB_{SA} = with black olives without brine; OB_{SA} = with black olives in brine)

lower AA concentration in the commercial dried apricots, and a higher AA value than the PLsw sample, despite similarly low AA values of the apricot and plum ingredients. The increase in AA content between AP and AL ingredients and the corresponding biscuits was of + 1060 and + 49% for the AP_{SW} and AL_{SW} samples, respectively. This result could be due to the large supply of sugars from the dried apricots in the AP_{SW} biscuit dough, which may have promoted stronger Maillard reactions during baking (Surma et al., 2018). On the other hand, the lower increase in AA content in the PL_{SW} sample (+ 379%) compared to that in AP_{SW} could be mainly due to the higher moisture and lower pH of the PL_{SW} biscuits. Indeed, significant negative and positive correlations were found between the values of moisture and pH of the biscuits and their AA concentrations with an *r*-value of –0.60 and 0.71, respectively. It is known that the rate of Maillard reactions is reduced at high moisture content and low pH conditions, reducing the concentration and interaction of AA precursors (Mesías & Morales, 2015; Schouten et al., 2022b). In addition, as for the PL_{SW} sample, the antioxidant activity of the dried plums may also have influenced the lowest AA concentration in this biscuit type. This is because the antioxidant compounds present in the biscuit matrix can react with the precursors of AA, reducing the formation of this toxic compound (AL-Ansi et al., 2019). Recent studies have confirmed that dried fruits can be considered a rich source of polyphenols with antioxidant activity (Rybicka et al., 2021; Srednicka-Tober et al., 2020). Srednicka-Tober et al. (2020) found a polyphenol content of 219.03-296.96 mg/100 g in dried apricots and

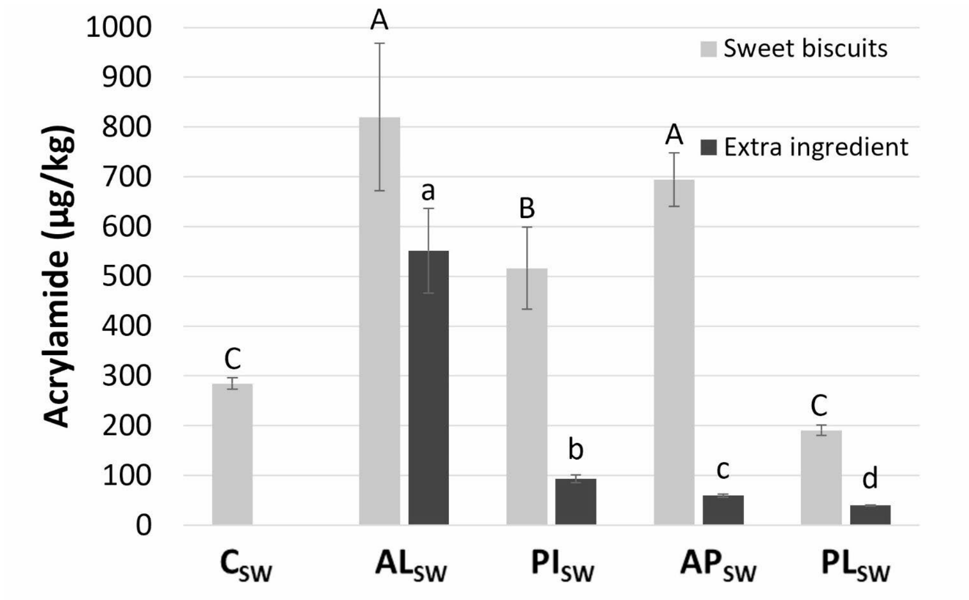

Fig. 2 Acrylamide concentration in baked sweet formulated biscuits (C_{SW} = sweet control; AL_{SW} = with peeled almonds; PI_{SW} = with roasted pistachios; AP_{SW} = with dried apricots; PL_{SW} = with dried plums) and in the respective additional ingredients. Different lower or capital letters indicate significant differences among the samples (Tukey's test, $p \leq 0.05$)

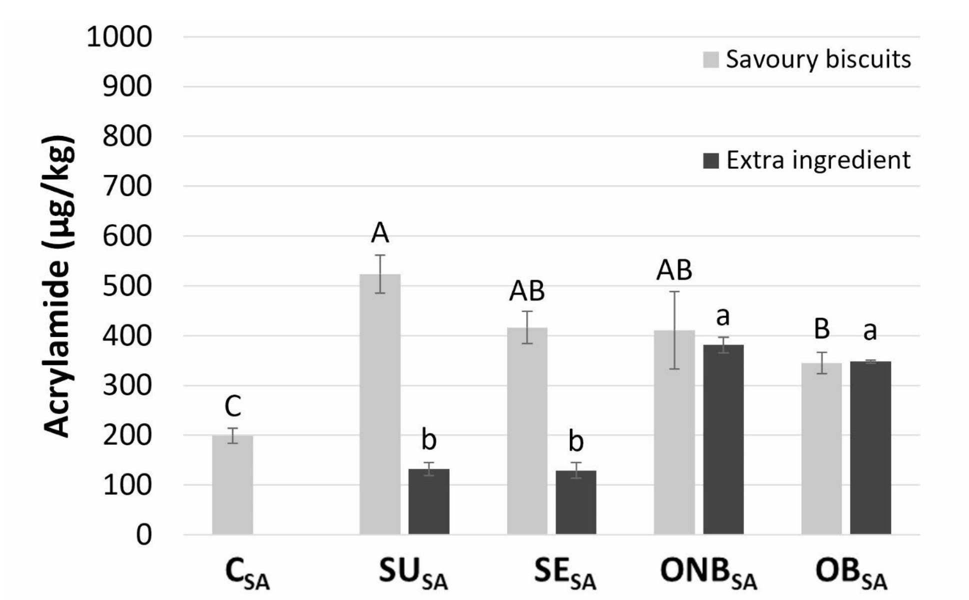

Fig. 3 Acrylamide concentration in baked savoury formulated biscuits (C_{SA} = savoury control; SU_{SA} = with sunflower seeds; SE_{SA} = with sesame seeds; ONB_{SA} = with black olives without brine; OB_{SA} = with black olives in brine) and in the respective additional ingredients. Different lower or capital letters indicate significant differences among the samples (Tukey's test, $p \leq 0.05$)

134.65-422.44 mg/100 g in dried plums. However, there are differences in total phenolic content and antioxidant activity depending on genotype, cultivation system (organic or conventional) and years, other than ripening time of the fruits (Ceccarelli et al., 2021; Rybicka et al., 2021; Srednicka-Tober et al., 2020). It is likely that the commercial plums used to prepare the PL_{SW} sample also have higher antioxidant activity than the apricots used for the AP_{SW} sample, apart from the higher moisture content and lower pH.

The concentrations of AA in savoury biscuits after baking and in the additional ingredients added are shown in Fig. 3. All savoury biscuits had a significantly lower mean concentration of AA than sweet biscuits (378.8 ± 119.2 vs. 501.1 ± 256.8 µg/kg); this is probably due to the lower proportion of sugars in the savoury biscuit recipes. The AA concentration determined in the control sample (C_{SA}) was 198.9 ± 15.6 µg/kg, similar to the concentration found in commercial salted crackers by Nematollahi et al. (2019), which averaged 200.7 µg/kg. All other enriched biscuits showed significantly higher AA concentrations than the control, namely 523.4 ± 38.5 µg/kg in SU_{SA}, 416.4 ± 32.2 µg/kg in SE_{SA}, 410.4 ± 77.9 µg/kg in ONB_{SA} and 344.9 ± 21.0 µg/kg in OB_{SA}. In general, also in this case, the higher AA content found in the enriched savoury biscuit samples can be partly attributed to the AA concentration of the additional ingredients.

Compared to C_{SA} biscuits, the addition of 10% of the sunflower and sesame seeds in the recipe resulted in an increase in the formation of AA by +160 and +110% in SU_{SA} and

SE_{SA} biscuits, respectively. Specifically, the commercial sunflower and sesame seeds had an AA concentration of 132.4 ± 13.1 and 129.2 ± 15.8 μg/kg, respectively, which is consistent with the concentration ranges determined by Liu et al. (2023), Nematollahi et al. (2020) and Berk et al. (2019). The AA in roasted or dried seeds is due to the presence of precursors and the heat treatments to which the seeds are subjected, although different concentrations may be obtained depending on the species and thermal conditions (Berk et al., 2019, 2020; Liu et al., 2023; Nematollahi et al., 2020; Salamatullah et al., 2021). In the study by Berk et al. (2020), no reducing sugars were detected in sesame seeds, leading to the consideration that some carbonyl compounds might be formed during thermal treatments as a result of sucrose decomposition. In addition, hydroxymethylfurfural (HMF) that can be formed during sesame seeds roasting, appears to be a critical intermediate of AA by reacting with free asparagine. Sesame and sunflower seeds are rich in fat (about 50%) but also in antioxidant compounds, so oxidation products may be considered a limited pathway for the formation of AA during their processing and use (Berk et al., 2020).

The savoury biscuits enriched with black olives (ONB_{SA} and OB_{SA}) also had significantly higher AA concentrations than the control sample of about + 110 and + 70%, respectively. The commercial table olives showed high AA concentrations of 381.1 ± 15.8 μg/kg and 357.7 ± 3.3 μg/kg for the ones without brine and with brine, respectively. These values are within the wide range of 172-1112 μg/kg found in a recent study by Duedahl-Olesen et al. (2022), which analysed several black sterilised table olives from the Danish market. The high content of AA in the commercial black olives used is due to the California-style process, a method used worldwide to preserve olives (Charoenprasert & Mitchell, 2014; Duedahl-Olesen et al., 2022). This type of olive processing includes treatment with lye, air oxidation, neutralisation with alkali, use of ferrous gluconate, heating to obtain a stable black colour and sterilization that can promote the AA formation. Studies examining specific processing steps have discovered that the application of minor changes during the curing process (e.g. washing the raw olives, shortening the oxygen exposure time, increasing the pH prior sterilisation, reducing the sterilisation temperature and time, using additives) can reduce the AA content in table olive products (Amrein et al., 2007; Casado et al., 2010; Martín-Vertedor et al., 2021). Since no correlation between reducing sugars and asparagine has been demonstrated (Amrein et al., 2007), it was hypothesised that some compounds formed during oxidation treatments could be converted to AA during sterilisation. However, the reaction mechanism for the formation of AA in olives is not yet fully understood (Duedahl-Olesen et al., 2022). Pérez-Nevado et al. (2018), Fernández et al. (2022) and Duedahl-Olesen et al. (2022) have demonstrated that heating black table olives in the oven increases the final AA concentration. Thus, some table olive products contained AA concentrations from below the detection limit up to 1100 μg/kg and after baking in the oven at 150-270 °C for time intervals of 7-21 min, the concentrations ranged from 24 to 18,300 μg/kg (Duedahl-Olesen et al., 2022). In the present study, both the concentration of AA found in the olive ingredients and the baking process could be the causes of the higher content of AA in the ONB_{SA} and OB_{SA} samples compared to the control samples (C_{SA}). Several studies have shown that the storage of olives in brine, in contrast to storage without brine, leads to a decrease in AA values during the shelf-life of the product, that was attributed to the fact that the toxic compound is diffused from the fruit to the liquid by diffusion (Charoenprasert & Mitchell, 2014; Fernández et al., 2022; Martín-Vertedor et al., 2020). However, in the present study, the commercial olives in brine had a slightly lower and non-significant concentration of AA compared to olives without brine, resulting in similar levels of AA in the biscuit samples enriched with olives.

Comparing the concentrations of AA in the seeds (SU, SE) and olives (ONB, OB) with those in the corresponding biscuits, it appears that both olives, despite their higher content of AA, contributed to a smaller increase of AA in the corresponding biscuits (on average + 7%) than seeds (on average + 260%). This result could be due to the fact that the olives-enriched biscuits had a significantly higher moisture content than the seeds-enriched biscuits (Table 4), which is also confirmed by a significant negative correlation between the concentrations of AA and the moisture content of the baked savoury biscuits (*r*-value of –0.71). However, in contrast to the sweet biscuits, no significant correlation was found between the pH values and the concentrations of AA in the baked savoury biscuits, as the pH values of the latter were similar.

Conclusion

From the present research, it can be concluded that the addition of 10% of enrichment ingredients such as apricots, plums, almonds, pistachios or sunflower seeds, sesame seeds and black olives to the formulation of sweet and savoury biscuits resulted in different final AA levels.

The sweet biscuits enriched with peeled almonds, roasted pistachios and dried apricots, exhibited an AA content higher than 350 μg/kg, the benchmark value set in Regulation (EU) 2017/2158 for this product category. Contrary to expectations, the sweet biscuits formulated with dried plums had an AA content of less than 350 μg/kg, similar to the control biscuit sample. The savoury biscuits had an average lower AA concentration than the sweet ones, which can be attributed to a lower sugars content in their recipes. The biscuits enriched with sunflower seeds, sesame seeds and black olives without brine slightly exceeded the AA benchmark value of 400 µg/kg for 'non-potato based crackers' (EU Regulation 2017/2158). The savoury biscuits with black olives stored in brine contained AA less than 400 µg/kg but more than the control sample.

The AA content found in the sweet and savoury biscuits can be attributed to the inclusion of the enrichment ingredients, which serve as sources of both AA precursors (i.e. asparagine and sugars) and AA itself, resulting from the processes to which they are subjected, such as dehydration, blanching, roasting, sterilisation, etc. Furthermore, these ingredients influenced some qualitative characteristics of the final products, including moisture and pH, which additionally contributed to affect the final AA content of the baked biscuits in different ways. However, the presence of AA free precursors and possible antioxidant compounds in the enrichment ingredients still needs to be verified by further research to confirm certain results and assumptions.

In summary, this study underlines how difficult it is to accurately predict the final AA content in bakery products knowing only the AA concentration in the ingredients used, as other factors related to general matrix properties must also be taken into account. This highlights the need to monitor not only the presence of AA in some ingredients, but also how these components react with other ingredients used in rich recipes during the different processing steps.

Authors' Contributions Maria Alessia Schouten: investigation, data curation, formal analysis and writing an original draft; Agnese Santanatoglia: investigation, methodology and data curation; Simone Angeloni: investigation, methodology and data curation; Massimo Ricciutelli: investigation and methodology; Laura Acquaticci: investigation and methodology; Giovanni Caprioli: validation of methodology and supervision; Sauro Vittori: validation of methodology and supervision; Santina Romani: conceptualization, purchase of necessary materials and supervision. All authors have reviewed and edited the manuscript.

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Declarations

Competing Interests The authors declare no competing interests.

Ethics Approval Not applicable.

Consent to Participate Not applicable.

Consent for publication The content of the submitted article has been carefully examined and approved by all authors who are all aware of its submission to this journal.

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