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Prediction of acrylamide formation in biscuits based on fingerprint data generated by ambient ionization mass spectrometry employing direct analysis in real time (DART) ion source

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ABSTRACT

The objective of this study is the evaluation of the potential of high-throughput direct analysis in real time–high resolution mass spectrometry (DART–HRMS) fingerprinting and multivariate regression analysis in prediction of the extent of acrylamide formation in biscuit samples prepared by various recipes and baking conditions. Information-rich mass spectral fingerprints were obtained by analysis of biscuit extracts for preparation of which aqueous methanol was used. The principal component analysis (PCA) of the acquired data revealed an apparent clustering of samples according to the extent of heat-treatment applied during the baking of the biscuits. The regression model for prediction of acrylamide in biscuits was obtained by partial least square regression (PLSR) analysis of the data matrix representing combined positive and negative ionization mode fingerprints. The model provided a least root mean square error of cross validation (RMSECV) equal to an acrylamide concentration of 5.4 μg kg^{-1} and standard error of prediction (SEP) of 14.8 μg kg^{-1}. The results obtained indicate that this strategy can be used to accurately predict the amounts of acrylamide formed during baking of biscuits. Such rapid estimation of acrylamide concentration can become a useful tool in evaluation of the effectivity of processes aiming at mitigation of this food processing contaminant. However, the robustness this approach with respect to variability in the chemical composition of ingredients used for preparation of biscuits should be tested further.

1. Introduction

Acrylamide (prop-2-enamide) is a processing contaminant formed in starch-rich heat-treated foods mainly from asparagine and reducing sugars and other reactive carbonyls via Maillard reaction (Arvanitoyannis & Dionisopoulou, 2014; Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002). Consumers' dietary exposure to acrylamide has become of high concern because of its classification as a probable human carcinogen by the International Agency for Research on Cancer (IARC) (IARC, 1994). The main pathway for acrylamide formation in heat-processed foods is the Maillard reaction in which free amino acids react with reducing sugars or other reactive carbonyls (Mottram et al., 2002; Stadler et al., 2002). The European Food Safety Authority (EFSA) has reported that acrylamide is present in many thermally-processed foods, including cereal based products such as biscuits, breakfast cereals and bread (EFSA, 2011). Based on the results of monitoring of 426 samples in 2010 by the European Union's member states, the mean concentration of acrylamide in biscuits, crackers, crisp breads and similar products was found to be 333 μg kg^{-1} (EFSA, 2012). The acrylamide concentrations found for 12% of the samples examined exceeded the indicative value of 500 μg kg^{-1} established by the European Commission Recommendation (2010/307/EU) (EC, 2011). Strategies to reduce acrylamide content in various food commodities have been summarised in the 'Toolbox' published by the Food Drink Europe (FDE, 2011). In any case, content of both precursors and acrylamide content should be controlled whenever raw material or processing conditions are changed.

A number of methods are available for the determination of acrylamide in foods. The most widely used approaches are based on chromatographic techniques coupled to mass spectrometric detection such as liquid chromatography–mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS) (Arvanitoyannis & Dionisopoulou, 2014). Despite their advantages of selectivity, sensitivity and accuracy, these methods may be

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time-consuming and expensive because of the need for extensive and laborious sample pre-treatment. Therefore, alternative workflows that allow faster assessment of acrylamide concentrations in food samples are needed. Studies describing the combined use of data obtained by rapid methods such as near infrared spectroscopy (NIR) (Pedreschi, Segtnan, & Knutsen, 2010), front face fluorescence spectroscopy (Rizkallah et al., 2008), or colour analysis (Lu & Zheng, 2012) with multivariate models have been reported for the prediction of acrylamide contents in foods.

The introduction of ambient ionization techniques, such as direct analysis in real time (DART) (Cody, Laramee, & Durst, 2005) and desorption electrospray ionization (DESI) (Takats, Wiseman, Gologan, & Cooks, 2004) has greatly simplified mass spectrometric analysis. Ambient mass spectrometry enabled sample examination in an open laboratory environment by exposing the sample to a stream of desorbing/ionising medium in proximity to the inlet of the mass spectrometer (Gross, 2014). This experimental setup typically requires only minimal sample preparation, omits the (chromatographic) separation and enables high-throughput measurements. In DART, excited-state metastable atoms or molecules of gas (helium or nitrogen) are used as the medium for ionisation. Sample introduction is typically performed with melting point capillaries that are used to transfer liquid or solid samples spread on their surface into the DART ionization region (Hajslova, Cajka, Vaclavik, 2011). This technique when coupled to high resolution mass spectrometry (HRMS) has been shown to have a great potential for rapid fingerprinting of various types of food samples (Cajka, Riddellova, Tomaniova, & Hajslova, 2011; Hrbek, Vaclavik, Elich, & Hajslova, 2014; Vaclavik, Belkova, Reblova, Riddellova, & Hajslova, 2013). The DART–HRMS fingerprinting workflow typically comprises the following steps: (i) preparation of samples employing a generic extraction procedure to isolate as many compounds as possible; (ii) instrumental analysis of the samples to record mass spectra characterising chemical composition of the test material; (iii) processing of the mass spectral records (*e.g.* background subtraction) followed by selection of ions across the examined sample set to form data matrix; and (iv) analysis of the data with chemometric tools.

In the present study, a procedure based on the DART–HRMS technique has been developed and applied to high-throughput MS fingerprinting of biscuit samples prepared by two recipes and baked under various conditions of time and temperature. The multidimensional data thus obtained were further analysed with multivariate regression analysis methods to accurately predict acrylamide concentrations in biscuits.

2. Materials and methods

2.1. Preparation of biscuits

The preparation of biscuits was identical with that described by Van Der Fels-Klerx et al. (2014). Biscuits were prepared using two different recipes, which were adapted from the American Association of Cereal Chemists (AACC) Method 10–54 dedicated for testing of baking quality of cookie flour (AACC, 2000). Biscuits prepared with recipe 1 (R_1) were prepared using standard T55/W150 flour (80 g), refined palm oil (20 g), sucrose (35 g), sodium bicarbonate ($NaHCO_3$, 0.8 g), water (17.6 g), ammonium bicarbonate (NH_4HCO_3, 0.4 g) and sodium chloride (NaCl, 1 g). Biscuits prepared with recipe 2 (R_2) contained all of the above ingredients with the exception of NaCl. Refined palm oil was first mixed with sucrose, NaCl and $NaHCO_3$ in a mixer. Mixing was completed in three consecutive steps of 1 min each. Dissolved NH_4HCO_3 was then added and mixed in three consecutive steps, 20 s each. Finally, flour was added and mixed during three consecutive steps of 10 s each. After each mixing step, the dough was peeled off of the mixer in order to achieve good homogeneity. The final dough was portioned at disks with a fixed diameter of 5 cm and a thickness of 3 mm. Biscuits were baked at temperatures of 180, 190 and 200 °C in a conventional oven for 8, 12 and 15 min. Baking experiments were performed in triplicate. Altogether 54 samples were available for analyses. Each replicate sample was examined by DART–HRMS and ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS) to obtain mass spectral fingerprints and concentrations of acrylamide, respectively.

2.2. DART–HRMS-based fingerprinting

A homogenised biscuit sample (2 g) was transferred into a 15 mL polypropylene centrifuge tube and hand-shaken for 2 min with 6 mL of methanol–water mixture (50:50, v/v). The extraction mixture was centrifuged (4 min, 11,000 rpm) and an aliquot of supernatant transferred into a well of a 96 deepwell micro-plate (Life Systems Design, Merenschwand, Switzerland).

DART–HRMS analyses were performed using a DART–HRMS system consisting of a DART SVP ion source (IonSense, Saugus, MA, USA) coupled to the Exactive benchtop orbital ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). A Vapur gas ion separator interface (IonSense, Saugus, MA, USA) was employed to hyphenate the ion source and the mass spectrometer. A low vacuum in the interface chamber was maintained by a membrane pump (Vacuubrand, Wertheim, Germany). The DART–HRMS instrument was operated in positive or negative ionization mode with the following settings: (i) DART ionization: helium flow: 2.5 L min^{-1}; gas temperature: 350 °C; discharge needle voltage: −5000 V; grid electrode: ±350 V; (ii) mass spectrometric detection: capillary voltage: ±60 V; tube lens voltage: ±110 V; capillary temperature: 250 °C. Sheath, auxiliary and sweep gases were disabled during the analysis. The mass spectra were recorded in the *m/z* range 50–1000 at an acquisition rate of 2 Hz. The mass resolving power of the instrument calculated at *m/z* 200 was 50,000 FWHM (full width at half maximum). The methanol-aqueous extracts of biscuits were delivered to the DART ionization region with the use of a 12 Dip-It tip scanner autosampler. Twelve Dip-It tips (IonSense, Saugus, MA, USA) were inserted into a holder and immersed in sample extracts placed in deepwell micro-plates. The holder was mounted on the body of the autosampler. Subsequently, the Dip-It tips automatically moved at a constant speed of 0.5 mm s^{-1} through the helium gas stream perpendicularly to the axis leading from the DART gun exit to the mass spectrometer inlet. The time of desorption from the surface of each tip was 12 s. Two parallel analyses were performed for each of the biscuit samples. The measurement sequence of the samples was random (*i.e.*, established by random number generation).

2.3. UHPLC–MS/MS analysis of acrylamide

The extraction of acrylamide from biscuits samples was performed using a procedure described by Gökmen, Morales, Ataç, Serpen, and Arribas-Lorenzo (2009). One biscuit per time point, recipe, baking temperature, and replicate was analysed. A homogenised sample (1 g) was extracted with 20 mL of 10 mM formic acid in three stages employing 10, 5, and 5 mL. The first extraction was carried out with 9 mL of 10 mM formic acid, 0.5 mL Carrez I and 0.5 mL Carrez II solutions. Each extract was centrifuged at 6080*g* for 10 min. Combined extracts were further centrifuged (11,180*g*, 5 min). An aliquot of supernatant (2 mL) was passed through an Oasis MCX solid phase extraction cartridge previously conditioned with 1 mL of methanol and 1 mL distilled water. First eight drops of the eluent were discarded and the remaining volume

was collected in an autosampler vial and analysed by UHPLC–MS/MS.

A Waters Acquity H Class UPLC System coupled to an Acquity TQD (Waters, Milford, MA, USA) triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operated in positive mode was used. The chromatographic separation was performed using an Acquity UPLC HSS T3 analytical column (100×2.1 mm i.d., 1.8 μm; Waters, Milford, MA, USA). The isocratic elution was performed with 10 mM aqueous formic acid containing 0.5% methanol at a flow rate of 0.3 mL min^{-1}. The autosampler and the column compartment were maintained at 10 and 40 °C, respectively. The following ESI settings were used: capillary voltage: 0.80 kV; cone voltage: 21 V; extractor voltage: 4 V; source temperature: 120 °C; desolvation temperature: 450 °C; desolvation gas (nitrogen) flow rate: 900 L h^{-1}. Argon was used as the collision gas at a flow rate of 0.25 mL min^{-1}. Data acquisition was performed in multiple reaction monitoring (MRM) mode by recording two MRM transitions: *m/z* 72 > 55 (quantifier, collision energy 9 V) and *m/z* 72 > 44 (qualifier, collision energy 12 V). A dwell time of 200 ms was used for both MRM transitions. Acrylamide was identified on the basis of retention time and the intensity ratio of the quantifier and qualifier transitions. The concentration of acrylamide was calculated on the basis of a linear regression equation obtained from the calibration curve constructed in the concentration range 1–100 ng mL^{-1}. The limit of detection (LOD) and limit of quantitation (LOQ) were 3 and 10 μg kg^{-1}, respectively. Each sample was analysed in triplicate and the resulting acrylamide concentrations were averaged.

2.4. Data processing and statistical analysis

The raw DART–HRMS data processing and elemental formulae calculations were performed with the use of the Xcalibur software (version 2.1, Thermo Fisher Scientific, San Jose, CA, USA). The DART mass spectra recorded across the desorption peaks were background-subtracted. Lists of 55 and 83 ions including their *m/z* values and intensities were obtained in positive and negative mode, respectively, based on a manual inspection of the mass spectra recorded during analysis of biscuits prepared and baked under different condition. Only ions with relative intensities ⩾5% in a record of at least one sample were selected. In the next step, normalisation using the constant row sum (*i.e.*, each ion intensity was divided by the sum of all intensities for each sample and multiplied by a factor of 1 $e10^4$) was performed for each sample/DART sample introduction. Data obtained by repeated analyses were averaged. The data were organised into an $\{x \times y\}$ matrix form containing the response for the *y* ions (variables) in each of *x* samples. Matrices were compiled either separately for data recorded in positive $\{54 \times 55\}$ and negative $\{54 \times 83\}$ mode or in combination (*i.e.*, with combined data recorded under both polarities $\{54 \times 138\}$).

Principal component analysis (PCA) was performed to screen combined positive and negative ionization mode multivariate data for outliers and to explore the presence of any clustering behaviour (Berruetta, Alonso-Salces, & Herberger, 2007). Partial least square regression (PLSR) and principal components regression (PCR) analyses were performed to construct regression models for the prediction of acrylamide concentrations in the biscuits. The data were divided into a training set (containing a random selection of 40 samples corresponding to approximately 75% of the samples) and a validation set (containing the remaining 14 samples), respectively. A stratified random sampling approach was followed to ensure a balanced number of biscuit samples from each combination of baking time and baking temperature and formulation. The training set of samples was used to develop the models. The optimal number of factors for each regression model was chosen based on the least root mean square error of cross validation (RMSECV). A "leave 10% out" cross-validation procedure was used for that purpose. However, when the RMSECV were not significantly different (F test, $p < 0.05$), the least number of factors was chosen. One optimised in terms of number of factors the models were then externally validated with the validation set. The accuracy of the models was evaluated by mean of the root mean standard error of prediction (SEP). Three different data transformation techniques, namely mean-centering, autoscaling (scaling to unit variance) and orthogonal signal correction (OSC) were tested. The models were developed based on either: (i) positive ionization mode data, (ii) negative ionization data, and (iii) combined positive and negative ionization data. PCA as well as PLSR and PCR models were performed in Pirouette 4.5 (Infometrix, Seattle, USA).

3. Results and discussion

As mentioned in Section 1, acrylamide represents one of the (minor) Maillard reaction products (Mottram et al., 2002; Stadler et al., 2002). A wide range of factors has been recognised to influence the reaction pathways resulting in formation of this processing contaminant. A rapid estimation of acrylamide concentrations may enable a flexible feedback within the mitigation process. The analysis of small molecules, such as acrylamide, in complex food matrices and at relatively low concentrations levels is not an easy task, specifically due to complicated sample preparation. Therefore, introduction of a simple, indirect sample screening for an extent of acrylamide formation has been considered as a challenge.

The approach presented in this study was based on an assumption that detection of a broad range of reactants, reaction intermediates, and products of heat-induced reactions may provide relevant information for estimation of acrylamide content in baked cookies. Acquisition of sample fingerprints by ambient mass spectrometry, *i.e.* by the technique allowing a significant simplification of sample preparation step and omitting chromatographic separation, has been identified as an approach of a choice. The method implementation and the use of generated data for construction of models for prediction of acrylamide are described in the paragraphs below.

3.1. DART–HRMS fingerprints

In the first phase of experiments, the composition of extraction solvent and DART settings were optimised. Since the primary aim of these experiments was to obtain mass spectra containing the highest possible amount of information on chemical composition of the samples, the extraction and ionization were tuned towards the highest number of detected ions. The method was optimised with the analysis of biscuits baked at 12 min at 180 °C and all the ions with relative intensities ⩾5% were considered when evaluating the number of signals detected in the DART mass spectra.

To avoid any discrimination of sample components available for the follow-up instrumental analysis, the extraction and removal of solid particles by centrifugation were the only sample preparation steps employed. Polar solvents were used to isolate the soluble components from biscuits because of rather polar nature of the majority of reactants, intermediates and products of the Maillard reaction (Martins, Jongen, & van Boekel, 2001). Pure methanol, deionised water and their mixture (50:50, *v/v*) were tested for this purpose. Only small differences in terms of the number of detected ions were observed among the DART–HRMS records of the extracts prepared using the above solvents. The highest number of detected ions was achieved in methanol–water mixtures providing 9–12% and 11–15% more ions in positive and negative ionization mode, respectively, as compared to pure methanol or aqueous extracts. The presence of organic solvent in methanol–water extracts

resulted in lower boiling points of the sample extracts and more effective thermo-desorption, thus providing absolute ion intensities 2–15-fold higher (depending on the particular ion and ionization mode) than those observed in extracts prepared with water only and analysed at identical ionisation gas temperature. The key parameter tuned within the optimisation of detection process was DART ionization temperature. In agreement with previous reports (Vaclavik, Mishra, Mishra, & Hajslova, 2013), this parameter had a dramatic impact on the DART mass spectra. Both the number and intensities of detected ions increased with the gas temperature up to 350 °C, which was chosen as an optimal setting. At higher gas temperatures, the number of ions decreased, probably due to thermal degradation of the components in the extract. No ions above *m/z* 500 were detected regardless the temperature tested. The mass spectra of extracts prepared from biscuits baked for 12 min at 180 °C (recipe R_1) recorded in positive and negative mode obtained by the optimised method are shown in Fig. 1.

The repeatability of the DART–HRMS measurements was characterised based on analyses of samples prepared by parallel extractions ($n = 12$) of a biscuit sample. The standard deviations (SD) of relative intensities of observed ions depended on the abundance of the particular ion (the higher the abundance, the lower the SD) and ranged from 2.6% to 22.5%. Under optimised conditions, the baseline width of the desorption peaks was at least 10 s which enabled recording of ~20 data points across each peak.

The manual examination of mass spectra collected across the entire sample set revealed numerous ions relative and/or absolute intensities of which increased or decreased with the prolonged heat-treatment during the baking of the biscuits. These dynamics of changes in relative intensities and absolute responses observed in extracted ion chronograms for ions detected at *m/z* 90.0550 and 127.0387 in positive ionization mode are shown in Fig. 2. The high mass resolving power of the mass spectrometer employed in this study allowed for estimation of their elemental compositions and tentative identification. Using the information on accurate masses, signals observed at *m/z* 90.0550 and 127.0387 were tentatively identified as $[M+H]^+$ ions of the amino acid alanine and hydroxymethylfurfural (HMF), a product of Maillard reaction and caramelisation. As can be seen in Fig. 2A, the relative intensity of the ion assigned as alanine decreased with prolonged baking time, probably due to reaction of this amino acid with available carbonyls within the Maillard reaction, while the intensity of the ion assigned as HMF increased. In addition to these "markers", some additional positive and negative mode ions that exhibited dynamic changes in their intensities, were also tentatively identified. These included amino acids, products and intermediates of Maillard reaction, saccharides and fatty acids. The overview of these ions and trends observed in their relative intensities during prolonged baking time are provided in Table 1. The increase in relative intensities of ions presumably arising from hexose and pentose can be attributed to the hydrolysis of sucrose (Ameur, Trystram, & Birlouez-Aragon, 2006), while the decrease in intensity of free fatty acids may be due to their heat-induced reactions, such as oxidation or polymerisation (Silvagni, Franco, Bagno, & Rastrelli, 2012). It is important to note that sample exposure to high temperatures during the DART thermo-desorption (~12 s) might have resulted in in-source decomposition of compounds or formation of some artifacts not originally present in the sample extracts. Despite this drawback, the fingerprint data reflected the chemical differences among biscuits prepared and baked under different conditions, as demonstrated with the use of multivariate statistics and as discussed in the paragraphs below.

3.2. Concentration of acrylamide in biscuits

The acrylamide concentrations in biscuit samples ranged from <10 to 214 μg kg^{-1} (see Table S1 in Supplementary material). As expected, acrylamide concentrations were positively correlated to both baking time and temperature. The highest amounts of acrylamide were therefore observed in samples baked at 200 °C for

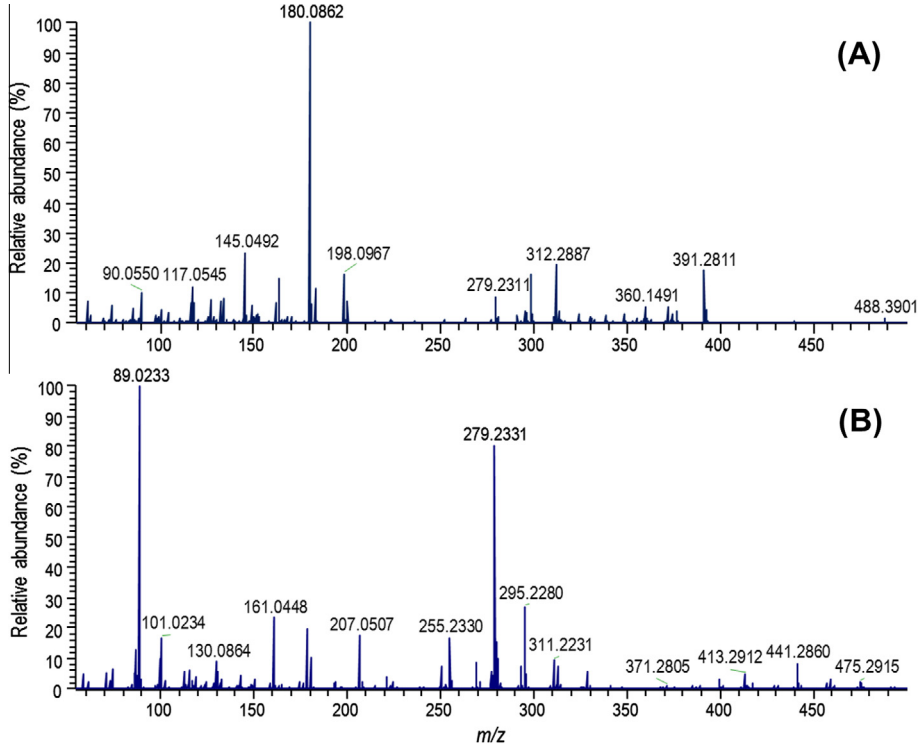

Fig. 1. DART mass spectra obtained at an ionization gas temperature 350 °C by analysis of an extract prepared from biscuits baked for 12 min at 180 °C (recipe R_1). (A) Positive ionization mode and (B) negative ionization mode.

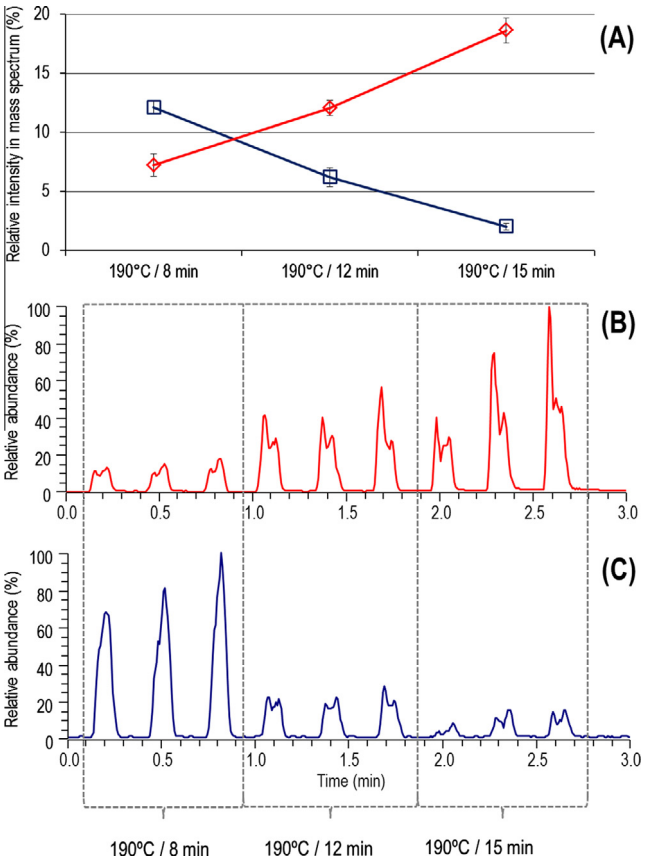

Fig. 2. Dynamic changes in intensities of ions *m/z* 90.0550 and 127.0387 tentatively identified as alanine and hydroxymethylfurfural (HMF), respectively. (A) Changes in relative intensity normalised to the highest peak in the DART mass spectra (average of three parallel determinations, error bars are ±standard deviation), (Δ) *m/z* 127.0387 (HMF), (□) *m/z* 90.0550 (alanine). (B) Extracted ion chromatogram of *m/z* 127.0387 (±2.5 ppm). (C) Extracted ion chromatogram of *m/z* 90.0550 (±2.5 ppm).

15 min, while the lowest were observed in those baked at 180 °C for 8 min. With respect to acrylamide concentrations in biscuits prepared using recipes R_1 and R_2, in samples baked at 180 and 190 °C, lower acrylamide amounts were observed in those prepared with R_1. No differences in acrylamide concentrations were observed between R_1 and R_2 at a baking temperature of 200 °C.

3.3. Principal component analysis of DART–HRMS data

An exploratory analysis of the DART–HRMS data matrix of {54 × 138}, which represented combined positive and negative ionization mode data, was carried with the use of principal component analysis (PCA). PCA is an unsupervised pattern recognition technique which allows reduction in the dimensionality of a multivariate dataset by retaining the maximum structure in the data. The PCA technique may also allow existing patterns or clustering behaviour to be uncovered in a data set without prior knowledge of the properties of the samples (Berruetta et al., 2007). The PCA scores plot defined by the first two principal components (PC) calculated based on the autoscaled data is shown in Fig. 3. The first two PC (PC1 and PC2) explained approximately 62% of the total data variance. No outliers were observed in the PCA scores plot (Fig. 3).

An apparent sample clustering behaviour according to the extent of heat-treatment was recognised in the PCA scores plot. Regardless of the recipe used for preparation of the biscuits, the baking process could be easily followed along the PC1 as the scores of samples decreased with increasing baking time at constant baking temperature and with increasing baking temperature at constant baking time. These differences in DART–HRMS fingerprints reflect the changes that take place in the chemical composition of the biscuits during heat-induced reactions (*i.e.*, Maillard reaction, caramelisation or lipid oxidation). With the exception of one sample, all three replicate samples prepared for each combination of baking time and temperature were located close in the scores plot defined by PC1 and PC2. This observation documents acceptable repeatability for both the baking process and the DART–HRMS fingerprinting.

Another interesting feature observed in the PCA scores plot (Fig. 3) is the fact that the samples representing biscuits prepared with the use of recipes R_1 and R_2 which differed only by the addition of NaCl were clearly discriminated along PC2 on the basis of their DART–HRMS fingerprints. This indicated that the presence/absence of NaCl in the biscuits had a considerable effect on the

Table 1
The overview of tentatively identified ions and trends observed in their relative intensities during prolonged baking.

Ionization mode	*m/z*	Ion type	Elemental composition	Mass error (ppm)	Tentative identification	Trend in relative intensity during prolonged baking
DART+	90.0550	$[M+H]^+$	$C_3H_8NO_2$	+0.5	Alanine	Decrease
	109.0284	$[M-H_2O+H]^+$	$C_6H_5O_2$	−0.1	Hydroxymethylfurfural	Increase
	127.0387	$[M+H]^+$	$C_6H_7O_3$	−2.1		
	116.0705	$[M+H]^+$	$C_5H_{10}NO_2$	−0.9	Proline	Decrease
	118.0861	$[M+H]^+$	$C_5H_{12}NO_2$	−1.3	Valine	Decrease
	132.1016	$[M+H]^+$	$C_6H_{14}NO_2$	−2.3	Leucine/isoleucine	Decrease
	145.0492	$[M+H]^+$	$C_6H_9O_4$	−2.3	Acetylformoin	Increase
DART−	131.0347	$[M-H]^-$	$C_5H_7O_4$	+2.3	3-Deoxypentos-2-ulose	Increase
	149.0445	$[M-H]^-$	$C_5H_9O_5$	−3.4	Pentose	Increase
	161.0446	$[M-H]^-$	$C_6H_9O_5$	−2.5	3-Deoxy-D-erythro-hexosulose/ 1-deoxyhexo-2,3-diulose	Increase
	161.0446	$[M-H_2O-H]^-$	$C_6H_9O_5$	−2.5	Hexose	Increase
	179.0552	$[M-H]^-$	$C_6H_{11}O_6$	−1.8		
	255.2327	$[M-H]^-$	$C_{16}H_{31}O_2$	+1.2	Palmitic acid	Decrease
	277.2170	$[M-H]^-$	$C_{18}H_{29}O_2$	+1.0	Linolenic acid	Decrease
	279.2327	$[M-H]^-$	$C_{18}H_{31}O_2$	+1.0	Linoleic acid	Decrease
	281.2483	$[M-H]^-$	$C_{18}H_{33}O_2$	+0.9	Oleic acid	Decrease
	305.0876	$[M-2H_2O-H]^-$	$C_{12}H_{17}O_9$	+1.1	Sucrose	Decrease
	323.0981	$[M-H_2O-H]^-$	$C_{12}H_{19}O_{10}$	+0.8		
	341.1086	$[M-H]^-$	$C_{12}H_{21}O_{11}$	+0.6		

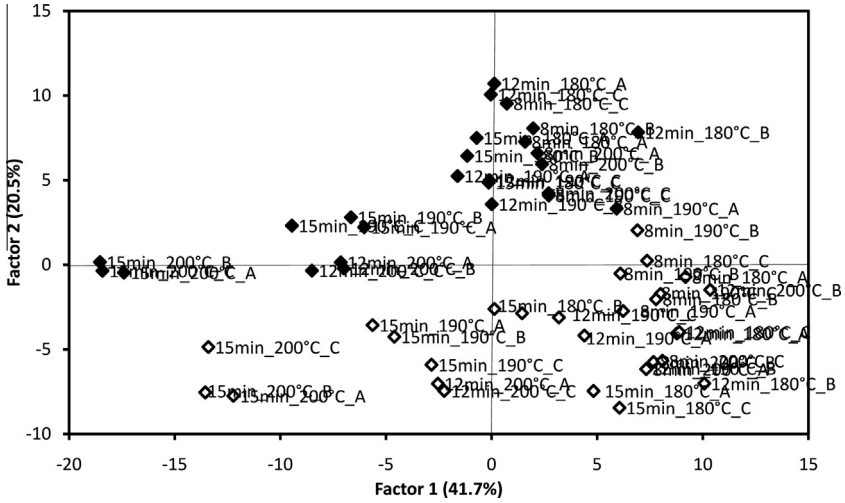

Fig. 3. Principal component analysis (PCA) scores plot obtained based on autoscaled data matrix combining positive and negative ionization mode DART–HRMS fingerprints of biscuits from formulation R_1 (♦) and formulation R_2 (◇). Samples code is: baking time (min)_baking temperature (°C)_replicate. Data for each sample were obtained by averaging results of two repeated DART–HRMS analyses.

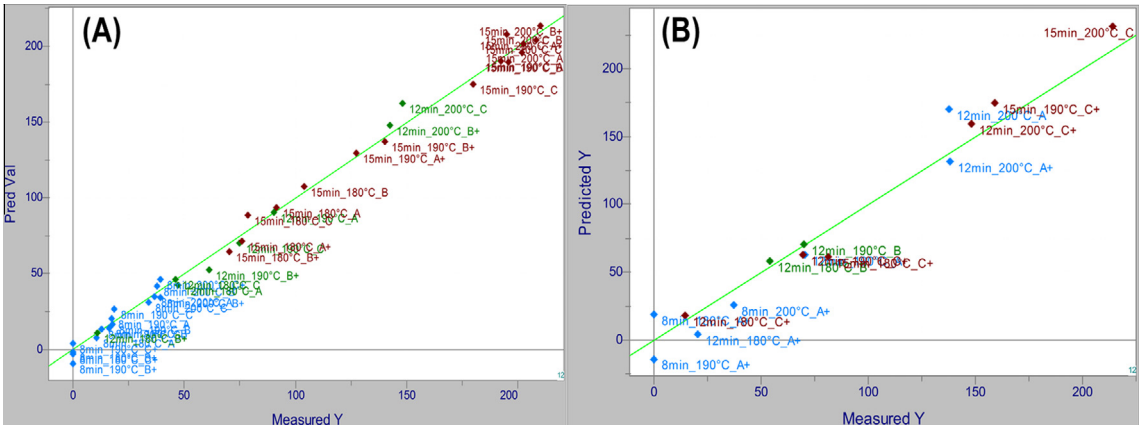

Fig. 4. Amount of acrylamide predicted by the PLSR model (autoscaled data, combined negative and positive ionization modes, 1 OSC) in internal validation (A) and in external validation (B) versus the concentration of acrylamide determined in the samples by UHPLC–MS/MS. Values represent concentration of acrylamide in $\mu g\,kg^{-1}$. Samples code is: baking time (min)_baking temperature_replicate. Samples with final "+" in the sample code were prepared with the use of the recipe R_1.

pattern of heat-induced reactions taking place during baking. The possibility that the observed differences between the fingerprints of R_1 and R_2 samples occurred not because of the impact of the availability of NaCl on heat-induced reactions, but due to the formation of adduct ions during the DART ionization was also considered. For this purpose, positive and negative records of R_1 samples were manually searched for $[M+Na]^+$ and $[M+Cl]^-$ species of corresponding $[M+H]^+$ and $[M-H]^-$ ions, respectively, in R_2 samples. However, no ion species matching such adduct ions were found.

The PCA loadings of the autoscaled data are presented in Table S2. The ions providing the most information for discrimination between samples prepared at different baking times/temperatures and according to different recipes had high and low scores along the PC1 and PC2, respectively.

3.4. Regression models for prediction of acrylamide concentrations

Considering the observations in the PCA plots reported here, the DART multivariate data appears to show good potential use in quantitative prediction of acrylamide content in biscuits. Needless to say, the information on acrylamide concentrations provided by a reference UHPLC–MS/MS method was needed to validate this assumption. Regression models needed to be developed for the prediction of the values of a continuous numerical variable. Two different algorithms, namely partial least square regression (PLSR) and principal components regression (PCR) analyses, and three pre-processing techniques were tested in this study. A PLS model developed on the whole dataset showed that one of the biscuit samples baked at 200 °C for 12 min simultaneously exhibited an extremely high studentised residual and leverage exceeding the computed threshold values and was therefore excluded from further modeling as outlier. The regression models were then developed using a training set of 39 samples. The calibration set was composed of samples randomly selected from each temperature and recipe group to ensure that the model calibration was performed on a sample set balanced in terms of acrylamide concentrations. The models were then externally validated with a sample set of the remaining 14 samples.

The most accurate regression model, that is, the model that provided the lowest standard error of prediction (SEP), was obtained by PLSR based on autoscaled data representing combined positive and negative ionization mode records and the application of one

orthogonal signal correction component. This model included 12 factors which explained approximately 70% of the total X variance. The model provided a RMSECV equal to an acrylamide concentration of 5.4 μg kg^{-1} and resulted in a SEP of 14.8 μg kg^{-1}. The amount of *Y* variance explained by the model (r2) in the external validation was 0.983. The *Y* fit for the model in cross validation and in external validation is shown in Fig. 4. As can be seen, a relatively high accuracy of prediction was achieved, especially at higher acrylamide concentrations. A plot of the residuals of the validation samples against the accurately measured acrylamide contents showed that at higher acrylamide concentration the model tended to slightly overestimate acrylamide content (data not shown). The most accurate PLSR models developed on the basis of only positive ionization mode data and negative ionization mode data achieved a SEP values of 16.4 and 35.0 μg kg^{-1}, respectively. The SEP values obtained in the present study are very similar to those reported by Rizkallah et al. (2008), who used front face fluorescence spectroscopy to predict acrylamide concentrations in cookies. However, SEP value in that study was obtained on the basis of internal cross-validation only. In another study, less accurate prediction of acrylamide content (RMSECV = 266 μg kg^{-1}) in potato chips was reported based on the data obtained by NIR (Pedreschi et al., 2010). The examination of the regression vectors enabled the recognition of the variables (ions) that significantly contributed to the prediction. These included ions *m/z* 99.0076, 129.0182, 130.0862, 161.0446, 175.0239, 257.0875, 341.1086, and *m/z* 399.275 in the negative ionization mode, and *m/z* 360.1491 in the positive ionization mode.

4. Conclusions

This study demonstrates the potential of the DART–HRMS technique to enable high-throughput fingerprinting of biscuit samples applicable in control of some quality/safety parameters. The presented strategy requires considerably shorter time for both sample preparation (~8 min per sample) and instrumental analysis (~0.4 min per sample in both positive and negative ionization mode), as compared to conventional, target acrylamide analysis by chromatographic methods. The multivariate analysis of fingerprint data (mass spectra of sample extract) provided insight into the chemical differences between biscuits prepared according to different recipes and made it possible to follow changes in the chemical composition of samples during the baking process. The concentrations of acrylamide formed during the baking of biscuit could be predicted with acceptable accuracy based on combined use of multivariate data obtained by positive and negative mode DART–HRMS and chemometric modeling. The PLSR prediction model developed in this study was proved to be robust against small changes in biscuit recipes (*i.e.*, presence/absence of NaCl at approximately 0.6%, *w/w*). However, the robustness of the model should be further investigated by studying variations in chemical compositions of raw materials such as flours used for the preparation of the biscuits. Studies on effects of varying contents of asparagine and reducing sugars, which are the main precursors of acrylamide, would be particularly helpful. A predictive model such as that reported in this study could be used for a rapid screening of biscuits to assess the impact of changes in processing conditions or formulation on acrylamide formation. To extend the applicability of this approach to other relevant food matrices, development of additional prediction models with the use of suitable sample sets is required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.09.151.

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