UNIVERSITY OF LATVIA
FACULTY OF CHEMISTRY

DEVELOPMENT AND APPLICATION OF MASS SPECTROMETRIC METHODS FOR THE DETERMINATION OF MAILLARD REACTION PRODUCTS IN FOODSTUFFS

DOCTORAL THESIS

MASSPEKTROMETRISKO METOŽU IZSTRĀDE UN PIELIETOŠANA MAIJĀRA REAKCIJAS PRODUKTU NOTEIKŠANAI PĀRTIKĀ

PROMOCIJAS DARBS

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Rīga
2016

The research for doctoral thesis was carried out at the Institute of Food Safety, Animal Health and Environment "BIOR" from 2012 to 2016.

The thesis contains the introduction, 3 chapters, conclusions and reference list.
Form of the thesis: dissertation in chemistry, analytical chemistry.

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The thesis will be defended at the public session of the Doctoral Committee of Chemistry, University of Latvia, at the Faculty of Chemistry of the University of Latvia (Jelgavas Str. 1, Riga, Latvia) on May 12, 2016 at 16:00.

The thesis is available at the Library of the University of Latvia, Raiņa Blvd. 19, Riga, Latvia.

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ABBREVIATIONS

AGC	Automatic gain control
arb	Arbitrary units
b.w.	Body weight
CE	Collision energy
CFU	Colony forming units
d.w.	Dry weight
DP	Declustering potential
EC	European Commission
ECD	Electron capture detector
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
FDA	Food and Drug Administration
FECFA	Joint FAO/WHO Expert Committee on Food Additives
FWHM	Full width half maximum
GC	Gas chromatography
HESI	Heated electrospray ionization
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HS	Headspace
IT	Injection time
JA	Jerusalem artichoke
L. sakei	*Lactobacillus sakei*
LAB	Lactic acid bacteria
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
ME	Matrix effect
MS	Mass spectrometry

MS/MS	Tandem mass spectrometry
MS2	Product ion scan
NCE	Normalized collision energy
Orbitrap-MS	Orbitrap mass spectrometry
P. acidilactici	*Pediococcus acidilactici*
P. pentosaceus	*Pediococcus pentosaceus*
PSA	Primary and secondary amines
QD	Quantum dots
QqQ	Triple quadrupole
RF	Radio frequency
RSD	Relative standard deviation
S/N	Signal-to-noise
SD	Standard deviation
SIM	Selected ion monitoring
SMF	Submerged fermentation
SPE	Solid phase extraction
SPME	Solid phase micro extraction
SRM	Single reaction monitoring
SSF	Solid-state fermentation
UPLC	Ultra-high performance liquid chromatography
WHO	World health organization

ABSTRACT

Development and application of mass spectrometric methods for the determination of Maillard reaction products in foodstuffs. Pugajeva I., supervisors Dr. Chem., Assoc. Prof. Bartkevičs V. and Dr. Chem., Prof. Vīksna A. Doctoral thesis in analytical chemistry, 101 pages, 23 figures, 16 tables, 155 literature references. In English language.

A new analytical methodology for two food toxicants formed by Maillard reaction has been elaborated. For the first time, the analytical capabilities of Orbitrap mass spectrometry were applied to the determination of acrylamide in coffee samples. This elaborated analytical method was demonstrated to be an important and powerful tool for accurate quantification of such small molecules as acrylamide in difficult matrices like coffee. The instrument ensured high resolution, very good sensitivity, linear range, and good reproducibility. A comparison of the elaborated method with the triple quadrupole method showed the superiority of high resolution mass spectrometry for this specific type of analysis. Application of the elaborated method for the analysis of real coffee samples showed that the acrylamide content in some samples exceeded the recommendations issued by the European Commission. HS-GC-MS/MS in the SRM scanning mode showed high sensitivity and selectivity for the analysis of furan, eliminating interference from other matrix components. The present study reports data on the occurrence of furan in baby foods and other foodstuffs in Latvia. Mitigation strategies for reducing the acrylamide content and possible precursors of furan in foods were proposed.

MAILLARD REACTION PRODUCT, ACRYLAMIDE, FURAN, ROASTED COFFEE, BABY FOODS, HIGH RESOLUTION MASS SPECTROMETRY, ORBITRAP, TANDEM MASS SPECTROMETRY

ANOTĀCIJA

Masspektrometrisko metožu izstrāde un pielietošana Maijāra reakcijas produktu noteikšanai pārtikā. Pugajeva I., zinātniskie vadītāji Dr. ķīm., asoc. prof. Bartkevičs V. un Dr. ķīm., prof. Vīksna A. Promocijas darbs, 101 lappuse, 23 attēli, 16 tabulas, 155 literatūras avoti. Angļu valodā.

Tika izstrādātas jaunas analītiskās metodes divu pārtikas produktos sastopamo toksikantu noteikšanai, kuri rodas produktos Maijāra reakcijas rezultātā. Pirmo reizi zinātniskajā literatūrā Orbitrap masspektrometra analītiskās spējas tika piemērotas akrilamīda noteikšanai kafijas paraugos. Izstrādātā metode uzrādīja selektīvu un precīzu detektēšanas spēju mazu molekulu, kā akrilamīds, kvantitatīvai noteikšanai sarežģītās matricās, piemēram, kafijā. Instruments nodrošina augstu izšķirtspēju, ļoti labu jūtību, lineāru diapazonu un labu atkārtojamību. Izstrādātās metodes salīdzinājums ar tandēma kvadrupola detektēšanas metodi uzrādīja pārākumu šim konkrētajam analīzes veidam. Kafijas paraugu analīze atklāja, ka akrilamīda saturs dažiem paraugiem pārsniedz Eiropas Komisijas uzrādītos ieteikumus. Tvaika fāzes līdzsvara gāzu hromatogrāfija ar tandēma masspektrometru selektīvajā skenēšanas režīmā, analizējot furānu, uzrādīja augstu jūtību un selektivitāti, novēršot traucējošos signālus no citiem matricas komponentiem. Šajā pētījumā ir noteikts furāna saturs bērnu pārtikā un citos Latvijā ražotos pārtikas produktos. Darbā tika izpētītas akrilamīda satura mazināšanas stratēģijas un iespējamie furāna veidošanās prekursori pārtikas produktos.

MAIJĀRA REAKCIJAS PRODUKTI, AKRILAMĪDS, FURĀNS, GRAUZDĒTA KAFIJA, BĒRNU PĀRTIKA, AUGSTAS IZŠĶIRTSPĒJAS MASSPEKTROMETRIJA, ORBITRAP, TANDĒMA MASSPEKTROMETRIJA

INTRODUCTION

Thermal processes are frequently used in food manufacturing in order to obtain safe products with prolonged shelf life and may have a strong impact on the final quality of foods. Baking, toasting, frying, roasting, sterilization and other types of treatment result in a range of desirable and undesirable effects, which all stem from certain chemical reactions, namely, Maillard reaction, caramelization, and to a minor extent, lipid oxidation occurring while the foods are heated. One of the purposes of thermal processes is to alter the sensory properties of foods, to improve palatability and to extend the range of colors, tastes, aromas, and textures in foods produced from similar raw materials. Maillard reaction is related to aroma, taste and color, in particular in traditional processes, such as the roasting of coffee and cocoa beans, the baking of bread and cakes, the toasting of cereals and the cooking of meat. After more than 100 years since the first paper described the reaction between sugars and amino acids leading to browning, is still at the very center of interest for scientists in various fields. The Maillard reaction has recently been linked to the formation of various carcinogens and mutagens in a variety of thermally processed foods. Acrylamide and furan are among those toxicants [1-3]. Since April 2002, when the Swedish National Food Administration published for the first time a dataset about high content of acrylamide in foods, several studies in different countries were dedicated to the assessment of acrylamide content in foods. The presence of acrylamide in various foodstuffs and its toxic impact stirred up an interest on a global scale about a possible risk to human health. Consequently, many countries have started to estimate the dietary intake of acrylamide. The main sources of acrylamide in the diet are primarily processed potato products, such as French fries and potato crisps, and also cereal products such as bread, breakfast cereals, cookies, and biscuits. Acrylamide is also formed during the coffee roasting process [4]. Two years later the US Food and Drug Administration (FDA) informed the public on the occurrence of another Maillard reaction product – furan in foods. This highly volatile water insoluble substance, so far known in the food industry as an irradiation indicator and coffee flavour constituent, was increasingly detected in heated, non-irradiated food. Already in 1993 furan was a subject to comprehensive toxicological evaluation within the US National Toxicology Program. Furan proved to be carcinogenic and mutagenic in animal testing.

The practical relevance of the problem. The selection of an appropriate analytical methodology for the determination of food toxicants is mostly based on the chemical and physical properties of analytes, and on the type of food matrix. The quantification of acrylamide and furan

in foods is a challenging task, because of the low molecular mass and high polarity of these contaminants.

Two analytical techniques, HPLC and GC coupled to mass spectrometric detector, are currently mainly used by laboratories for the analysis of acrylamide in food samples. A GC–MS method that involves a bromination procedure has the advantage of adequate sensitivity with multiple ion confirmation. The application of GC–MS/MS or coupling to a high resolution MS would even further decrease the detection threshold. On the other hand, the determination of acrylamide using LC–MS/MS can avoid derivatization and provides an appropriate sensitivity and reproducibility [5-7]. However, several papers indicate the existence of significant problems related to the matrix suppression phenomenon and the presence of interfering co-extractives during the determination of acrylamide in coffee samples [8-11]. High-resolution mass spectrometry (HRMS) is particularly suitable for the detection of low molecular weight food contaminants, and it can provide some analytical advantages over other MS techniques. The detection of acrylamide by HRMS in full scan mode was compared to LC coupled to tandem mass spectrometry method and the conclusion was drawn that it provides results perfectly in line with those obtained by LC-MS/MS [12]. However, a challenge still remains with respect to the development of stable, reliable, and robust methods for difficult food matrices, such as cocoa powders, coffee, and high salt flavorings.

Because of the high volatility of furan, headspace sampling is the most common method for its analysis. To avoid losses of furan during the sample preparation, sampling and extraction should be performed under cold conditions [13]. The food samples could be weighed directly in the headspace vial. After mixing and homogenization with water, the vial is sealed and heated to promote volatilization of the analyte in the headspace, thereby achieving an equilibrium between the liquid and gaseous phases [14]. Furan is also analyzed by a solid-phase microextraction (SPME) method in combination with GC-MS. SPME allows the concentration of analyte and affords minimum interference from matrix compounds [15]. However, this technique has some essential drawbacks: an appropriate stationary phase, stable SPME fiber, and optimized coating techniques are crucial for the extraction of trace amounts of furan, making this method more expensive compared to other methods. Direct headspace sampling appears to be a simple, fast, and economical procedure, but the limits of quantification are too high to obtain exact furan concentration in food samples. Baby food was of particular interest as a high proportion of samples sold in jars and cans contained furan, and such foods may form the entire diet of many babies.

Many researchers reported non-detection of furan because of relatively high limits of detection, for example, 2.4 μg kg^{-1} [16], 4.0 μg kg^{-1} [17,18], 0.17 μg kg^{-1} [19], 0.12 μg kg^{-1} [20], 0.60 μg kg^{-1} [21]. Therefore, the development of new methods with higher sensitivity for furan in baby foods is still a very important task.

The aim of the work. Several aims were proposed during this work:

i. The elaboration and application of mass spectrometric methods for the determination of acrylamide and furan in foodstuffs;

ii. The investigation of new mitigation strategies to reduce acrylamide content in cereal based products and the possible precursors of furan in various baby food types;

iii. Estimating the occurrence of furan and acrylamide in Latvian products and risk assessment for these contaminants.

The approach used. To achieve the aim of the work, several tasks were proposed:

i. The elaboration of an efficient and reliable analytical method using high resolution Orbitrap mass spectrometry and the examination of the efficiency of several possible scan modes: full MS scan, targeted-SIM (selected ion monitoring), and targeted-MS2 (product ion scan) for the analysis of low concentrations of acrylamide in such difficult matrices as coffee;

ii. The optimization of single reaction monitoring (SRM) detection method and comparison in terms of sensitivity and selectivity with selected ion monitoring (SIM) for the analysis of furan, using HS-GC-MS/MS technique;

iii. Development of an efficient sample extraction and clean-up procedure, and the selection of optimal instrumental conditions for the analysis of compounds of interest;

iv. Validation of the elaborated methods for acrylamide in coffee matrix and furan in baby foods;

v. Application of the developed analytical methodology for the analysis of selected samples from the Latvian market;

vi. Assessment of mitigation strategies for acrylamide reduction in cereal based products (bread and biscuits).

Scientific novelty.

i. The application of a new type of hybrid quadrupole – Orbitrap mass spectrometry in the analysis of acrylamide;

ii. The elaboration of a sensitive and selective furan detection method by using the HS-GC-MS/MS technique;

iii. Assessment of Latvian population exposure to acrylamide intake from food products;

iv. Proposals for new mitigation strategies to reduce acrylamide contamination in bread and biscuits.

Practical application of the work. The elaborated analytical methods are used for the analysis of acrylamide in a coffee samples and furan in various matrices in the Institute of Food Safety, Animal Health and Environment "BIOR". The proposed mitigation strategies for acrylamide contamination can be applied in bread and biscuit production.

Scientific publications.

1. Bartkiene, E.; Jakobsone, I.; **Pugajeva, I.**; Bartkevics, V.; Zadeike, D.; Juodeikiene, G. Reducing of acrylamide formation in wheat biscuits supplemented with flaxseed and lupine. *Food Sci Technol-LWT*[1] **2016**, *65*, 275-282.

2. **Pugajeva, I.;** Jaunbergs, J.; Bartkevics, V. Development of a sensitive method for the determination of acrylamide in coffee using high-performance liquid chromatography coupled to a hybrid quadrupole Orbitrap mass spectrometer. *Food Addit Contam. A*[2] **2015**, *32(2)*, 170-179.

3. Bartkiene, E.; Jakobsone, I.; Juodeikiene, G.; Vidmantiene, V.; **Pugajeva, I.**, Bartkevics, V. Influence of the addition of Helianthus tuberosus L. fermented with different lactobacilli on acrylamide content in biscuits. *Int J Food Sci Tech*[3] **2015**, *50(2)*, 431-439.

4. **Pugajeva, I.**; Zumbure, L.; Melngaile, A., Bartkevics, V. Determination of acrylamide levels in selected foods in Latvia and assessment of the population intake. 9th Baltic Conference on Food Science and Technology "Food for consumer well-being", FOODBALT-2014, Conference Proceedings **2014,** 111-116.

5. Bartkiene, E.; Jakobsone, I.; Juodeikiene, G.; Vidmantiene, V.; **Pugajeva, I.**, Bartkevics, V. Study on the reduction of acrylamide in mixed rye bread by fermentation with bacteriocin-like inhibitory substances producing lactic acid bacteria in combination with Aspergillus niger glucoamylase. *Food Control*[4] **2013,** *30(1)*, 35-40.

6. Bartkiene, E.; Jakobsone, I.; Juodeikiene, G.; Vidmantiene, V.; **Pugajeva, I.**, Bartkevics, V. Effect of fermented Helianthus tuberosus L. tubers on acrylamide formation and quality properties in wheat bread. *Food Sci Technol-LWT*[1] **2013,** *54(2)*, 414-420.

7. Bartkiene, E.; Jakobsone, I.; Juodeikiene, G.; Vidmantiene, V.; **Pugajeva, I.**, Bartkevics, V. Effect of lactic acid fermentation of lupine wholemeal on acrylamide content and quality characteristics of wheat-lupine bread. *Int J Food Sci Nutr*[5] **2013**, *64 (7)*, 890-896.

8. **Pugajeva I**.; Rozentale, I.; Viksna, A.; Bartkiene, E.; Bartkevics, V. The application of headspace gas chromatography coupled to tandem quadrupole mass spectrometry for the analysis of furan in baby food samples. *Food Chemistry*[6], *FOODCHEM-D-15-03810*. (Under review 06.09.2015).

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[1] *Peer reviewed journal, imprint of Elsevier (IF=2.416 (2014)), ISSN: 0023-6438*
[2] *Peer reviewed journal, imprint of Taylor & Francis (IF=2.341 (2013)), ISSN: 1944-0057*
[3] *Peer reviewed journal, imprint of Wiley (IF= 1.384 (2014)), ISSN: 1365-2621*
[4] *Peer reviewed journal, imprint of Elsevier (IF= 2.806 (2014)), ISSN: 0956-7135*
[5] *Peer reviewed journal, imprint of Taylor & Francis (IF= 1.206 (2014)), ISSN: 1465-3478*
[6] *Peer reviewed journal, imprint of Elsevier (IF= 3.391(2014)), ISSN: 0308-8146*

List of conferences.

1. 73rd University of Latvia conference, Riga, Latvia, 2015. **Pugajeva, I.**; Rozentāle, I.; Začs, D.; Vīksna, A.; Bartkevičs, V. Gāzu hromatogrāfijas - tandēma masspektrometrijas izmantošana furāna noteikšanai pārtikas produktos (oral presentation).

2. 72nd University of Latvia conference, Riga, Latvia, 2014. **Pugajeva, I.**; Začs, D.; Vīksna, A.; Bartkevičs, V. Orbitrap masspektrometriskās noteikšanas parametru optimizācija akrilamīda noteikšanai kafijā (oral presentation).

3. 9th Baltic Conference on Food Science and Technology, FOODBALT-2014, Jelgava, Latvija, 2014. **Pugajeva, I.**; Zumbure, L.; Melngaile, A., Bartkevics, V. Determination of acrylamide levels in selected foods in Latvia and assessment of the population intake (in book of abstracts/poster).

4. 7th Baltic Conference on Food Science and Technology, FOODBALT-2012, Kaunas, Lithuania, 2012. Bartkiene, E.; Jakobsone, I., Juodeikiene, G., Vidmantiene, D., **Pugajeva, I.**, Bartkevics, V., Onisciukas, M. Acrylamide formation in wheat bread produced by fermented lupine products (in book of abstracts).

5. 7th Baltic Conference on Food Science and Technology, FOODBALT-2012, Kaunas, Lithuania, 2012. Bartkiene, E.; IJakobsone, I., Juodeikiene, G.; Vidmantiene, D.; **Pugajeva, I.**; Bartkevics**,** V.; Krungleviciute, V.; Rekstyte, T. Acrylamide reduction in mixed wheat-rye bread by using lactic acid fermentation in combination with Aspergillus niger glucoamylase (in book of abstracts).

6. Proceedings of the 6th International Congress Flour-Bread '11. 8th Croatian Congress of Cereal Technologists, Opatija, Croatia, 2011. Schönberg, N.; Podjava, A.; **Pugajeva, I.**; Bartkevics, V.; Mekss, P.; Jakobsone, I.; Henle, T. Determination of 5-hydroxymethyl-2-furfural and acrylamide in bread (in book of abstracts).

1. LITERATURE REVIEW

1.1. Thermally induced chemical reactions in foods

Thermal food processing is focused on three main targets: enzyme inactivation, microbial destruction, as well as the development of desirable sensory characteristics. From this perspective, heat treatment of food is clearly beneficial. However, safety remains a primary objective within the food industry. Conversely, palatability is also a crucial aspect to the commercial success of food products and cannot be neglected [22].

As shown in Figure 1.1., various types of reactions are promoted in foods during heating. The extent, variety, and balance of chemical reactions and compounds generated vary as a function of several factors. The development of aromas, colors and flavors generated as a means of controlling or improving sensory outcome has been extensively studied. Despite the years of research, much remains unknown as to the mechanisms of formation of specific compounds that impart characteristic organoleptic properties to foods. However, recent evidence clearly indicates that ideal conditions producing desirable aromas could also generate potential food toxicants [23].

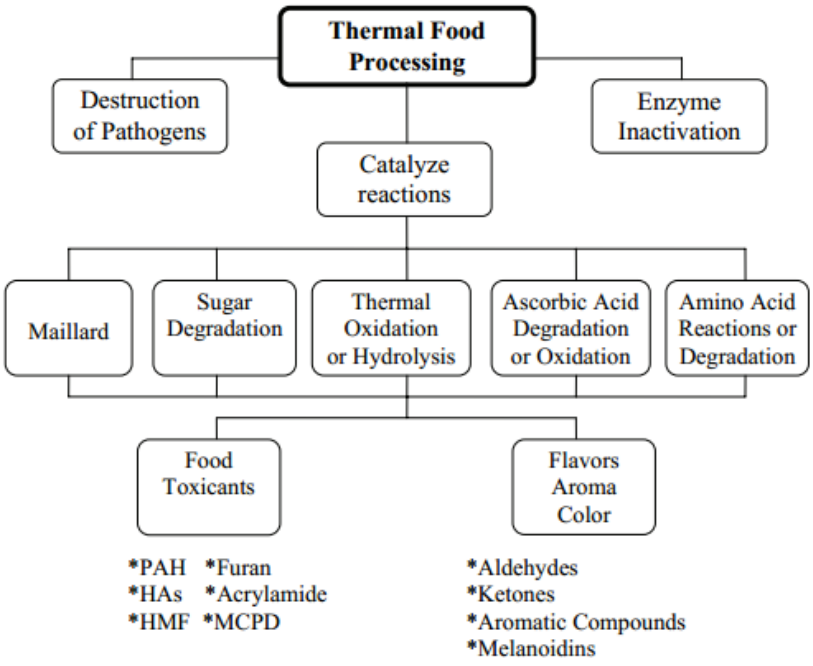

Fig. 1.1. **An overview of the implications of thermal food processing**

The main purpose of industrial food processing is to provide safe and high quality foods as demanded by consumers. The application of thermal processing in an appropriate way is the key factor for obtaining safe foods with enhanced nutritional functionality, compared to the raw materials [24]. This can be offset by decreased digestibility and possibly the formation of toxic and mutagenic compounds, but the properties of foods can also be improved by the formation of antioxidative compounds [3]. The information on the main food processing toxicants and their approximate mean intake is summarized in Table 1.1. It is apparent that the daily intake amounts of acrylamide and furan are the highest among all food products, therefore a special attention should be paid to better understanding of these compounds [25].

Table 1.1.

Selected chemical contaminants resulting from heating or other processing of foods [25]

Contaminant	Examples of foods	Approximate mean intake (µg/day)
Acrylamide	Heated potato and cereal products (e.g., French fries, potato chips, cookies, crackers), coffee	21-60
Furan	Canned soups, stews, and sauces; jarred baby foods; coffee	15
Heterocyclic amines (HAAs)	Cooked meat or fish	≤ 1
3-Monochloropropane- 1,2-diol (3-MCPD)	Acid-hydrolyzed vegetable protein, soy sauce and related sauces, heated cereal products (e.g., cookies, crackers)	3-25
N-Nitroso compounds	Meats cured with sodium nitrite or nitrate	≤ 1
Polycyclic aromatic hydrocarbons (PAHs)	Grilled and smoked foods, vegetable oils (also fruits, vegetables, and mollusks, through environmental deposition)	2-3
Ethyl carbamate	Fermented foods and beverages	1-4

1.2. Description of the Maillard Reaction

In 1912, the French chemist Louis Camille Maillard was the first scientist to study and describe changes resulting from thermal reactions between amino acids and sugars, also noting the variations in chemical and aroma formation as a function of the amino acids involved. For as long

as food has been cooked, the Maillard reaction has played an important role in improving the appearance and taste of foods. It has been a major and even central challenge in food industry, since the Maillard reaction is related to aroma, taste, and color, particularly in traditional processes such as the roasting of coffee and cocoa beans, the baking of bread and cakes, the toasting of cereals and the cooking of meat [3].

1.2.1. Chemistry of the Maillard reaction

The chemistry underlying the Maillard reaction is very complex. It encompasses not just a single reaction pathway, but a whole network of various reactions. The original comprehensive reaction scheme of Hodge (Figure 1.2.) has been developed and checked by food technologists ever since, so the understanding of the reaction is advancing steadily [26]. Nevertheless, the Maillard reaction is notoriously difficult to control. Various factors involved in food processing influence it, and these factors can be considered as food processing variables. The kinetic approach tends to present a complementary view of this mechanism, because it considers the rate-determining steps of the reaction [3].

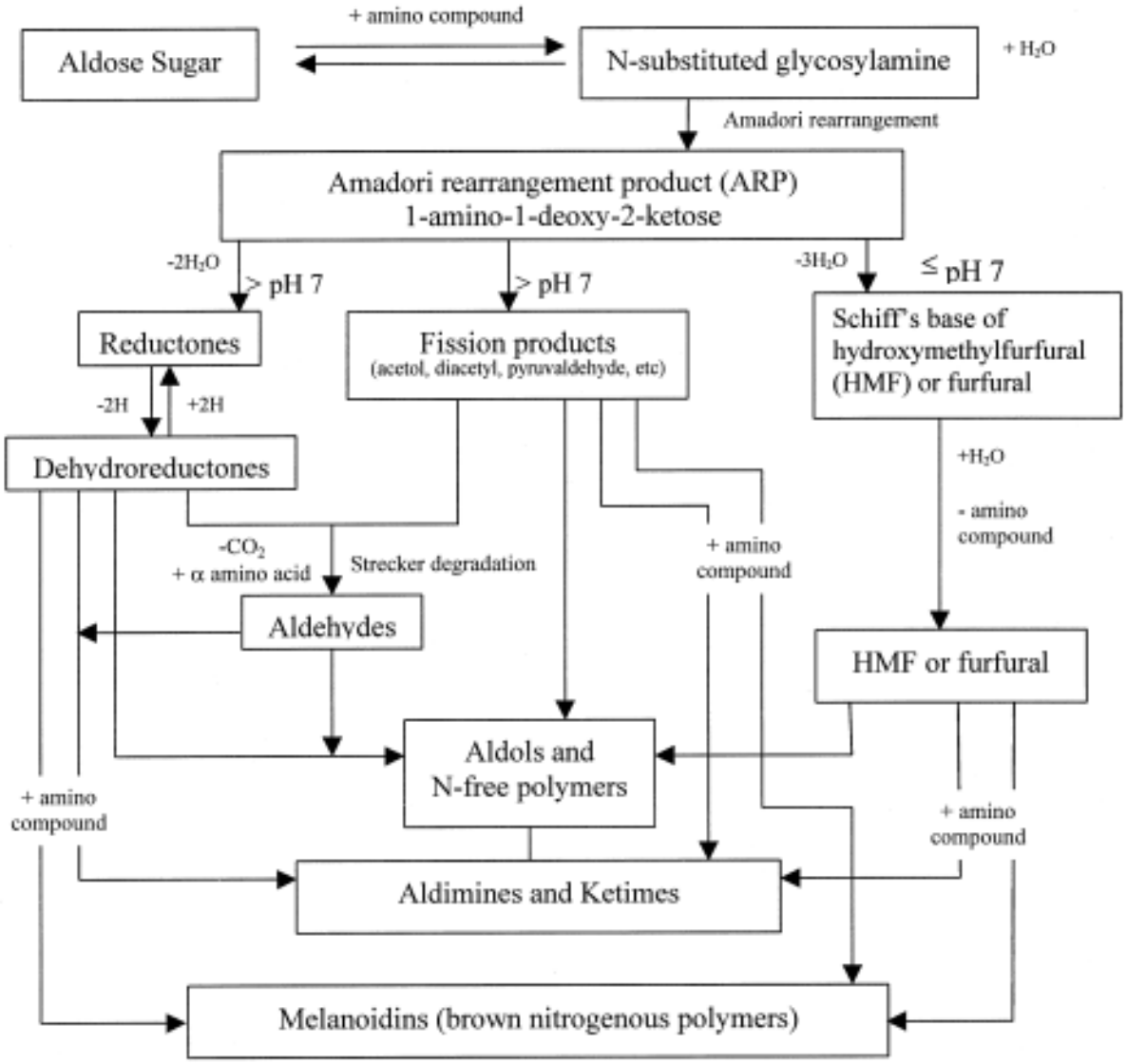

Fig. 1.2. **The Maillard reaction scheme adapted from Hodge [26]**

In essence, this scheme states that in an early stage, a reducing sugar, such as glucose, condenses with a compound possessing a free amino group (an amino acid or, in proteins, mainly the ε-amino group of lysine, but also the α-amino groups of terminal amino acids) to give an *N*-substituted glycosylamine as condensation product, which rearranges to form the Amadori rearrangement product. The subsequent degradation of the Amadori product is dependent on the pH of the system. At pH 7 or below, it undergoes mainly 1,2-enolization with the formation of furfural (when pentoses are involved) or hydroxymethylfurfural (when hexoses are involved). At pH >7 the degradation of the Amadori compound is thought to involve mainly 2,3-enolisation, where reductones, such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3-one, and a variety of cleavage products, including acetol, pyruvaldehyde and diacetyl are formed. All these compounds are highly

reactive and take part in further reactions. Carbonyl groups can condense with free amino groups, which results in the incorporation of nitrogen into the reaction products. Dicarbonyl compounds will react with amino acids with the formation of aldehydes and α-aminoketones. This reaction is known as the Strecker degradation. Subsequently, at an advanced stage, a range of reactions takes place, including cyclizations, dehydrations, retroaldolizations, rearrangements, isomerizations and further condensations, which ultimately, at the final stage, lead to the formation of brown nitrogenous polymers and co-polymers, known as melanoidins [3].

1.2.2. The influence of Maillard reaction products on food properties

Besides the positive effects, some detrimental consequences of thermal processes must be carefully evaluated. The loss of heat labile compounds such as vitamins, as well as essential amino acids (lysine, tryptophan) and/or the formation of undesired tastes and off-flavors are well established phenomena bringing about a loss in the nutritional value and sensory quality of heated foods. All the same, the major concern arising from heating processes come from the formation of hazardous compounds, the so-called foodborne toxicants, i.e., compounds that are not naturally present in foods, but that may develop during heating or preservation and that reveal harmful effects, such as mutagenic, carcinogenic, and cytotoxic effects. Well-known examples of these foodborne toxicants are heterocyclic amines, nitrosamines, and polycyclic aromatic hydrocarbons [24].

The Maillard reaction has recently been linked to the formation of various carcinogens and mutagens in a variety of thermally processed foods. Acrylamide, furan, and heterocyclic amines are among those toxicants. Recently acrylamide and furan have attracted a great interest because of their high toxicological potential and common occurrence in foods [27].

1.3. Acrylamide

Acrylamide is an industrial chemical used in products for water purification, grouts, packaging, and scientific research.

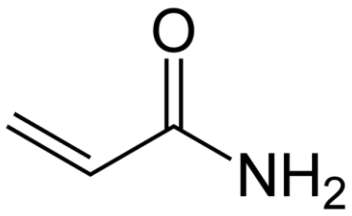

Fig. 1.3. **The structure of acrylamide**

Scientists have discovered that acrylamide is forming in food as a result of a heat-induced reaction between two naturally occurring ingredients, the amino acid asparagine and reducing sugars [1, 27].

Acrylamide has been added to the list of foodborne toxicants since the Swedish National Food Administration in 2002 found substantial amounts of acrylamide in several heat treated, carbohydrate-rich foods, such as potato chips and crisps, coffee, and bread [28]. After these findings, it has been clearly established that the major pathway for acrylamide formation in foods is a Maillard reaction with free asparagine as the main precursor [1, 27, 29, 30]. Asparagine can thermally decompose by deamination and decarboxylation, but when a carbonyl source is present, the degradation of asparagine to acrylamide provides a much better explanation for the high concentration of acrylamide detected in foods rich in reducing sugars and free asparagine, such as fried potatoes and bakery products [27, 31, 32]. Other minor reaction routes for acrylamide formation in foods have been postulated, i.e., from acrolein and acrylic acid [33], and from wheat gluten [34]. Finally, acrylamide can be generated by deamination of 3-aminopropionamide [35]. 3-Aminopropionamide is an intermediate in Maillard reaction, it can also form by enzymatic decarboxylation of free asparagine and can yield acrylamide upon heating even in the absence of a carbonyl source [36].

Acrylamide formation occurs primarily at elevated cooking temperatures when frying or baking (above 120°C) and in low moisture conditions. There are also several foods in which acrylamide appears to form in high-moisture conditions at lower temperatures, such as prune juice and canned ripe black olives. Acrylamide has been found primarily in plant-based foods, notably potato products such as French fries and potato chips; cereals such as cookies, crackers, breakfast cereals, toasted bread, and coffee [37].

1.3.1. The toxicity of acrylamide

In 1994, the International Agency for Research on Cancer classified acrylamide as "Group 2A" (probably carcinogenic to humans) [38], due to its neurotoxicity, carcinogenicity, and genotoxicity [39, 40]. Subsequently, acrylamide was classified as a Category 2 carcinogen and Category 2 mutagen by European Commission [41] as well as a substance of "very high concern" by European Chemical Agency in 2010 [42].

The 72nd meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has systematically summarized and updated the studies of acrylamide throughout the world since their 64th meeting [43, 44]. The resulting document indicated that multi-organ tumors were discovered in experimental animals after the exposure to acrylamide, although no significant association between dietary acrylamide intake and increase in multiple cancers has been established from epidemiological research [45, 46]. The genotoxicity of acrylamide can be presented in two ways. First, acrylamide can be converted to its metabolite glycidamide, which is a 3 times stronger mutagen compared to acrylamide and can induce point mutations in various systems [47]. Secondly, acrylamide can act as a Michael acceptor to form adducts with thiol, hydroxyl, and amino groups in DNA, which leads to DNA damage [39, 48]. Workers who experience occupational exposure to acrylamide suffer from the damage of both peripheral and central nervous systems, since the neurotoxic effects of acrylamide are cumulative and chronic [49]. Acrylamide is toxic to humans at various levels via inhalation, dermal absorption, and ingestion. Rodent studies have proven a high level exposure to be neurotoxic and even deadly [50]. The no-observed-adverse-effect level (NOAEL) for morphological changes in the nervous systems of rats was 200 μg kg^{-1} b.w. per day. Tardiff *et al*. (2010) reported that the tolerable daily intake of acrylamide was 40 μg kg^{-1} per day for neurotoxicity, and 2.6 μg kg^{-1} per day for carcinogenicity [51].

However, the low levels of food-derived acrylamide are clearly not expected to produce an acute response. Daily exposures to acrylamide across surveys and age groups were estimated at 0.4 to 1.9 μg kg^{-1} b.w. per day and 0.6 to 3.4 μg kg^{-1} b.w. per day, respectively, far below the threshold of acute toxicity. Therefore, the health concern lies mainly upon the possible mutagenic or carcinogenic effects of low dose, long term exposure [52].

1.3.2. The occurrence of acrylamide in foods and dietary exposure

Many European countries are engaged in acrylamide monitoring programs since 2002. In 2007, the EC launched a Recommendation that the Member States should perform the monitoring of acrylamide in foodstuffs that are known to contain high acrylamide levels and/or contribute significantly to human exposure. Based on the results of the monitoring in the Member States from 2007–2011, the EC has set "indicative values" for acrylamide in various foodstuffs. The "indicative values" are not safety thresholds, but only intended to indicate the need for an investigation if the values are exceeded in order to explore whether appropriate measures have been taken to control the formation of acrylamide [53].

The Panel on Contaminants in the Food Chain (CONTAM Panel) evaluated a total of 43,419 analytical results from food commodities collected and analyzed since 2010 and reported by 24 European countries and six food associations. The data provided by the European countries and by food associations gave generally consistent and complementary information. Acrylamide was found at the highest levels in "Coffee substitutes (dry)" (average levels of 1499 μg kg^{-1}) and "Coffee (dry)" (average levels of 522 μg kg^{-1}). However, due to dilution effects, lower levels are expected in "Coffee beverages" and "Coffee substitute's beverages" as consumed by the population.

High levels were also found in "Potato crisps and snacks" (average level of 389 μg kg^{-1}) and "potato fried products (except potato crisps and snacks)" (average level of 308 μg kg^{-1}). Lower acrylamide levels were found in "Processed cereal-based baby foods" (average level of 73 μg kg^{-1}), "Soft bread" (average level of 42 μg kg^{-1}) and "Baby foods, other than cereal-based" (average level of 24 μg kg^{-1}). The CONTAM Panel explored the possibility of performing a temporal trend analysis of the acrylamide concentrations in certain foodstuffs across Europe on the basis of the data submitted to EFSA by the European countries. Because of gaps in the databases and the fact that results for different years are not always comparable, a reliable Europe-wide temporal trend analysis is not feasible. However, a dataset of manufacturers' measurements of acrylamide levels in 40,455 samples of fresh sliced potato crisps from 20 European countries for the years 2002 to 2011 showed a substantial downward trend for the mean levels of acrylamide, from 763 ± 91 μg kg^{-1} in 2002 to 358 ± 25 μg kg^{-1} in 2011. For other food categories, a similar downward trend was not observed.

An estimation of human exposure to acrylamide revealed that infants, toddlers, and other children were the most exposed groups. Depending on the survey and age group, chronic dietary exposure of children was estimated to be on average between 0.5 and 1.9 μg kg^{-1} b.w. per day and the 95th percentile was between 1.4 and 3.4 μg kg^{-1} b.w. per day. Chronic dietary exposure of adolescents, adults, elderly, and very elderly was estimated to be on average between 0.4 and 0.9 μg kg^{-1} b.w. per day and the 95th percentile was between 0.6 and 2.0 μg kg^{-1} b.w. per day depending on the survey and age group [52].

1.3.3. Methods for analytical detection of acrylamide

Acrylamide has been quantified in various foods by using chromatographic techniques, such as gas chromatography (GC) or liquid chromatography (LC) in combination with appropriately selective and specific detectors. Another useful technique for acrylamide determination can be capillary electrophoresis. Bioanalytical methods, such as immunoenzymatic tests and biosensors were also found to be sufficiently sensitive and selective [54]. However, analysis of acrylamide is currently generally performed by one of two methodologies: LC-MS/MS or GC-MS.

HPLC coupled with MS is the most preferred method for separation and quantification of acrylamide in foods, due to its sensitivity, selectivity, and versatility. Proficiency tests (total amount n=10) organized by the Food Analysis Performance Assessment Scheme (FAPAS) revealed that LC-QqQ-MS/MS is a reliable method and has broad applications [55].

The detection of acrylamide by HRMS in full scan mode was compared to LC coupled to tandem mass spectrometry method, and the conclusion was drawn that it provides results perfectly in line with those obtained by LC-QqQ-MS/MS [12]. The Orbitrap mass analyzer was described for the first time in the year 2000 by Makarov [56], and has now reached the status of a mainstream mass spectrometry technique. It shows an insignificant trade-off in sensitivity versus resolving power, distinguishing it from other high-resolution analyzers [57].

Ultra-performance liquid chromatography (UPLC), an alternative to the conventional HPLC, requires higher pressures and relies on lower flow rates. UPLC allows better separation of mixture components in a shorter time and with high sensitivity. Such a separation is achievable by using reversed-phase columns with much thinner film and particle size than in typical HPLC columns. Chromatographic separation of acrylamide on a traditional column filled with 5 μm particles suffers from weak retention and separation of polar compounds such as acrylamide and

deformation of the peak shapes. The application of a UPLC method solved these problems, because it improved the analyte peak symmetry. Besides, the smaller size of bed particles (1.7 µm) renders the analysis faster, but not less efficient. Other points worthy of emphasis in this regard are the advantages of UPLC, including the minimized solvent consumption in separations resulting from low flow rates (0.2 mL min^{-1}), substantially shorter time of separation compared to traditional liquid chromatography techniques, more symmetric peaks of separated compounds and considerably smaller sample volumes [54].

For the chromatographic separation of acrylamide, most researchers use reversed-phase chromatography. Hypercarb column (5µm, Thermo Electron, San Jose, CA, USA) has been the most frequently used column. However, it is difficult to choose an appropriate mobile phase to achieve good analyte elution with a reasonable retention time, because of the high polarity of acrylamide. Most chromatography experts have experienced problems retaining and separating polar compounds, similar to acrylamide, when using conventional reversed-phase chromatography. These difficult-to-analyze compounds either pass through the column unretained or, if retained at all, co-elute at the beginning of the chromatogram. Waters Atlantis columns (Waters, Milford, MA, USA) are designed for these types of challenging separations. Atlantis dC18 columns are a silica-based line of difunctionally bonded C18 columns that provide the optimal balance of retention for polar and non-polar compounds in reversed-phase chromatography [5].

An alternative to reversed-phase columns is ion-exchange chromatography. In this case, an IonPac ICE-AS1 column (Dionex, Sunnyvale, CA, USA) could be used that combines ion exchange with size exclusion chromatography. The advantage is that there is a significant increase of the *k*' value compared to reversed-phase columns, leading to a good separation of acrylamide from matrix compounds even for untreated sample extracts [5]. However, hydrophilic interaction liquid chromatography (HILIC) is one of the most successful approaches for the retention and separation of polar compounds. The primary advantage of HILIC as a separation technique is the strong retention of polar, hydrophilic compounds that are unretained under conventional reversed phase conditions, without the requirement for an ion-pair additive in the mobile phase.

Quantitative assays of acrylamide or its derivatives contained in foods were also performed by gas chromatography coupled with mass spectrometry. In the case of gas chromatography, derivatization of the analyte is required in order to increase volatility, selectivity, sensitivity and retention time. The most popular method of acrylamide derivatization is its bromination prior to the analysis [5]. This technique is highly selective and improves the assay precision, it also

effectively compensates for the difficult and time-consuming derivatization process. Another method of acrylamide derivatization consists of its silylation followed by solid phase microextraction (SPME), which can be used in the analysis of polar and non-polar compounds in gases, liquids, and solids [54].

Both GC based methods with and without derivatization were developed, showing a satisfactory agreement with LC-MS for the detection of acrylamide in various foods. A GC method coupled with MS following the derivatization was proposed for the detection of trace levels (<50 $\mu g\ kg^{-1}$) of acrylamide in cereal-based foods due to its high sensitivity (LOD 2 $\mu g\ kg^{-1}$) and great recoveries (93-104%) [58]. It is important to note, however, that the derivatization is not necessarily essential and some scientific groups have chosen to eliminate this lengthy procedure using a more polar GC phase, although this approach does not provide sufficiently low limit of detection in comparison to the derivatization techniques [7, 59, 60]. The main drawback of GC–MS without derivatization is the lack of characteristic ions in the mass spectrum of underivatized acrylamide and the interference caused by matrix decomposition. Due to the high background noise, a low limit of detection is impossible to obtain. The application of gas chromatography coupled to tandem mass spectrometry (GC–MS/MS) allows to decrease the interference, which results in a larger acrylamide peak area. The values of LOQ and LOD in the GC–MS/MS method for baby foods are below 5 $\mu g\ kg^{-1}$ and 1.5 $\mu g\ kg^{-1}$, respectively [54].

Lee *et al*. (2007) detected acrylamide in aqueous matrices using direct immersion solid-phase microextraction (SPME) coupled with gas chromatography - positive chemical ionization tandem mass spectrometry, which showed very high sensitivity (0.1 $\mu g\ kg^{-1}$) [61].

The other detectors used in a tandem with gas chromatographs to quantify acrylamide have been flame-ion detectors (FID) or electron capture detectors (ECD). A GC–ECD method with prior derivatization by $KBrO_3$ and KBr was used to determine the amounts of brominated acrylamide derivative in fried foods. Electron capture detectors are selective and very sensitive. For a GC–ECD method, the values of LOD and LOQ for potato crisps, potato chips, and fried chicken wings, were around 0.1 $\mu g\ kg^{-1}$ and 3 $\mu g\ kg^{-1}$, respectively, which provides evidence of a good precision when working with this relatively new method. Besides, the cost of instruments in a GC–ECD system is lower compared to GC–MS/MS, while their sensitivity is comparable [54].

Capillary electrophoresis is a powerful alternative for analyzing organic compounds based on charge-to-mass ratio differences with high separation efficiency. Two in-line preconcentration capillary zone electrophoresis methods (field amplified sample injection and stacking with sample

matrix removal) have been evaluated for the analysis of acrylamide in foodstuffs after being derivatized with 2-mercaptobenzoic acid, both of which showed similar sensitivity and precision compared to chromatography-based methods [62, 63]. A laser-induced fluorescence detection method mediated by capillary electrophoresis was studied for the detection of acrylamide in potato crisps with good recovery (90-95%) and precision (RSD <5.7%) [64].

Enzyme-linked immunosorbent assay (ELISA) is a rapid method based on the recognition of antigen-antibody binding with high specificity and affinity, as well as the signal readout through optically detecting colored products catalyzed by enzyme labels. Because of the high specificity/affinity of antigen-antibody recognition and the high efficiency of enzymatic catalysis, ELISA has many advantages, such as good sensitivity, selectivity, high throughput, and theability of coupling to other technologies, for example, biotin-avidin amplification and chemiluminescence. The application of ELISA for acrylamide quantification requires synthesis of specific antibodies, which has been troublesome because of the low molecular mass of this compound. In general, substances with molecular mass below 1000 Da are not immunogenic and do not elicit synthesis of antibodies. Preston *et al.* proposed to use polyclonal antibodies for this purpose [65]. The isolation of acrylamide binding antibodies enabled quantitative determination of acrylamide in foods. Because of the low molecular mass and the lack of strong epitope groups, acrylamide cannot by itself elicit synthesis of specific antibodies, however, its coupling (as a hapten) to immunostimulating carrier proteins was found to be an effective method for the stimulation of antibody synthesis. Then, the antibody of acrylamide can be produced via immunoreactions stimulated by the complex antigen [66].

Therefore, ELISA has attracted an increasing attention for detecting acrylamide in foods. In this new field, the two key issues are the development of appropriate antigens to obtain high affinity antibodies, and signal amplification. Figure 1.4 illustrates the preparation of a complete antigen, antibody, and performing ELISA for acrylamide detection.

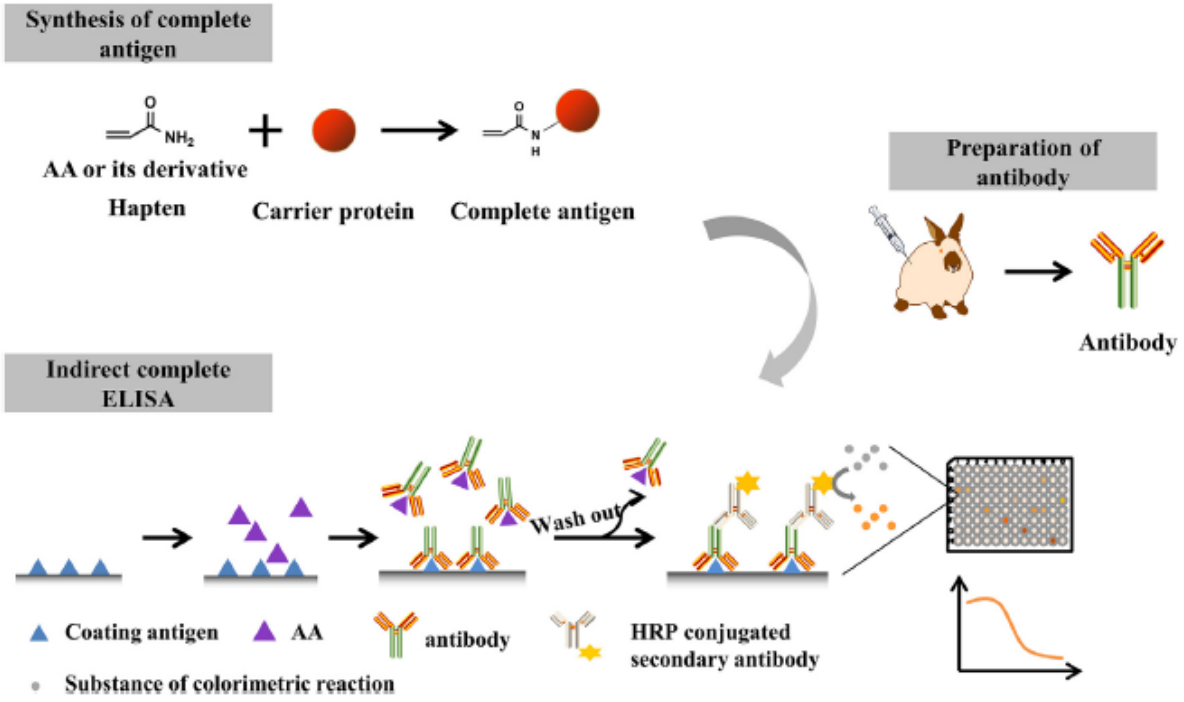

Fig. 1.4. **Schematic representation of the preparation of complete antigen, antibody and competitive indirect ELISA for acrylamide analysis**

Compared to the common analytical techniques, ELISA is a simpler method with similar sensitivity, lower cost, shorter detection time, and does not require expensive equipment or complex sample preparation. Therefore, ELISA kits available for commercial application are of significant interest. For example, an ELISA kit was tested and gave good recovery (from 92 % to 96 %) with a LOD and a linear range of 5 µg kg^{-1} and 10-10000 µg kg^{-1}, respectively [67]. However, ELISA still needs to be improved and the analytical results should be confirmed by other robust methods. Meanwhile, the ways to obtain stable antibodies of acrylamide with high affinity are still the key issue in this field.

Electrochemical biosensing methods have been proposed to detect acrylamide in foods or complex matrices only in the past few years. A biosensor is a device used to detect an analyte that combines a biological component (bioreceptor) with a physicochemical detector (transducer). Electrochemical biosensors, with electric signal (such as current and potential) output, have shown the advantages of speed, simplicity, suitability for automation, and sensitivity, leading to broad applications in food safety. The first trial for determining acrylamide in wastewater was based on biocatalysis from microbial metabolism, including respiration and enzyme reactions. Ignatov et al. (1997) first quantified acrylamide by defining specific respiratory activity as the difference

between the rate of oxygen consumption of *Brevibacterium sp.* and endogenous cell respiration after the introduction of acrylamide. The reduction of oxygen concentration and the current created from the metabolism of acrylamide could be used to detect acrylamide [68]. Silva *et al.* (2011) designed an electrochemical biosensor based on the electron transfer of a direct biochemical interaction between acrylamide and whole cells of *Pseudomonas aeruginosa* containing intracellular amidase, which catalyzed the hydrolysis of acrylamide, producing ammonium ions and acrylic acid. The use of biochemical reactions from microbial metabolism can lead to real-time detection of acrylamide. Good stability, a long working life, and the presence of various enzymes in cells are the advantages of microbial biosensors. However, the dependence on a living organism elicits long response time. Also, protecting the bacteria against aggressive environments is another problem [69].

Recently, a novel fluorescent sensing method based on acrylamide polymerization and the unique photophysical properties of quantum dots (QDs) was proposed to detect acrylamide [70], as shown in Figure 1.5. In this study, QDs containing carbon-carbon double bonds after modification of *N*-acryloxysuccinimide polymerized under UV irradiation, resulted in the decrease of the distance between QDs and the fluorescence intensity [71, 72]. In the presence of acrylamide, the distance of QDs became larger due to the participation of acrylamide in the polymerization reaction, resulting in an increase of the fluorescence intensity (Figure. 1.5). Therefore, a correlation was established between the concentration of acrylamide and changes of fluorescence intensity after UV irradiation. The linear interval and LOD were in the range of 35-350000 µg kg^{-1} and 35 µg kg^{-1}, respectively. Compared to the standard methods and electrochemical biosensing, the lower sensitivity of this method limits its applicability to the detection of acrylamide in various food samples.

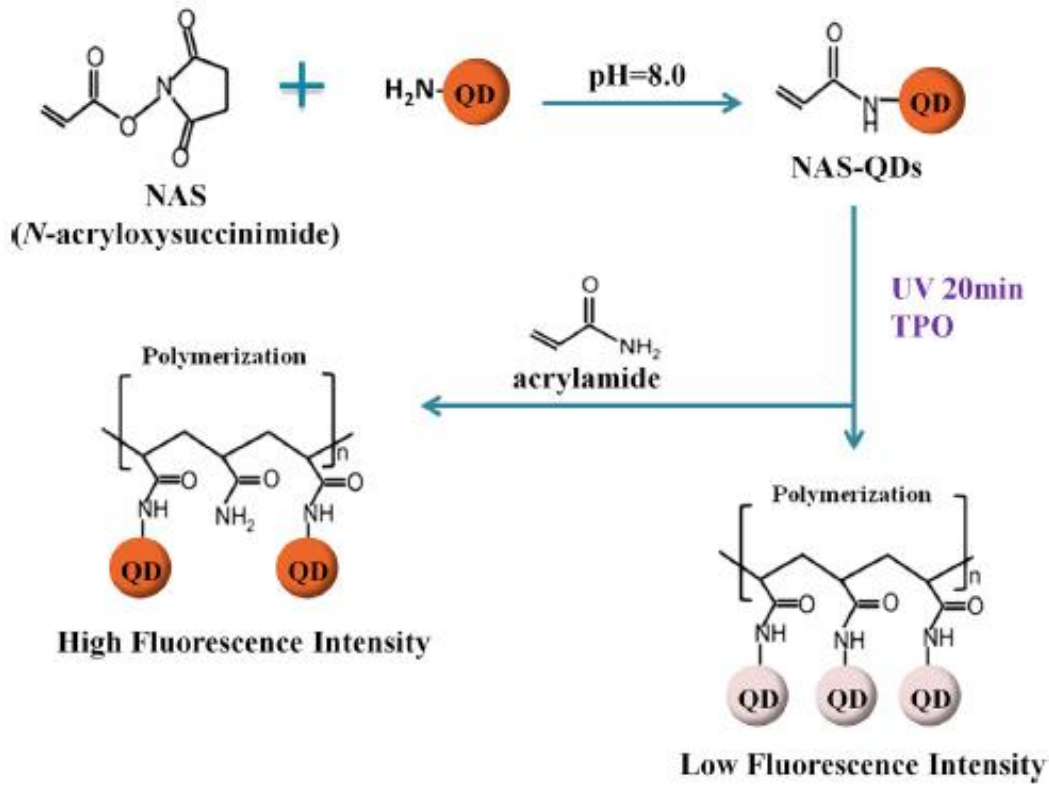

Fig. 1.5. **Schematic representation of the fluorescent sensing method for AA detection based on CdSe/ZnS quantum dots [70]**

For LC-MS/MS and GC-MS methods, the general procedures of pretreatment include homogenization, spiking with an internal standard, extraction, defatting, deproteinization, purification by SPE cartridges, and derivatization (for GC-MS).

The addition of an internal standard in food samples can allow the compensation for recovery and to keep track of the possible loss during the whole sample preparation procedure, which improves the accuracy, precision, and repeatability of measurements. Most of the published studies used $^{13}C_3$-acrylamide as an internal standard for acrylamide detection, which has very similar properties to acrylamide. However, D_3-acrylamide, $^{13}C_1$-acrylamide, *N*,*N*-dimethylacrylamide, propionamide, and methacrylamide also have been used as internal standards [73, 74].

Extraction is a critical procedure in food pretreatment prior to the detection of acrylamide. The hydrophilic character of acrylamide allows for an application of water or organic solvents, such as methanol and acetonitrile, for its extraction from foods [75, 76]. Water can minimize the co-extraction of hydrophobic compounds from foods, but other hydrophilic interferences still remain that should be removed afterwards [5]. In comparison to water, organic solvents have the advantages of easier separation even without centrifugation and more convenient evaporation. The

combination of water (salt solutions) and organic solvents improved the extraction efficiency in some studies [74, 77]. Depending on the food matrix, a defatting step with an organic solvent or a deprotonating step could be necessary to remove interfering components [10]. For protein-rich food samples, Carrez reagents ([I] potassium ferricyanide and [II] zinc sulfate), acetone, ethanol, or methanol were used to precipitate and remove proteins [78].

Solid phase extraction (SPE) has been used extensively in purifying the extracts of food samples due to its simplicity, stability, suitability for automation, accuracy, precision, and the repeatability of instrumental analysis. Depending on the selection of SPE cartridges used for purification of acrylamide from the extracts of food samples, two strategies have been developed. One strategy relies on acrylamide absorption from the complex extract by the cartridges through hydrogen bonding, π-π interaction, and cation exchange, followed by elution of acrylamide using other polar solvents. Some common cartridges are Oasis HLB, Oasis MCX, Isolute Multi-Mode, ENVI-Carb, and Isolute ENV+. The second strategy is applied to retain the interferences and collect the eluent containing acrylamide by using Oasis HLB cartridges coupled with Bond Elut-Accucat and a custom made SPE column filled with a mixture of C18, SCX, and SAX sorbents. Among these cartridges, Oasis HLB and Isolute Multimode are the most preferred. Recently, novel filling materials such as carbon nanotubes, magnetic chitosan, and molecule imprinted polymers were used, resulting in an effective purification [5, 9, 79, 80].

Selection of an appropriate analytical methodology for the determination of acrylamide is mostly based on the type of food matrix. Its quantification in food is difficult because of the low molecular mass (71.08 Da), lack of chromophores or fluorescent functional groups, high polarity, very good water solubility (2.1 g mL^{-1}), high reactivity, and low volatility. Quantification of acrylamide in complex matrices rich in interfering compounds is an especially demanding task [54]. The analysis of coffee samples to determine the acrylamide content has high importance because the exposure to acrylamide from coffee has been found to contribute substantially to the overall dietary acrylamide exposure. Several papers describe the determination of acrylamide in coffee samples using LC-MS/MS or GC-MS, indicating the existence of significant problems related to the matrix suppression phenomenon and the presence of interfering co-extractives [8-11], therefore a challenge still remains with respect to the development of stable, reliable, and robust methods for difficult food matrices, such as cocoa powders, coffee, and high salt flavorings. High-resolution mass spectrometry (HRMS) is particularly suitable for the detection of low molecular weight food contaminants and it can provide some analytical advantages over other MS

techniques. Analysis of acrylamide in roasted coffee with hybrid quadrupole – Orbitrap mass spectrometry was not previously described in the scientific literature.

1.3.4. Mitigation strategies to reduce acrylamide content in foods

Several research projects have been focused on ways to mitigate formation of acrylamide in foods. Temperature is a key factor that has been repeatedly shown to influence acrylamide levels. Microwaving and boiling generates minimal amounts, whereas harsher treatments such as frying and baking may lead to high levels [81].

The proposed techniques generally fall into one of several areas: reducing the availability of free asparagine or reducing sugars, modifying other ingredients, and changing the cooking time or temperature. Different techniques appear to be useful for certain types of products: for potato products, selecting potatoes low in reducing sugars, controlling storage conditions, and reducing the time or temperature of cooking are frequently cited methods, while for cereal products, modifying the time or temperature of cooking, avoiding the use of ammonium bicarbonate, and selecting materials low in asparagine may be more relevant. Other suggested techniques for acrylamide mitigation include the use of minor ingredients (e.g., amino acids, calcium, and citric acid) that interfere with acrylamide formation, and the use of asparaginase to reduce asparagine levels prior to cooking. No appropriate techniques have been identified for reducing acrylamide in coffee while preserving the taste. Importantly, most of the proposed measures for reducing acrylamide are still commercially unproven. Additionally, manufacturers need to know whether these measures affect the taste, stability and safety of products [82-84].

Particularly in the case of potatoes, soaking them in water prior to baking or frying significantly reduces acrylamide levels, as it leaches away its precursors from surface. Further reduction has also been observed after the addition of salt, citric acid or amino acids (glycine, lysine, cysteine) to the soaking solutions [85-87]. The addition of amino acids such as glycine to dough used for making snacks also has a negative impact on acrylamide formation [88].

One of the most promising tools to control acrylamide content in heat-treated foods is the addition of the enzyme asparaginase. Asparaginase (L-asparagine amidohydrolase) is an enzyme promoting the hydrolysis of asparagine to aspartic acid and ammonia, thus lowering the content of acrylamide precursor asparagine. Asparaginase has been successfully applied on a lab scale both to potato products [30] and cereal-based products [2, 89, 90] resulting in acrylamide reduction by

up to 85-90% and no effect on the taste and appearance of foods. Asparaginase is being already used for some products on industrial scale [91].

The modification of acrylamide precursors by using biotechnological tools [92] is regarded as an interesting way of acrylamide reduction. Fermentation of dough allows for the control of the acrylamide formation rate related to the consumption of reducing saccharides or asparagine [93]. Reduction of acrylamide content in cereal products can be achieved by prolonged fermentation as a consequence of extensive asparagine consumption by yeast [94, 95]. One of the possibilities for reducing the levels of acrylamide and thus ensuring the safety of bakery products [96, 97] is the use of bacteriocin type inhibitory substances produced by lactic acid bacteria (LAB) with high proteolytic activity [98]. These LAB are generally fastidious on artificial media, but they grow readily in most food substrates and lower the pH rapidly to a point where competing organisms are no longer able to grow. The formation of lactic acid is ensured by acidification below pH 4.2, which is a safety factor, and also gives a nice taste to whey products [99]. The leavening by LAB is associated with yeast forming a softer and more palatable bread crumb, and modifying flour components, e.g. swelling and partial hydrolysis of proteins and polysaccharides [100]. The highly promising results of these studies underline the important role that functional bacteriocins of certain LAB strains play in the food industry as starter cultures, co-cultures, or bioprotective cultures, in order to improve food quality and safety [97]. The use of lactic acid bacteria sourdough is a way to enhance dough properties, to improve flavor, texture, and microbiological shelf life of bread [101].

1.4. Furan

Furan is a small, heterocyclic organic molecule that has been identified in a number of food products [102], especially jarred and canned food items that undergo heat treatment in a sealed container, but furan has also been detected in other food items such as meat, beer, and nuts [103]. It is a lipophilic, highly volatile colorless liquid with a boiling point of 31.4°C [14].

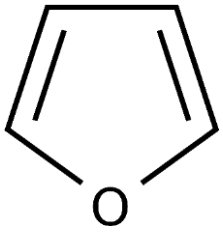

Fig. 1.6. **The structure of furan**

Before 2004, the presence of furan due to heating had been reported in a limited number of foods, with the highest levels found in coffee [102]. Although the presence of furan in foods has been known for years, the interest in this compound has increased after the International Agency for Research on Cancer in 1995 classified this compound as a possible human carcinogen (Group 2B) based on an extensive research study on animals [104].

In May 2004, the US Food and Drug Administration (FDA) published survey results concerning the presence of furan in products that undergo heat treatment. Detectable furan levels were found in a variety of foodstuffs (e.g., canned and jarred foods, baby foods, coffee, soups and sauces, etc.). [105]. Furan was also subsequently identified in certain low moisture foods as well, including crackers, potato chips, and tortilla chips [106].

The formation of furan is not as well understood as that of acrylamide. Multiple mechanisms have been proposed. Possible pathways for the formation of furan in foods were assessed by several researchers. These mechanisms are mainly related to:

i. The Maillard reaction between sugars and amino acids such as aspartic acid, alanine and threonine that can generate only acetaldehyde [60, 107];
ii. Thermal degradation of carbohydrates such as glucose, lactose, and fructose [102, 107, 108, 110];
iii. The thermal degradation of certain amino acids, such as serine and cysteine, capable of forming acetaldehyde and glycolaldehyde, which are precursors of furan through aldol condensations followed by cyclization reactions [107, 109];
iv. The thermal oxidation of ascorbic acid [60, 111];
v. The oxidation of polyunsaturated fatty acids [107, 111];
vi. The oxidation of carotenoids [112].

Studies on the formation of furan and the effect of interaction with can coatings revealed no interaction with the cans and their coating [113]. When comparing specific heating techniques, differences were observed between heating in a pot and heating in microwave oven. In most cases the furan levels decreased when heating in a pot, but this was not always the case when heating in a microwave oven [114].

Possible pathways of furan formation from ascorbic acid, sugars, carbohydrates, amino acids and polyunsaturated fatty acids are illustrated below in the Figure 1.7 [103].

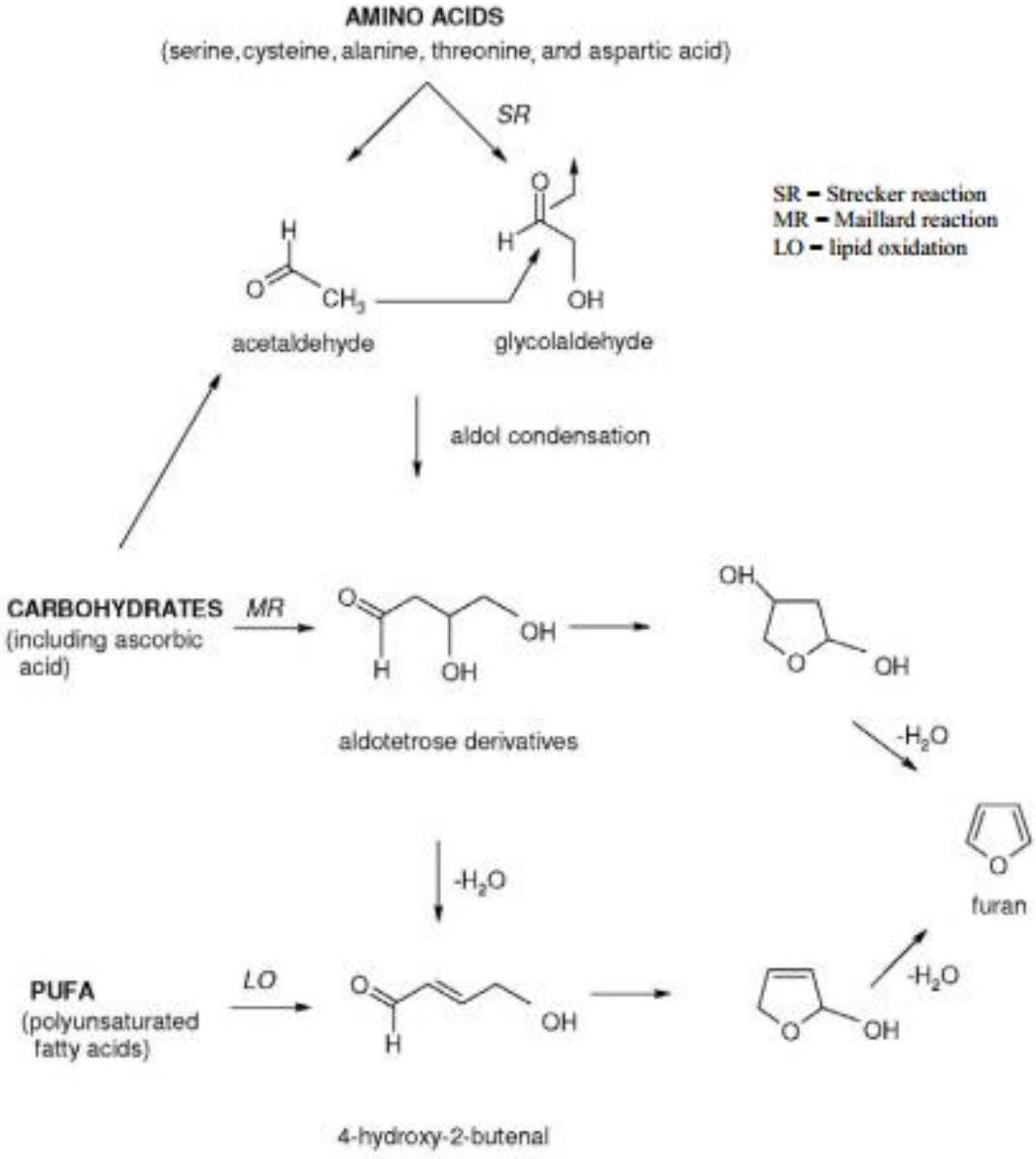

Fig 1.7. **The proposed pathways leading to the formation of furan from three main groups of sources, i.e., amino acids, carbohydrates, and polyunsaturated fatty acids**

Reaction conditions such as temperature, time, and pH can significantly affect the formation of furan [115, 116]. For instance, Fan *et al.* demonstrated a time- and temperature-dependent increase in the formation of furan in fresh apple cider. Heat treatment at 90ºC for 10 min produced no measurable furan, whereas heating to 120ºC resulted in a time-dependent increase in furan production. Importantly, the effect of pH on furan formation may depend on the precursor from which furan is formed. In a study investigating the influence of pH on furan formation from sugars, ascorbic acid, and fatty acids, the highest amount of furan was formed from linoleic acid at pH 6. In contrast, thermally induced furan formation in a solution of ascorbic acid was significantly higher at pH 3 than at pH 6 [116].

The reduction of furan in foods is likely to be more challenging compared to the mitigation of other process contaminants, for two reasons. First, there may be little room for maneuver to lower heating times and temperatures, because the processes of pasteurization and sterilization are optimized for the microbiological safety of foods. Second, furan has a wide range of precursors [13].

1.4.1. The toxicity of furan

Furan in food is a concern due to its carcinogenic properties, therefore, it is listed by the US Department of Health and Human Services Report on Carcinogens, based on animal tests, and it is considered possibly carcinogenic to humans by the International Agency for Research on Cancer. A European Food Safety Authority review concluded that there is a relatively small difference between possible human exposures and doses that caused cancer in animals, although emphasizing that both toxicity and exposure data were limited and that more data were needed to draw conclusions [117].

Furan is a potent hepatotoxin and hepatocarcinogen in rodents, causing hepatocellular adenomas and carcinomas in rats and mice, and high incidences of cholangiocarcinomas in rats at doses ≥ 2 μg kg^{-1} b.w. There is therefore a relatively low margin of exposure between estimated human exposure and doses that cause a high incidence of tumors in rodents. Since a genotoxic mode of action cannot be excluded for furan-induced tumorigenesis, the present exposures may indicate a risk to human health that requires mitigation [118].

1.4.2. The occurrence of furan in foods and dietary exposure

In 2011, EFSA has done an update on all data sampled and analyzed between 2004 and 2010, and has presented exposure estimates for different populations. The report includes a total of 5,050 analytical results for furan content in foods submitted by 20 countries. The highest furan levels were found in coffee with the mean values varying between 45 μg kg^{-1} for brewed coffee and 3,660 μg kg^{-1} for roasted coffee beans. The highest 95th percentile was reported for roasted coffee beans at 6,407 μg kg^{-1}. In non-coffee categories, the mean values ranged between 3.2 μg kg^{-1} for infant formula and 49 μg kg^{-1} for jarred baby food containing vegetables only, the latter also with the highest 95th percentile of 123 μg kg^{-1}. Mean furan exposure across surveys was estimated to range between 0.03 and 0.59 μg kg^{-1} b.w. per day for adults, between 0.02 to 0.13 μg kg^{-1} b.w. per day for adolescents, between 0.04 and 0.22 μg kg^{-1} b.w. per day for other children, between 0.05 to 0.31 μg kg^{-1} b.w. per day for toddlers and between 0.09 and 0.22 μg kg^{-1} b.w. per day for infants. A major contributor to the exposure of adults was brewed coffee with 85% of the total furan exposure on average. Major contributors to furan exposure in toddlers and other children were fruit juice, milk-based products and cereal-based products, whereas in addition for toddlers jarred baby foods were major contributors [119].

1.4.3. Methods for analytical detection of furan

The FDA published the first quantitative method for determination of furan in foods [103]. It used sample preparation under cold conditions and headspace sampling following incubation at 80ºC. Chromatographic separation used a PLOT (porous layer open tubular) type capillary columns with mass spectrometric detection in selected ion monitoring mode. The selected PLOT-Q column had a bonded polystyrene-divinylbenzene based phase, which effectively separates small volatile molecules. Quantification was based on standard additions and used deuterated furan as internal standard. Due to the increasing interest in testing for furan, several variations of this procedure were designed and introduced by other laboratories.

Because of the volatility of furan, headspace sampling is the obvious method for this analysis. To avoid losses of analyte, the food samples typically need to be chilled (+4ºC) before handling, and to be homogenized briefly but effectively by using a chilled blender with the sample sitting in an ice bath. Puree and liquid samples can be weighed directly into the headspace vial and mixed

with chilled water prior to addition of internal standard and spiking solutions. Solid samples may need homogenization with cold water using a top drive homogenizer [13].

Partitioning of furan from the food sample into the vial headspace is affected by time, temperature, and the mobility of the sample. Effective partitioning has been ensured by prolonging the equilibration incubation time [60], or improving the efficiency of automated shaking by adding glass beads to the headspace vial [113]. In any event, sufficient water must be present or added to ensure that the sample is completely mobile before analysis.

Normal headspace procedures involve heating the sample to promote volatilization of analyte into the headspace gas phase. With furan, excessive heating is both undesirable and unnecessary. Becalski *et al.* (2005) showed that increasing the headspace incubation temperature from 30 to 50ºC caused only a 50% increase in the furan peak area of an aqueous standard. Addition of salt more than doubled the furan signal. In view of the danger of forming extra furan even at quite low temperatures, most scientists have settled on an incubation temperature of 50ºC or below, and this gives adequate sensitivity, at about 1 µg kg^{-1} [60].

Senyuva and Gokmen (2005) demonstrated furan formation in green coffee (4 µg kg^{-1}) and in tomato and orange juices on incubation at 40ºC for 30 min. For any new sample type, it is necessary to check that the analytical procedure, and especially the headspace incubation temperature used, does not cause the formation of extra furan [120].

Furan can also be analyzed by a SPME method in combination with GC-MS. In direct headspace analysis a portion of the headspace gas is taken and injected directly into the GC-MS instrument. In contrast, during SPME procedure a needle coated with a polymeric material is first exposed to the headspace vapors to absorb volatiles (for 10-60 min in the references cited), and is then desorbed thermally (1-5 min; 90-300ºC) in the injection port of the gas chromatograph to drive off volatiles onto the GC column. SPME uses fibers coated with a liquid (polymer), a solid (sorbent), or a combination of both [13, 14]. SPME increases the concentration of analyte and enables high sensitivity and minimum interference from matrix compounds [15]. Kim *et al.* (2010) concluded that SPME is a simple and fast technique for the analysis of furan in processed foods. However, this technique has some essential drawbacks: an appropriate stationary phase, stable SPME fiber, and optimized coating techniques are crucial for the extraction of trace amounts of furan that make it more expensive compared to other methods [20].

Several authors have focused their studies on the occurrence of furan in baby foods due to the susceptibility of this population group. Reports have been published describing relatively high

concentrations of furan up to 153 µg kg^{-1} in Switzerland [121], 141 µg kg^{-1} in Italy [122], and 95.5 µg kg^{-1} in Brazil [16] for vegetable and meat based baby foods. At the same time, furan levels in fruit based baby foods are very low, below 5 µg kg^{-1} on average. Many researchers reported non-detection of furan because of relatively high limits of detection, for example, 2.4 µg kg^{-1} [16], 4.0 µg kg^{-1} [17, 18], 0.17 µg kg^{-1} [19], 0.12 µg kg^{-1} [20], 0.60 µg kg^{-1} [21]. Therefore, the development of new methods with higher sensitivity for furan in baby foods is still a very important task. Direct headspace sampling appears to be a simple, fast, and economical procedure, but, as it was mentioned above, the limits of quantification sometimes are too high for measuring the accurate furan concentrations in samples. Analysis of furan with tandem mass spectrometry was not previously described using direct headspace sampling and gas chromatography separation.

2. EXPERIMENTAL PART

2.1. Sampling and storage

All samples analyzed for acrylamide and furan contents were purchased from local supermarkets in Riga, Latvia. Samples were chosen of different brands containing various product groups, such as meat, vegetables, cereals, fruits, potato chips, bread, confectionery, coffee and others. 106 samples were sampled for furan analysis and 457 samples for acrylamide analysis (Table 2.1).

For determination of acrylamide samples were homogenized and stored in refrigerator until analysis. For determination of furan the samples were stored unopened and refrigerated until analysis.

Table 2.1.

Summary of furan and acrylamide analysis

Product group	Amount of samples	
	Furan	Acrylamide
Baby food	35	
Canned vegetables	7	
Jam	8	
Honey	6	
Sauce	15	
Canned beans	8	
Juice	10	
Soups	4	
Bread	1	244
Coffee	12	40
Snacks		77
Pastry		96
Total:	106	457

2.2. Chemicals and materials

Analytical standards of acrylamide (99%), acrylamide-d_3, furan (99%) and furan-d_4 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol was of a gradient grade for HPLC. Formic acid (98%) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was prepared by a Milli-Q (Millipore, Billerica, MA, USA) water purification

system. Sodium chloride and anhydrous magnesium sulfate were purchased from Scharlau (Barcelona, Spain). Primary secondary amine (PSA) sorbent was purchased as a bulk sorbent from United Chemical Technologies (Brockville, ON, Canada). Strata NH_2 cartridges (500mg/6ml) were obtained from Phenomenex (Torrance, CA, USA).

Stock and working standard solutions of acrylamide and acrylamide-d_3 were prepared in acetonitrile. Stock solutions of furan and furan-d_4 were prepared at the concentration of 1 mg mL^{-1} in chilled methanol. Working standard solutions were freshly prepared before analysis at the concentrations of 0.1 and 1 µg mL^{-1}.

2.3. Analytical method for determination acrylamide in foods

2.3.1. Sample preparation and clean-up, QuEChERS method

Sample (2 g), the internal standard (volume, corresponding to the concentration in sample 100 ng g^{-1}) and 5 mL of hexane were added into a 50 mL centrifuge tube, then the tube was vortexed. Distilled water (10 mL) and acetonitrile (10 mL) were added followed by the QuEChERS extraction salt mixture (4.0 g anhydrous $MgSO_4$ and 0.5 g NaCl). The sample tube was shaken for 1 min vigorously and centrifuged at 4500 rpm for 5 min. The hexane layer was discarded, and 1 mL of the acetonitrile extract was transferred to a tube containing 50 mg of PSA-sorbent and 150 mg of anhydrous $MgSO_4$. The tubes were vortexed for 30 s and then the purified extract was analyzed by the ultra-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-QqQ-MS/MS).

2.3.2. HPLC-QqQ-MS/MS system

The quantitative analysis of acrylamide was performed by HPLC-QqQ-MS/MS using Acquity Ultra Performance LC system (Waters, Milford, MA, USA) coupled to a QTrap 5500 (AB Sciex, MA, USA). The separation of acrylamide was achieved with Luna 3u HILIC column (100×2.00 mm; Phenomenex, Torrance, USA). Methanol (6%) solution in acetonitrile acidified with 0.1% formic acid was used as a mobile phase (flow rate 0.25 mL min^{-1}, column temperature 40°C and injection volume 10 µL). The conditions for the detection by MS/MS were as follows: 30 psi curtain gas (CUR), 5500 V ion spray voltage, 400°C temperature, 40 psi ion source gas

(GS1), 50 psi ion source gas (GS2), 60 V declustering potential (DP). Acrylamide was quantified in food samples using the product ion *m/z* 55 (precursor ion *m/z* 72) for the analyte and *m/z* 58 (precursor ion *m/z* 75) for the internal standard (acrylamide-d3). The quantification was based on the peak area of analyte compared to that of the deuterated internal standard. The limit of quantification for acrylamide was 10 μg kg^{-1}.

The HPLC-QqQ-MS/MS method for determination of acrylamide in food was validated. The laboratory has participated in the inter-laboratory proficiency testing organized in 2011 by FAPAS analyzing a biscuit test material and has received a Z-score -0.3. Furthermore, the laboratory quality control sample was also used in each series of analysis to determine recovery of acrylamide during the sample preparation procedure.

2.4. Analytical method for determination acrylamide in coffee

2.4.1. Sample preparation and clean-up

The sample preparation procedure entailed the following steps: weighing 1 g of thoroughly homogenized sample into a 50 mL centrifuge tube and adding internal standard at 200 μg kg^{-1} (20 μL of 10 ng μL^{-1} standard solution of acrylamide-d_3 in acetonitrile). Deionized water (10 mL) and acetonitrile (10 mL) were added, followed by anhydrous $MgSO_4$ (4.0 g) and NaCl (0.5 g) as a pre-weighted salt mixture. The tube was sealed immediately thereafter and shaken vigorously for 1 min by hand to prevent the formation of crystalline agglomerates and to ensure sufficient solvent interaction with the entire sample. Then the tube was centrifuged for 5 min at 4500 rpm. Acetonitrile extract (4 mL) was transferred to a 15 mL centrifuge tube containing 200 mg PSA and 600 mg $MgSO_4$. The extract was vortexed for 30 s and centrifuged at 3500 rpm. Strata NH_2 SPE cartridges were activated with acetonitrile (3 mL), dried under vacuum, and 2 mL of extract was passed through the sorbent into a new tube without application of vacuum. The eluate was transferred into an autosampler vial for the HPLC-Q-Orbitrap-HRMS analysis.

2.4.2. HPLC-Q-Orbitrap-HRMS system

The chromatographic separation of acrylamide was carried out using an HPLC Accela system 1250 (Thermo Fisher Scientific, San Jose, CA, USA) consisting of a degasser, a quaternary pump,

a thermostated autosampler, and a column oven. Chromatographic separation was performed on a HILIC analytical column (100 x 3.0 mm, 5 µm) from Phenomenex (Torrance, USA). The mobile phase was delivered at the flow rate of 0.3 mL min^{-1} consisting of (A) 0.1% formic acid in water and (B) 0.1 % formic acid in acetonitrile. A gradient program was used: 100% of mobile phase (B) was used from 0 to 3 min, 100% (B) to 50% (B) from 3.0 to 3.5 min, maintained at 50% (B) from 3.5 to 5.0 min, than increased (B) to 100% from 5.0 to 5.5 min and finally the column was re-equilibrated with 100% (B) from 5.5 to 10 min. A 10 µL aliquot of the extract was injected. The column and autosampler were maintained at 30 °C and 10 °C, respectively.

The HPLC system was coupled to a Q-Orbitrap-HRMS (Thermo Fisher Scientific) equipped with a heated electrospray ionization probe (HESI II) operating in the positive ionization mode. Nitrogen was used for spray stabilization, collision-induced dissociation experiments in the higher energy collision dissociation (HCD) cell, and as the damping gas in the C-trap. The following ionization parameters were applied: electrospray voltage 4 kV, heater temperature 350°C, capillary temperature 300°C, sheath gas (N_2) 40 arbitrary units (arb), auxiliary gas (N_2) 10 (arb), and S-Lens RF level at 35 (arb). The AGC target was set to 1×10^6, the maximum IT was set to 20 ms, the number of microscans to be performed was set at 2. Fragmentation mass spectra were recorded at a mass resolving power of 35000 FWHM with a quadrupole isolation window of 1.0 Da for precursor ions (72.0444 Da for acrylamide and 75.0632 Da for acrylamide-d_3), the use of normalized collision energy (NCE) of 60% and stepped NCE of 30%. The scan range for daughter ions was from 50 to 80 *m/z*.

Before analysis, the mass analyzer was externally calibrated by introducing a calibration solution that consisted of caffeine, Met-Arg-Phe-Ala (MRFA), Ultramark 1621, and acetic acid in acetonitrile/methanol/water solution (2:1:1, v/v/v).

The data processing was carried out with Xcalibur 2.2. and TraceFinder EFS software (Thermo Fisher Scientific).

2.5. Analytical method for determination of furan

2.5.1. Sample preparation and clean-up

The sample preparation procedure was selected taking into account the published information from various researchers in relation to the amount of sample to be weighed [20], type and optimal

amount of salt [15, 123], and temperature of incubation [123]. After summarisation of all available information, the following sample preparation procedure was chosen.

A portion of 5 g of homogenous sample was weighed into a chilled 20 mL headspace vial, then 2.0 g of NaCl and 10 mL of deionised water were added. After adding internal standard at 25 µg kg^{-1} concentration, vials were immediately closed to avoid analyte losses, and were shaken for 1 min to obtain a homogenous mass. Laboratory quality control samples at furan concentration of 10 µg kg^{-1} were prepared along with the study sample.

2.5.2. HS-GC-MS/MS system

Furan was analysed using GC equipped with a TriPlus RSH headspace autosampler, coupled with a TSQ Quantum XLS tandem mass spectrometer (ThermoScientific, MA, USA). The capillary column used was 60 m × 0.32 mm × 1.8 µm Rtx-624 (Restek, Bellefonte, USA). Analysis was performed in the splitless mode. Operating conditions: carrier gas was helium (GC grade, 99.999%) at a constant flow rate of 1.7 mL min^{-1}, injector temperature of 200°C, MS transfer line temperature was 250°C, and the ion source temperature was 200°C. The initial oven temperature was 35°C (held for 2 min), then increased to 230°C at a rate of 20°C min^{-1} and held for 20 min. The total analysis time was 13 min. Injection volume was 1.5 mL, incubation for 10 min at 70°C. The emission current was set at 50 µA and electron energy at 70 V and 35 V, respectively, for selected ion monitoring (single ion scan) and selected reaction monitoring (daughter ion scan) modes. The collision gas pressure for SRM mode was set at 1.3 mTorr. Two scan types were compared: SIM and SRM for the detection of furan and the internal standard.

2.6. Determination of Brix and pH

The content of soluble solids was estimated using an Atago RX-5000α refractometer (Tokyo, Japan). The pH of each sample was measured using WTW inoLab 730 pH meter (Frimley, Surrey, UK).

2.7. Determination of sugars (fructose, glucose, saccharose, and lactose)

The concentration of sugars in baby food samples was tested by high performance liquid chromatography with refractometric detector in accordance to the DIN 10758:1997-05 standard [124], with minor changes made to include the determination of lactose in the same procedure.

2.8. Determination of β-carotene

The sample preparation for β-carotene analysis was performed according to Schrieber *et al.* (2002) with some minor changes [125]. A 1:1 mixture of acetone and hexane by volume (20 mL) was added to a 5 g portion of homogenous sample. After separation, the hexane layer was washed with 20 mL of 10% sodium chloride solution. The extract was finally dried with sodium sulphate and then evaporated under nitrogen stream. The residue was reconstituted in 1 mL of mobile phase.

Quantitative analysis of β-carotene was performed by HPLC-QqQ-MS/MS using an Acquity HPLC system (Waters, Milford, USA) coupled to a QTrap 5500 mass spectrometer (AB Sciex, MA, USA). The separation of β-carotene was achieved with a 50 × 3.00 mm, 1.7 µm C18 column maintained at 30°C. Elution of the analyte was performed with mobile phase of 0.1 M aqueous ammonium acetate (A) and methanol/isopropanol (1:1, v:v) (B). The mobile phase system consisted of a 1 min linear gradient from 80% to 99% B, held at 99% for 1.5 min, and then returned to 80% B for 2.5 min to re-equilibrate. The flow rate was 0.6 mL min^{-1}, with injection volume of 10 µL.

A QTrap 5500 mass spectrometer was used for the analysis with atmospheric pressure chemical ionisation performed in the positive ion mode. The nitrogen gas flow was set at 30 psi for curtain gas and 50 psi for ion source gas. Temperature of the heated nebulizer was 450°C with a nebulizer current of 4 µA. The mass transitions for β-carotene were selected as follows: *m/z* 537 → 321 and *m/z* 537 → 177.

2.9. Determination of asparagine

Asparagine was determined in non-fermented JA, fermented JA samples, and wheat flour and biscuits samples. L-Asparagine monohydrate (≥99.9%) was purchased from Sigma-Aldrich. The formic acid was obtained from Fluka (Buchs, Switzerland). A liquid chromatography–tandem

mass spectrometry method described by Nielsen *et al.* with slight modifications was applied in this study [126].

A 1 g sample was weighed into a 50-mL centrifuge tube and 40 mL of water was added and shaken on the shaker (Multi Rotator RS-60, BioSan, Riga, Latvia) for 30 min. Afterwards centrifuged (centrifuge "Multifuge 3L-R", Thermo Fisher, Vilnius, Lithuania) at 4000 rpm for 10 min. Subsequently, 10 mL of supernatant was filtered through a paper filter, and additional water (10 mL) was added. SPE C18 500 mg per 6-ml cartridges (Strata, Phenomenex, Torrance, USA) was conditioned with 4 mL of methanol and 4 mL of water and washed with 1 mL of extract. Then, 2 mL of extract was slowly filtered through cartridges, and 10 µL of the solution was injected for detection by Qtrap 5500 tandem quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source coupled with an Acquity HPLC system. Conditions used for liquid chromatography analysis: Hypercarb column (Thermo Scientific), 100 × 2.1 mm 5 µm; mobile phase A (0.1% formic acid in water); mobile phase B (methanol); isocratic conditions: 60% A and 40% B; flow rate: 0.3 mL min1; column temperature 40 °C; 10 µL injection volume. Tandem mass spectrometry conditions were: electrospray ionization in the negative mode; 4000 V voltage; source temperature 500 °C; DP 50 V; CE 20 V; MRM was acquired with the characteristic fragmentation transitions m/z: 131 →113, 131 → 70. Asparagine was quantified using linear calibration graph with standard solutions of asparagine dissolved in water. The calibration range of the applied method covered the content interval 10–500 mg kg^{-1}, and the limit of detection and quantification was 3 mg kg^{-1} and 10 mg kg^{-1}, respectively.

2.10. Preparation of fermented products

The fermented product (65% moisture content) for mixed rye bread has been prepared using 300 g of extruded rye wholemeal flour and 450 mL of water (50ºC). Saccharification was made with or without addition of a selected amount of *A.niger* glucoamylase. After 30 min LAB cell suspension (5 mL) containing about 9.2 CFU mL^{-1} was added, followed by fermentation for 24 h at temperatures optimal for the strains. The sourdough for control bread was made following the same formulation using rye flour and hot water (95ºC), saccharified at 30ºC temperature for 3 h, and fermented under the same conditions as the test sourdough without addition of starter cultures (spontaneously). The pH value of sourdough was measured and recorded by a pH electrode. Prepared fermented product (750 g) was used for bread production.

Preparation of fermented products with lupine wholemeal: lupine wholemeal (300 g), tap water (160 g) and LAB cell suspension (10 g), containing on average of 10.2 $\log_{10}$ CFU g^{-1} of the above individual LAB strains were used to prepare 460 g of lupine sourdough following the fermentation at appropriate temperatures for 24 h. Final LAB colony number in the sourdoughs was on an average of 7.28 $\log_{10}$ CFU g^{-1}. Spontaneous lupine sourdough was prepared from lupine wholemeal (300 g) and water (160 g) without LAB starter followed by the incubation at 30ºC for 24 h.

Fermentation of JA tubers: LAB *P. acidilactici KTU05-7, P. pentosaceus KTU05-9, L. sakei KTU05-6* were used for JA fermentation. Water content was calculated with reference to moisture content of the raw materials and water absorption capacity; required humidity of the end product for solid-state fermentation (SSF) was 450 g kg^{-1}, and for submerged fermentation (SMF), it was 650 g kg^{-1}. Fermentation was carried out for 48h at optimal temperatures for LAB cultivation: 32°C (*P. acidilactici KTU05-7*), 35°C (*P. pentosaceus KTU05-9*) and 30°C (*L. sakei KTU05-6*). Using different LAB and different fermentation technologies (SSF and SMF), six different sourdoughs were obtained. The pH values of fermented JA were measured and recorded with a pH electrode (PP-15, Sartorius, Gottingen, Germany).

Fermentation of plant material: flaxseed or lupine flour (200 g) were mixed with water and 2% (w/v) of pure culture of *P. acidilactici (P. acidilactici KTU05-7), P. pentosaceus (P. pentosaceus KTU05-9), L. sakei (L. sakei KTU05-6).* Water content was calculated with a reference to moisture content of the raw materials and required humidity of the end product for SSF (moisture content 45%) and for SMF (moisture content 65%). Fermentation was carried out for 48 h at optimal temperatures for LAB cultivation: 32ºC (*P. acidilactici*), 35 ºC (*P. pentosaceus*) and 30 ºC (*L. sakei*). Six different sourdoughs from each plant were made using different LAB and different fermentation technologies (SSF and SMF).

2.11. Bread and biscuit experimental preparation

Experimental bread-making (3.4.1) was done according to the traditional procedure used for sourdough bread making in Lithuania and Latvia. Breads, containing 15% flour basis of *P. acidilactici, L. sakei or P. pentosaceus* fermented product, were made from 1 kg of wheat and 700 g of rye flours, 750 g of fermented product and 700 mL of tap water. The recipe of the control bread without addition of fermented product was based on 2 kg of flour weight (1 kg wheat flour

and 1 kg of rye flour): salt 1.7%, fresh compressed yeast 2.5% and tap water 56%. The dough was then mixed at a low regime in a mixer (Diosna SP25, Osnabrück, Germany) for 10 min, fermented at 27ºC and 75% relative humidity for 30 min. Then the dough was shaped and proofed at 35ºC and 80% relative humidity for 40 min. Dough loafs of 1000 and 500 g were formed and baked in a deck oven (MIWE Michael Wenz GmbH, Germany) at 240ºC for 50 and 35 min, respectively. Breads were cooled at room temperature and bread crumb was subjected to acrylamide analysis.

Experimental bread-making (3.4.2) was carried out according to the traditional procedure used for bread making in Lithuania and Latvia. The recipe of the control bread without sourdough was based on 2 kg of wheat flour: salt 34 g, fresh compressed yeast 50 g and tap water 1100 g. Test breads, containing lupine wholemeal (15% of flour basis), spontaneous lupine sourdough and *P. acidilactici KTU05-7, L. sakei KTU05-6,* and three different *P. pentosaceus strains (KTU05-8, KTU05-9, KTU05-10)* sourdoughs (460 g) were prepared following the same formulation with the adjustment of water (dough moisture 46%). The dough was then mixed in a spiral mixer (Diosna SP25, Osnabruk, Germany) for 10 min, fermented at 28 ºC and 75% relative humidity for 30 min. Then the dough shaping and proofing at 35 ºC and 80% relative humidity for 60 min was performed. Dough loaves of 500 g were baked in a deck oven (MIWE Michael Wenz GmbH, Arnstein, Germany) at 230 ºC for 25 min. Breads were cooled at room temperature, and bread crumb was subjected for acrylamide analysis. Determination of the texture and sensory evaluation of breads were performed 24 h after baking.

Main formula for biscuit preparation (3.4.3): wheat flour 300 g, margarine 100 g, saccharose 80 g, eggs 80 g, salt 1 g, baking powder 1.5 g. Test samples were prepared by addition of JA fermented with each of LAB using different processing (SSF and SMF) to the main biscuit dough at levels of 25 g, 50 g and 75 g. With each bacteria six biscuit samples were prepared. Control sample was prepared with 50 g of non-fermented JA. Saccharose and fat were creamed in a mixer (Guangzhou R & M Machinery, Guangdong, China). Eggs were added to this cream and mixed for 0.5 min to obtain a homogeneous cream. Finally, flour was added and mixed for 1 min to obtain a homogeneous plain dough (for control samples) and dough with different levels of LAB-fermented (SSF and SMF) JA additives. Biscuits were formed manually by rolling with rolling machine 'Roll S5B' (Vicenza, Italy), the thickness of the dough was 2.5 mm, and stamping by hands. Biscuits were baked in a deck oven (MIWE, Michael Wenz, Germany) at 220°C for 10 min.

Main formula for biscuit preparation (3.4.4): wheat flour 300 g, margarine 100 g, saccharose 80 g, eggs 80 g, salt 1 g, baking powder 1.5 g. Test samples were prepared by addition of flaxseed

and lupine fermented with each of LAB using different processing (SSF and SMF) to the main biscuit dough at levels of 25 g, 50 g and 75 g. With each bacteria six biscuit dough samples with flaxseed and six with lupine were prepared. Control samples were prepared without plant additives with 25 g, 50 g and 75 g of non-fermented plants.

2.12. Quality control and quality assurance

Quality control of the method was performed through routine analysis of laboratory quality control samples, as well as reagent blanks in each sample sequence. To compensate for the losses of analytes during the extraction and clean-up steps, each sample was spiked before the sample preparation procedure with deuterated internal standard. Five to six point calibration graphs were used for the quantification of furan or acrylamide concentrations in each sample run.

The following internal quality control criteria for the positive identification of analytes of interest were applied:

i. the retention time of native compound should be within a window of ± 0.2 min compared to the corresponding internal standard;
ii. the signal-to-noise ratio should be equal to or greater than 3 ($S/N \geq 3$);
iii. recovery of laboratory quality control samples should be in the range of 60 – 120%;
iv. the coefficient of determination (R^2) should be ≥ 0.99.

2.13. Collection of food consumption data

The comprehensive Latvian National Food Consumption database was developed in 2007-2009 and was updated from 2012 to 2013 to facilitate:

i. risk assessment in relation to chemical and biological hazards in food;
ii. estimation of nutrient intakes by target population;
iii. development of scientifically based food policy documents.

During the first stage of the fieldwork, a total of 2000 individuals aged 19 to 64 years old and living in private households in Latvia were interviewed. Three age groups (19-35, 36-50, and 51-64) were created. The information on food intake for each age group was collected using two non-consecutive 24-hour dietary recalls in combination with food frequency questionnaire. Additional

data on sociodemographic and lifestyle characteristics were obtained in face-to-face interviews. Nutritional data were processed with the help of software for the storage and analysis of food consumption and composition data that was developed within the framework of this project.

The design and methodology of the food consumption survey was based on „European Food Consumption Survey Method" (EFCOSUM) recommendations, developed within the framework of the EU Programme on Health Monitoring to provide a common method for monitoring of food consumption in Europe in a comparable way [127].

Strictly standardized procedures were used in order to harmonize research methodology (including the sample size, data analysis, and data presentation) and to obtain data comparable with the data from other EU countries. For the quantification of portion sizes, the food picture book was used. For presentation of the data, parameters of interest were mean, median, quartiles, P5, and P95.

The exposure assessment based on the National consumption data has been conducted for evaluating chemical contamination risk and the related nutrition risk, in order to develop scientifically based conclusions and to ensure relevant risk management and communication activities.

3. RESULTS AND DISCUSSION

3.1. Development of method for determination of acrylamide in coffee using HPLC-Q-Orbitrap-HRMS

3.1.1. Optimization of HRMS conditions

One of the tasks of the thesis is devoted to the optimization of HRMS parameters and exploration of the efficiency of different scan modes to detect acrylamide in coffee samples. HESI source parameters such as gas flow rate, capillary and heater temperatures and spray voltage were optimized to obtain an enhanced analytical signal of acrylamide. The final parameters are described above in 2.4.2 section of the Experimental part.

The transmission of ions through S-lens was optimized during the initial stages of method development. The S-lens is a radio frequency (RF) device (known as progressively spaced stacked ring ion guide) that captures and efficiently focuses the ions into a tight beam. Decreasing the S-lens RF level theoretically should decrease the fragmentation of fragile ions and increase the transmission of low *m/z* ions. Since the acrylamide molecule has a low *m/z* ratio, the S-lens RF level could have a significant impact on the analysis of acrylamide. The signal-to-noise ratio of acrylamide peak was examined at the S-lens RF level of 25, 35, 50, and 75 units. The best peak intensity was observed at the level of 35 arbitrary units.

3.1.2. Comparison of different HRMS scan modes

The efficiency of several possible scanning modes were examined: full MS scan, targeted-SIM (selected ion monitoring), and targeted-MS2 (product ion scan). In difficult matrices where the matrix effect had a major impact on the signal and noise intensity, the critical value was the signal-to-noise (S/N) ratio that was compared in further experiments.

The peak intensity obtained in three different MS operation modes is compared in Figure 3.1. It can be seen that the S/N ratio for the targeted-MS2 mode is significantly better in comparison to the full MS scan and targeted-SIM modes. No substantial difference between the latter two scan modes was observed in this study.

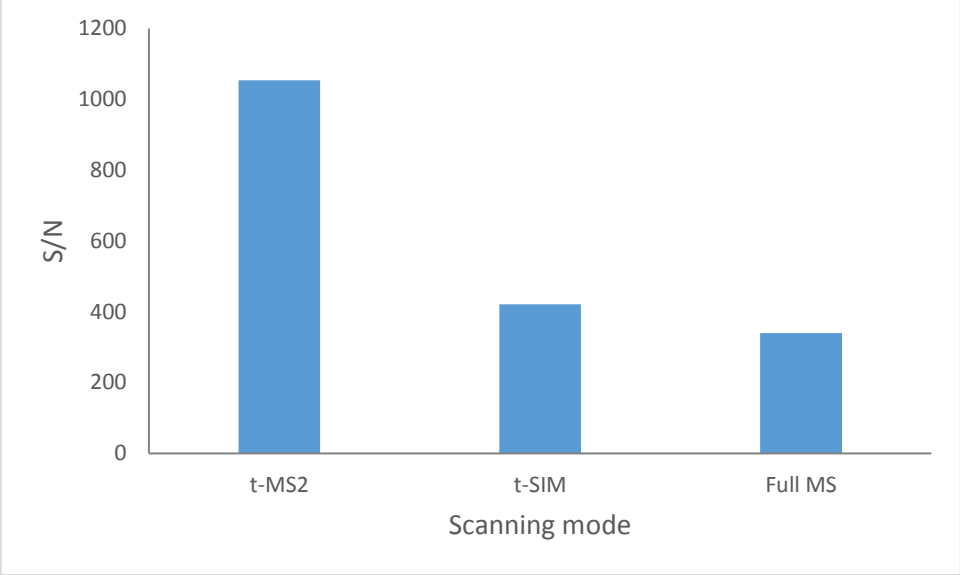

Fig. 3.1. **The signal-to-noise ratio of acrylamide peak in extract of roasted coffee using different scanning modes of Q-Orbitrap HRMS system (targeted-MS2, targeted-SIM and full-MS scan)**

As the next step, the impact of matrix effect on the signal-to-noise ratio of acrylamide peak in purified acetonitrile extracts from unroasted and roasted coffee samples with acrylamide content of 0.06 ng μL^{-1} was compared with the same concentration of acrylamide standard in acetonitrile. It was observed that the matrix effect had a major impact on the S/N ratio in all scan modes (Figure 3.2.).

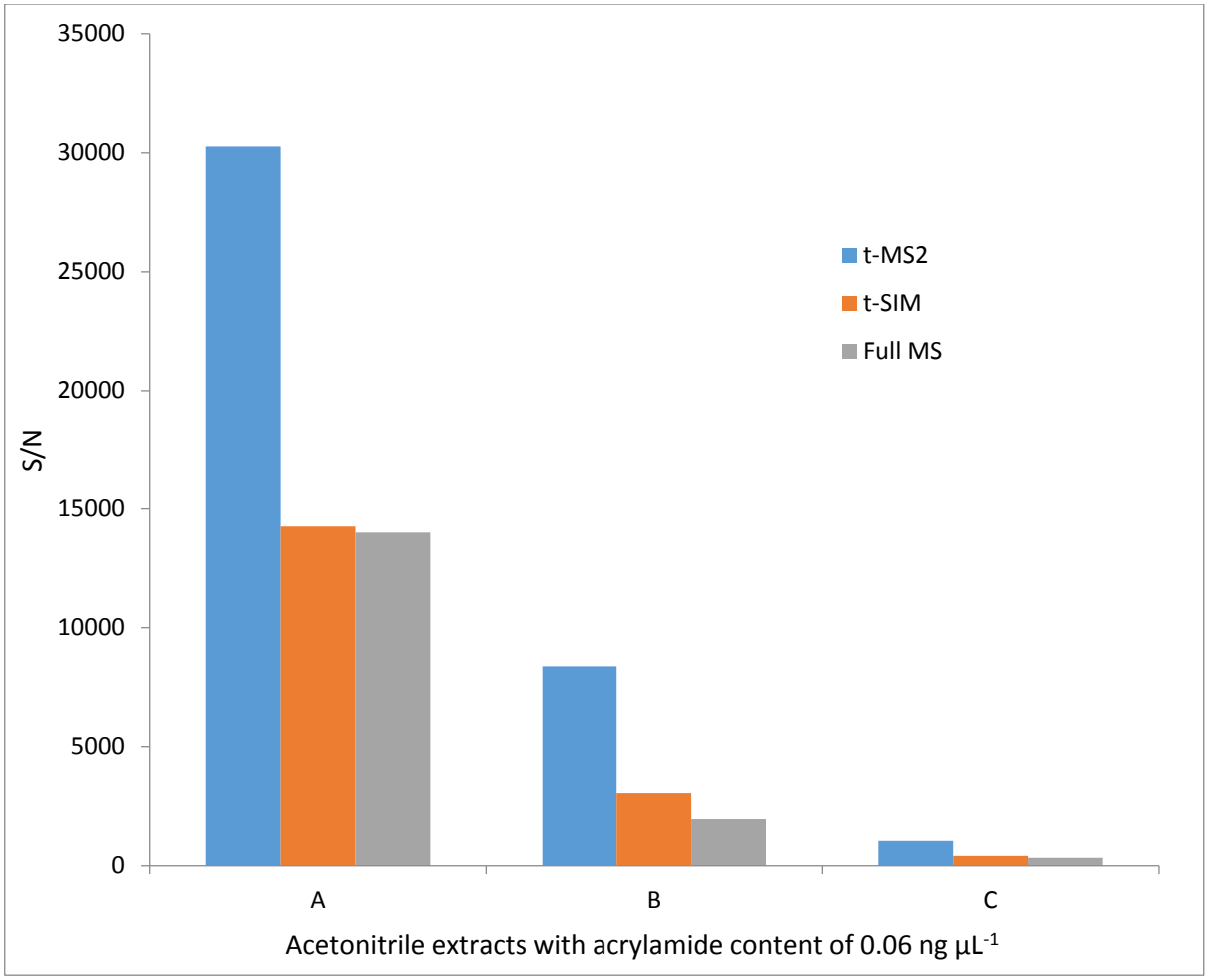

Fig.3.2. **The impact of matrix effect on the signal intensity in different MS scanning modes: (A) 0.06 ng µL^{-1} acrylamide standard solution in acetonitrile; (B) acetonitrile extract of unroasted coffee, spiked with 0.06 ng µL^{-1} of acrylamide; (C) acetonitrile extract of roasted coffee spiked with 0.06 ng µL^{-1} of acrylamide (the signal-to-noise value for roasted coffee was calculated from spiked sample minus the signal-to-noise ratio of the natural acrylamide content in the corresponding sample)**

The results obtained within the current study showed that the best sensitivity in the analysis of acrylamide can be achieved in the targeted-MS2 scanning mode. This mode was selected and further optimized for the acrylamide determination in coffee samples.

In the targeted-MS2 mode the precursors specified in the inclusion list are selected by quadrupole, fragmented in HCD cell with the specific fragmentation energy and collected in the C-trap. The number of targeted-MS2 scan events depends on the number of precursor ions in the inclusion list eluting at the same time, which means that the number of data points (scans) per chromatographic peak largely depends on the data in the inclusion list. From that point of view, the targeted-MS2 scan mode is less appropriate for the multi-compound analysis in comparison to the full-MS mode, but it is very effective for single residue methods.

The mass difference between the theoretically calculated mass of daughter ion and the experimental value is 0.78 mDa (Figure 3.3).

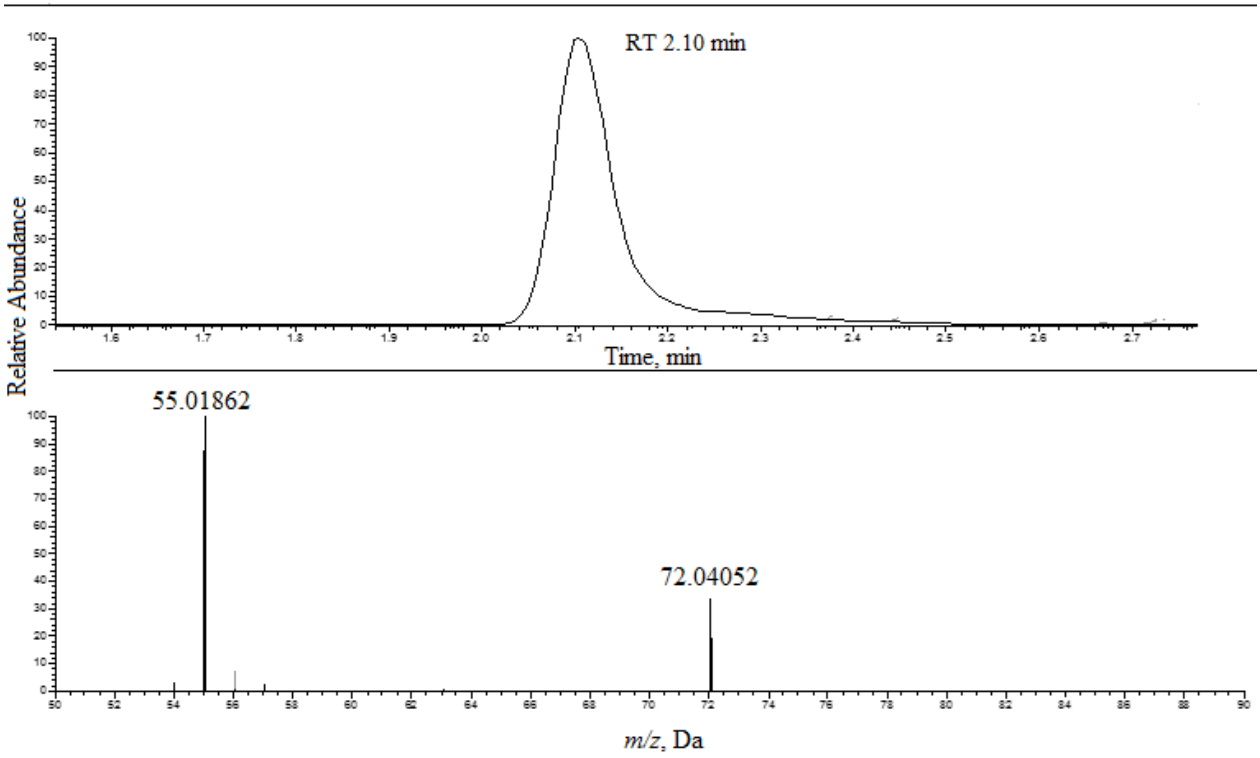

Fig. 3.3. **The extracted ion chromatogram and mass spectrum of the precursor and fragment ions**

The mass extraction window was set to 1 mDa or MMU (milli-mass unit), which was sufficiently wide to detect fragmentation ions, while avoiding false positive results due to the presence of interfering ions. If the selected extraction window would be too narrow, the ions due to fragmentation of acrylamide molecules could be missed, thereby resulting in false negative results.

Resolution is one of the most critical parameters in the analysis of "difficult" matrices by HRMS techniques. High resolving power helps to avoid false results. The instrument used in our study can work with one of four resolution settings: 17500, 35000, 70000, and 140000. For the Orbitrap mass analyzer the resolution depends on the acquisition time. An increased mass resolution brought a higher mass accuracy and thereby better selectivity, but excessively high resolution (such as 140000 FWHM) would significantly affect the sensitivity due to the reduced scanning speed. To achieve the higher resolution, ions have to spend more time in the analyzer, and the time between consecutive analyses is longer – consequently, fewer data points per peak can be obtained. Experiments were carried out at all four resolution settings. The higher intensity

of the peak was achieved with the resolving power 17500 and 35000. Application of higher resolution settings significantly decreased the sensitivity of assay. In complex matrices the possible interfering endogenous matrix components can co-elute with near isobaric compounds. In that case resolution of 17500 might be too low, especially at low concentrations, therefore all analysis was carried out with the resolution of 35000 units.

In ion-trap based instrumentation, such as Orbitrap systems, limitation of sensitivity can occur in cases of high matrix load. Ions are injected into the Orbitrap in packets. Before injection, the ions accumulate in the C-trap, which represents a gas-filled curved quadrupole. The operation of the C-trap and the time of injection are controlled by two main parameters: IT (injection time, expressed in milliseconds) and AGC target (automatic gain control, expressed by number of charges). The most important requirement for any ion trap device is the ability to control the ion population within the trap. When the ion population is not accurately maintained, it can result in large variations of data quality, such as a shift in mass accuracy and decrease in resolution. This process is maintained by the automatic gain control settings. For sensitive quantitative analysis a larger amount of ions is needed to enter the mass analyzer. Ions are injected until the IT or the AGC target is reached, depending on which happens first. Because of this mechanism, the response in more "difficult" matrices can be lower. To improve the results for acrylamide in coffee samples, experiments were carried out with different AGC target values: 2×10^5, 5×10^5, 1×10^6, 3×10^6, and 5×10^6 (the resolution was set at 35000 and the injection time value (IT) at 200 ms). The best results were obtained with the maximum AGC target value of 1×10^6, due to the increased number of ions entering the Orbitrap. In general, both changes: decreasing of the scan range and increasing of the AGC target had a beneficial influence on the peak shape and peak area reproducibility. A maximum injection time of 20 ms resulted in an adequate number of data points for our chromatographic peak, ensuring acceptable integration and quantification performance.

The proposed sample preparation and instrumental method indicated an excellent sensitivity. The signal-to-noise ratio for the acrylamide peak at the lowest calibration point of 10 μg kg^{-1} was 1445, therefore this method has a clear potential for measurements even at lower concentrations (Figure 3.4).

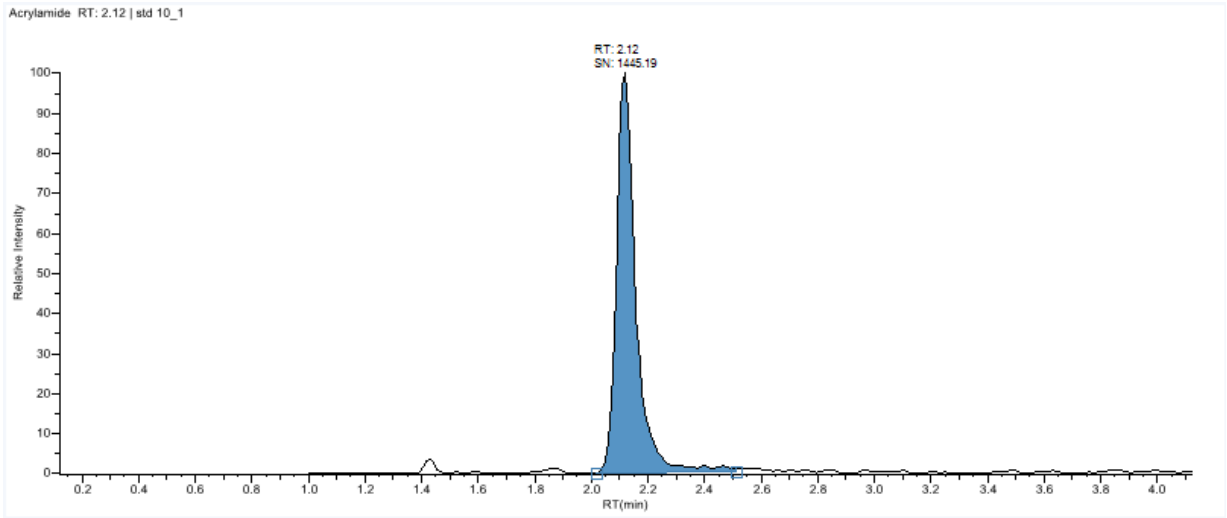

Fig. 3.4. **Chromatogram of acrylamide standard at the calibration level 10 µg kg^{-1} acquired in targeted-MS2 scanning mode**

The AGC target was set to, the maximum, the number of microscans to be performed was set at 2. Fragmentation mass spectra were recorded at a mass resolving power with a quadrupole isolation window of 1.0 Da for precursor ions (72.0444 Da for acrylamide and 75.0632 Da for acrylamide-d_3), the use of normalized collision energy (NCE) of 60% The scan range for daughter ions was from 50 to 80 *m/z*.

3.1.3. Optimization of the sample preparation procedures

QuEChERS based sample preparation for the determination of acrylamide was proposed by Mastovska and Lehotay, who evaluated the required sample amount versus the volume of water and acetonitrile to be added for better extraction [128]. Various combinations of $MgSO_4$ and NaCl and different dispersive-SPE mixtures were tested for the most effective removal of interfering matrix components, in order to achieve sufficient separation of water and acetonitrile layers and to transfer acrylamide as completely as possible into the acetonitrile layer. In order to optimize this sample preparation method for detection with hybrid quadrupole – Orbitrap detector, it was decided to skip the defatting step, since coffee contains very low fat concentrations, and to add an additional clean-up step for the remaining part of the matrix using NH_2 columns after the dispersive SPE. The efficiency of extract purification on the amino columns was checked gravimetrically. A 1 mL aliquot of sample extract in acetonitrile after each purification step was evaporated and weighed. The weight of matrix components in acetonitrile extract after each purification step is shown in Figure 3.5.

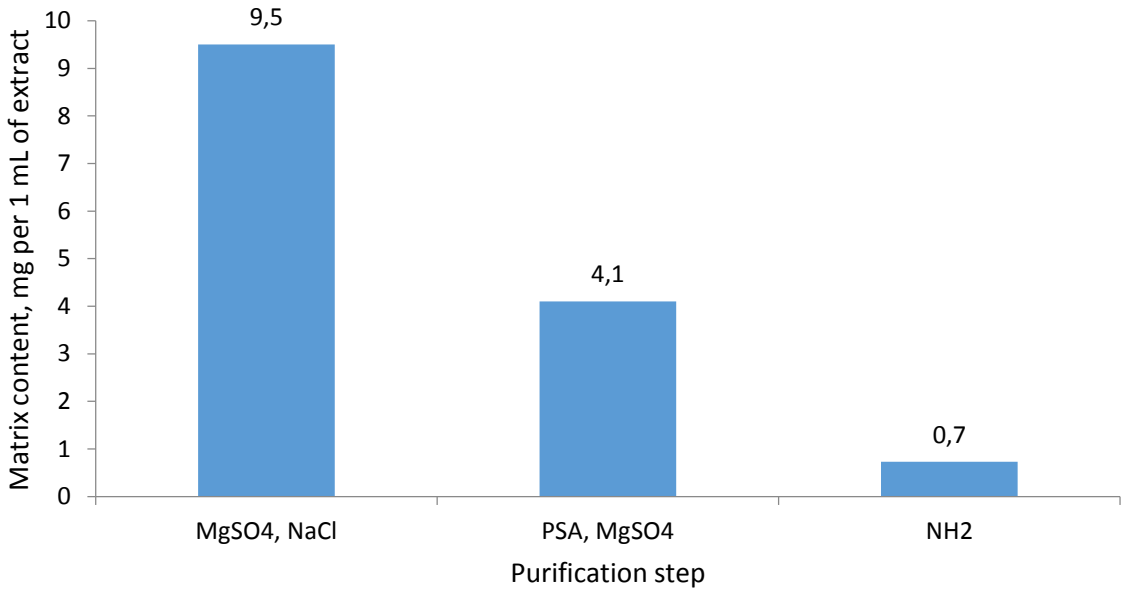

Fig. 3. 5. **The remaining load of matrix components (mg) in 1 mL of acetonitrile extract after each step of purification**

From the experimental data it can be concluded that the last treatment with amino columns had the most crucial effect. The final extract obtained after the amino column was clear and colorless, and therefore suitable for injection into the HPLC-HRMS system without the risk of contamination of ionization capillary and ion source.

It can be seen from chromatogram (Figure 3.6.) that there are peaks of co-extractable compounds located pretty close to the retention time of the acrylamide peak. After the purification by amino column these co-extractible peaks became insignificant and the signal-to-noise ratio increased by almost 5 times.

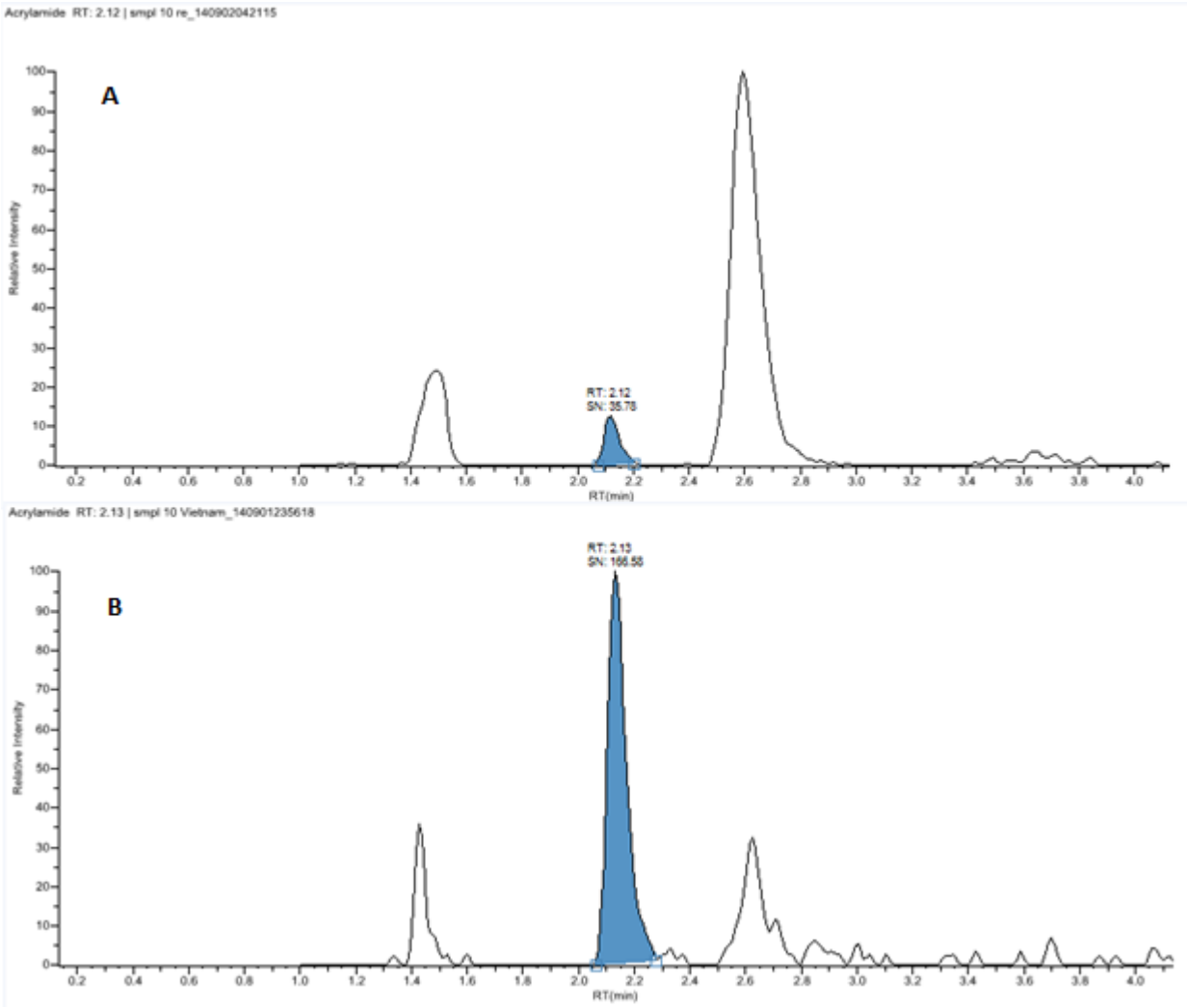

Fig. 3.6. **Chromatogram of coffee sample without (A) and with (B) clean-up steps using the amino column**

The optimum chromatographic separation was achieved on HILIC column (hydrophilic interaction chromatography). Polar compounds tend to elute without retention from C18 reversed-phase columns, while a HILIC column separates compounds by eluting with strong organic phases where the elution is driven by increasing of the water content in the mobile phase. The highly volatile organic mobile phase such us acetonitrile used in HILIC ensures low column backpressure as well as an increase of ionization efficiency for mass spectrometric detection [57].

The selected gradient program facilitated the efficient removal of matrix constituents, and as a result, reduced the noise level and the risk of carry-over effects.

3.1.4. Comparison of HRMS and MS/MS

The advantage of the high-resolution mass spectrometric detection in the assay of such difficult matrices as coffee was highlighted by the comparison with results obtained for the same coffee sample while using a low resolution quadrupole mass-spectrometric detector. Figure 3.7 shows the peaks of acrylamide (A) and acrylamide-d_3 (B) obtained with triple quadrupole AB Sciex QTrap 5500 mass spectrometer operating with an electrospray ionization source. The sample clean-up procedure and chromatographic parameters were the same to those described in this study. The MRM transitions were *m/z* 72.0→54.9 for acrylamide and 75.0→58.0 for acrylamide-d_3. It can be clearly concluded from the presented chromatogram that signal-to-noise ratio is low and the impact of matrix effect is quite pronounced.

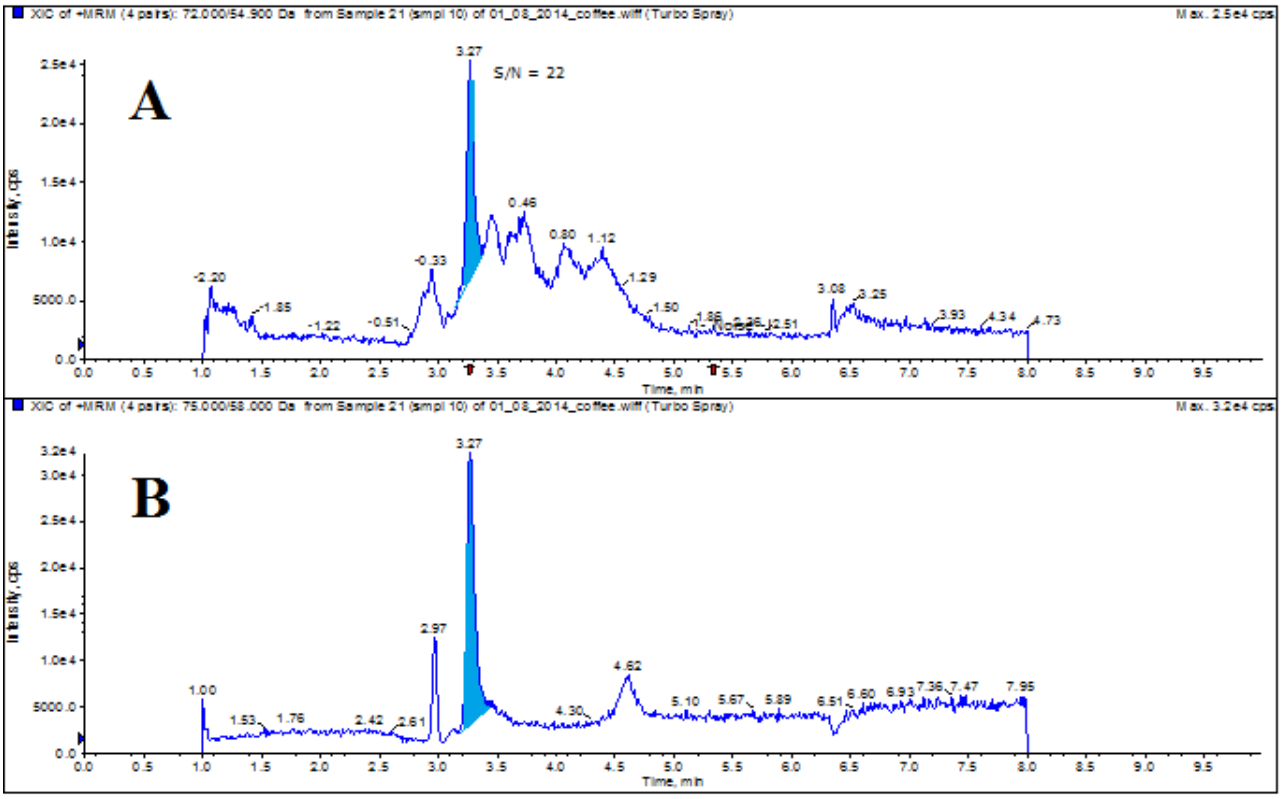

Fig. 3.7. **The chromatogram of acrylamide (A) and acrylamide-d_3 (B) in coffee sample obtained with the HPLC-QqQ–MS/MS**

The same coffee sample was analyzed in accordance with the method described in this study (Figure 3.8). It can be concluded that the high resolution mass spectrometric detector offers more

reliable results, significantly mitigates the matrix effect and therefore provides a lower quantification limit in the analysis of acrylamide.

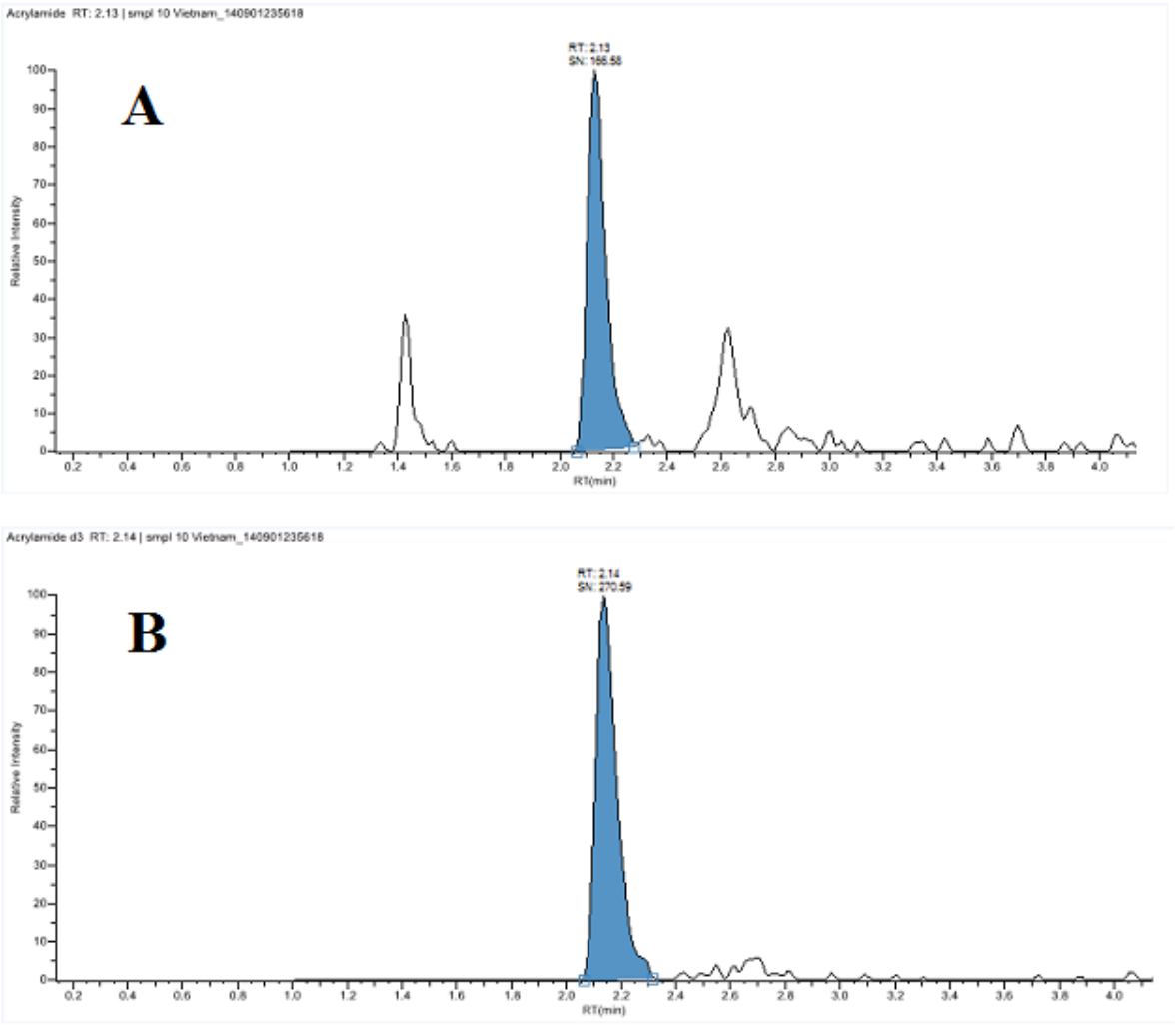

Fig. 3.8. **The chromatogram of acrylamide (A) and acrylamide-d_3 (B) in coffee sample obtained with HPLC-Q-Orbitrap HRMS**

3.1.5. Validation of HPLC-Q-Orbitrap HRMS method

The method performance was tested for quality parameters such as selectivity, linearity of the calibration range, repeatability, recovery, memory effect, matrix effect, LOD and LOQ. Validation experiments were carried out on three different days and consisted of six replicates for

each concentration level. Matrix-match calibration graph consisted of nine calibration points in the range from 10 to 500 µg kg^{-1}. The data were evaluated by carrying out linear regression with origin point included and with weighting 1/x to establish the coefficient of determination (R^2).

The selectivity of the method was checked by the analysis of reagent blank and sample of unroasted coffee beans. No interfering peaks were observed at the retention time of acrylamide. Memory effect was assessed by injecting blank sample (solvent) after the analysis of sample at the highest calibration point. No interfering peaks at the retention time of acrylamide were detected. The calibration graph within the concentration range of 10-500 µg kg^{-1} showed a good linearity with R^2 between 0.9984 and 0.9996. Repeatability of the method expressed as mean RSD of 18 replicates ranged from 2.3% to 7.8% and recovery ranged from 100% to 111% (Table 3.1). LOD and LOQ calculated based on the S/N ratio were 0.07 µg kg^{-1} and 0.21 µg kg^{-1}.

Table 3.1.

Validation data for the analysis method of acrylamide

Spiking level	10 µg kg^{-1} (n=18)	50 µg kg^{-1} (n=18)	200 µg kg^{-1} (n=18)	450 µg kg^{-1} (n=18)
Recovery (%)	111	102	104	100
RSD (%)	7.8	4.6	3.5	2.3

The matrix effect was investigated by calculating the percentage (ME%) of signal enhancement or suppression, according to equation (1),

$$ME(\%) = 100 \times \left(\frac{a_m}{a_s} - 1 \right)$$

where a_s is a slope of the calibration plot with calibration solutions in solvent and a_m is the slope of the calibration plot with matrix-matched calibration solutions. In cases when the suppression effect was higher than 20%, it was important to use the matrix-matched calibration or spiking with standard. The difference of slope values between the matrix-matched calibration graph and standard calibration graph in the current study displays a significant matrix effect ME = 30% (Figure 3.9).

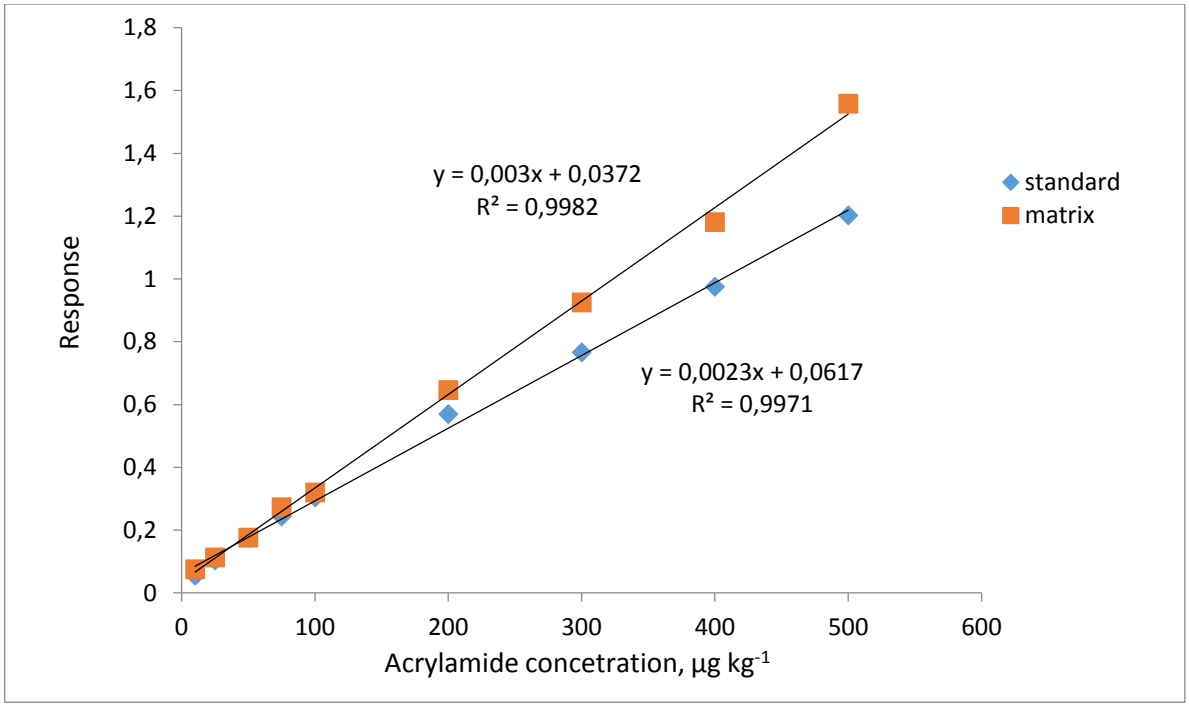

Fig. 3.9. **Matrix-matched calibration and standard solution calibration graphs**

A positive value of the ME criterion reflects an enhancing effect of the investigated matrix. As a result of these findings, we conclude that the matrix-matched calibration approach should be used for quantification even in the case of using high resolution mass spectrometer. Since no appropriate matrix blank of roasted coffee for acrylamide is available, the recovery should be calculated for each sample batch using the laboratory quality control sample – the analyzed sample spiked with known amount of acrylamide. The concentration of all samples must be recalculated by taking into account the recovery value.

The accuracy of the method was proven by the analysis of the Reference Material of toasted bread (ERM – BD273) with certified value of acrylamide 425 µg kg^{-1} purchased from the Institute of Reference Materials and Measurement. The toasted bread was chosen because no Reference Materials of coffee matrix was available. Concentration of 433 µg kg^{-1} was obtained for the reference material using the proposed methodology.

3.1.6. Analysis of coffee samples

The method developed during the current study was applied to the analysis of roasted coffee samples. Twenty-two samples of roasted coffee samples were purchased from local supermarkets.

Two most popular types of coffee bean varieties - Arabica and Robusta - were used for this part of the study. The performance characteristics of the method were estimated with laboratory quality control samples by spiking samples with acrylamide at 200 μg kg^{-1} concentration (Table 3.2). The recoveries of spiked samples were calculated using a standard calibration graph and subtracting the blank value.

Table 3.2.

The recovery yields of acrylamide using HPLC-Q-Orbitrap-HRMS

Coffee sample	Acrylamide content (μg kg^{-1})		Recovery yield (%)
	Before spiking	After spiking with 200 μg kg^{-1}	
1	447	644	99
2	244	460	108
3	228	446	109
4	196	401	103

Results shown in Table 3.3 indicate that the content of acrylamide in samples varied from 166 to 503 μg kg^{-1} with no specific pattern dependent on the origin or type of coffee. The concentration of acrylamide in some samples were close or even exceeded the recommendation issued by the EC (450 μg of acrylamide per kg of ground coffee) [129].

Table 3.3.

Average concentration of acrylamide in 22 locally purchased roasted coffee samples

Origin / Brand	Variety of coffee beans	Region / Country / Manufacturer	Color value	Acrylamide content (μg kg^{-1})
Costa Rica, Tarrazu	Arabica	Central America, Costa Rica	110	185
Santo Domingo, Organico	Arabica	Latin America, The Dominican Republic	118	166
India monsooned Malabar	Arabica	Asia, India	91	453
Nicaragua Maragogype	Arabica	Nicaragua, Central America	115	260
FazendaLagoa	Arabica	South America, Brazil	110	228
Gurmans, Irish cream flavoured coffee beans	Arabica	Gurman's tea and coffee world	87	323

Table 3.3 continued

Origin / Brand	Variety of coffee beans	Region / Country / Manufacturer	Color value	Acrylamide content ($\mu g\ kg^{-1}$)
Gurmans, Rum flavoured coffee beans	Arabica	Gurman's tea and coffee world	88	447
Gurmans, Caramel flavoured coffee beans	Arabica	Gurman's tea and coffee world	89	271
Julius Meinl, JubilaumBohne	Arabica	Julius Meinl	113	256
LavAzza, caffe espresso	Arabica	Luigi LavazzaS.p.A.	113	314
LavAzza, qualitaoro	Arabica	Luigi LavazzaS.p.A.	109	244
Colombia	Arabica / dark	South America, Colombia	89	182
Colombia	Arabica / light	South America, Colombia	105	179
Brazil Santos	Arabica / dark	South America, Brazil	106	218
Brazil Santos	Arabica / light	South America, Brazil	120	196
Indonesia Java	Robusta	Asia, Indonesia	117	503
India Plantation	Arabica / light	Asia, India	136	299
India Plantation	Arabica / dark	Asia, India	125	272
Vietnam wet polished	Robusta	Asia, Vietnam	128	283
Blend Triage Arabica	Arabica	Africa	138	408
Vietnam	Robusta	Asia, Vietnam	135	361
Ethiopian Sidamo	Arabica	Africa, Ethiopia	123	267

Additionally the degree of coffee roasting (values indicated in Table 3.3) was measured using the color measuring device "Colorette 3a" (Probat Burns, USA). The coffee is illuminated using two wavelengths (red light and infra-red light) via LEDs at a specific frequency. The total light reflected in two wavelengths is evaluated by software and indicated as a color value. Measurement scale of Colorette 3a is 200 scale divisions and measuring accuracy +/- 3 scale divisions. For example, the value 130 indicates light grey and 70 – dark grey color. No correlation was found between the acrylamide content and degree of roasting coffee.

3.2. Development of method for determination of furan in baby foods using HS-GC-MS

3.2.1. Comparison of MS scanning modes and the optimization of HS-GC-MS/MS analysis

Two different MS scanning modes were compared at the beginning of the method development procedure. Five ions were monitored in the SIM scanning mode: three for furan (*m/z* 68 as a quantifier, *m/z* 39 and *m/z* 40 as qualifier) and two characteristic ions for furan-d_4 (*m/z* 72 as quantifier and *m/z* 42 as qualifier). Three ion transitions were monitored in the SRM scanning mode: two for furan (*m/z* 68 → 39 as quantifier and *m/z* 68 → 40 as qualifier) and one for furan-d_4 (*m/z* 72 → 42). Taking into account the small mass and a low retention of a furan molecule, the selectivity of analysis is crucial, since a lot of potentially interfering substances could have the same retention time as furan, thus decreasing the sensitivity and selectivity of instrumental detection. Figure 3.10 shows a chromatogram of baby food sample spiked at 1 µg kg^{-1} level, acquired in the SIM and SRM modes.

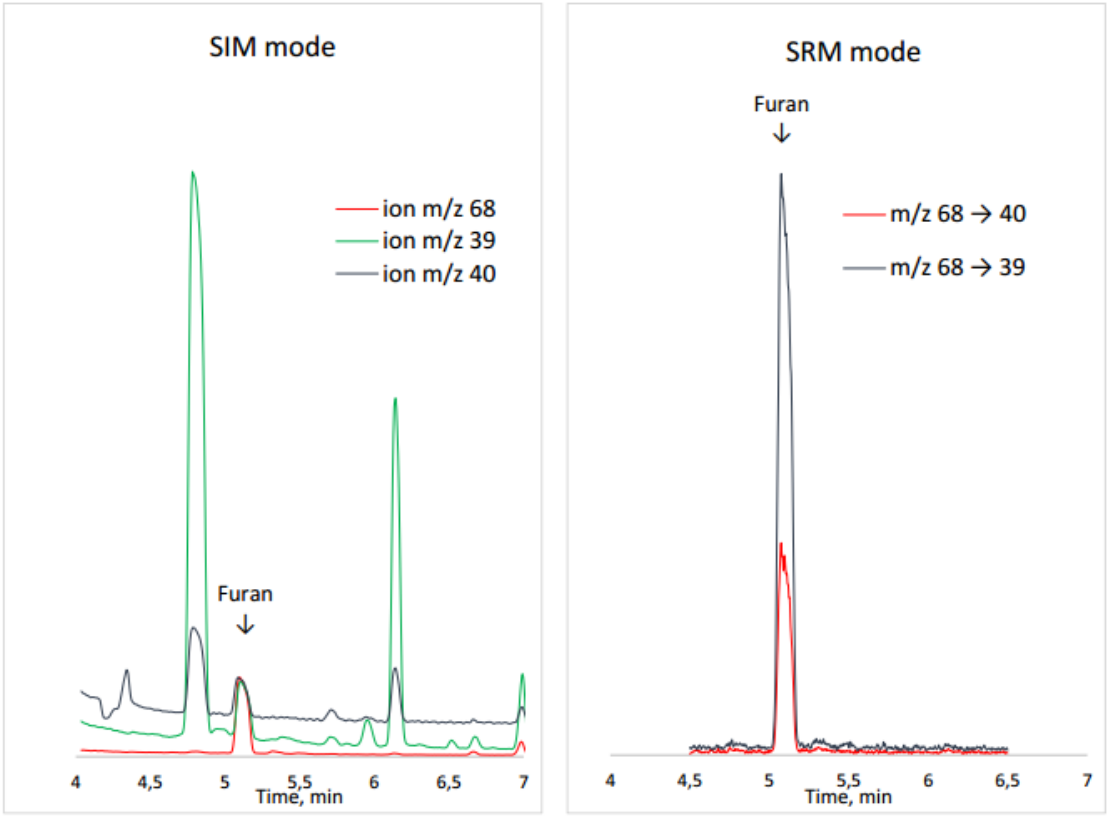

Fig. 3.10. **Chromatograms of furan at spiked level of 1µg kg^{-1} in baby food acquired in SIM and SRM mode**

As it can be seen, SIM mode provides a relatively high background noise level with interferences from other matrix compounds. Especially high level of background can be observed for the qualifier ions. From the chromatogram of the same sample acquired in the SRM mode, it is apparent that the proposed methodology provides a much higher selectivity for the determination of furan, and no interferences from other matrix components are observed. The obtained results showed that the SRM method is more sensitive and selective in comparison to the SIM scanning mode.

It was noticed during the method development that a better sensitivity in the SRM mode could be obtained by decreasing the electron energy parameter to 35 V. The optimal value of electron energy was checked experimentally in the range from 20 V to 70 V with a step of 5 V.

3.2.2. Validation of HS-GS-MS/MS method

The method performance was tested for quality parameters such as selectivity, linearity of the calibration range, repeatability, recovery, memory effect, LOD and LOQ. Validation experiments was performed using a homemade fruit based sample and were carried out on three different days and consisted of five replicates for each concentration level. Matrix-match calibration graph consisted of six calibration points in the range from 0 to 100 µg kg^{-1}. The data were evaluated by carrying out linear regression with origin point included and with weighting 1/x to establish the coefficient of determination (R^2).

The selectivity of the method was checked by the analysis of reagent blank and homemade fruit based sample. No interfering peaks were observed at the retention time of furan. Memory effect was assessed by injecting blank sample (solvent) after the analysis of sample at the highest calibration point. No interfering peaks at the retention time of furan were detected. The calibration graph within the concentration range of 0-100 µg kg^{-1} showed a good linearity with R^2 between 0.9982 and 0.9997. Repeatability of the method expressed as mean RSD of 15 replicates ranged from 4.0% to 11% and recovery ranged from 89% to 109% (Table 3.4). LOD and LOQ calculated based on the S/N ratio were 0.03 µg kg^{-1} and 0.09 µg kg^{-1}.

Table 3.4.

Validation data for the analysis method of furan

Spiking level	1 µg kg^{-1} (n=15)	10 µg kg^{-1} (n=15)	30 µg kg^{-1} (n=15)
Recovery (%)	102	95	89
RSD (%)	11	6.0	4.0

3.2.3. Analysis of baby food samples

The applicability of the validated method for the determination of furan in baby food samples was checked by analysis of 30 commercial baby food samples available on the Latvian retail market. An example of real sample chromatogram obtained within this study is shown in Figure 3.11. The chromatogram represents the lowest furan concentration detected in a baby food sample and demonstrates a very good selectivity and sensitivity.

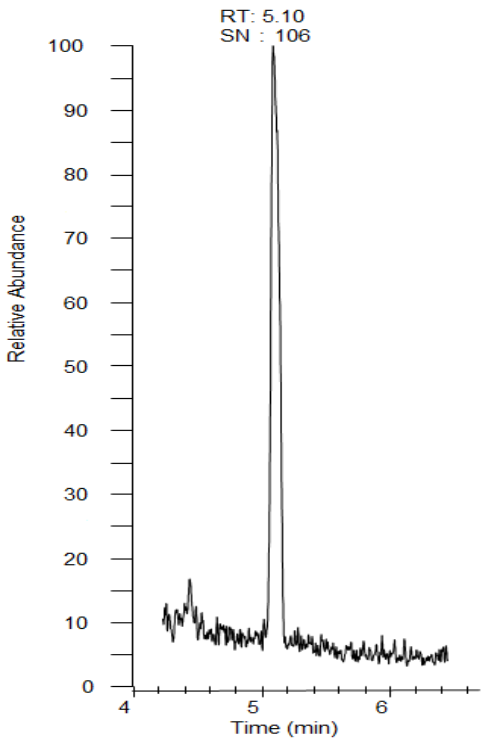

Fig. 3.11. **Chromatogram of furan (*m/z* 68→ 40) in blueberry puree (0.45 µg kg^{-1})**

The furan levels in samples available on the Latvian market are summarized in Table 3.5. All jarred baby foods showed a furan content between 0.45 to 82 μg kg^{-1}. These concentrations agree with those reported in the literature for baby food, for example, from 3.4 to 102 μg kg^{-1} [20], from not detected (LOQ 2.4 μg kg^{-1}) to 96 μg kg^{-1} [16], from not detected (LOQ 4.0 μg kg^{-1}) to 65 μg kg^{-1} [17], and from not detected (LOQ 0.18 μg kg^{-1}) to 87 μg kg^{-1} [19].

Table 3.5.

Furan levels, pH and Brix content in baby food samples

Number	Product	Ingredients	Furan (μg kg^{-1})	pH (20°C)	Brix (20°C,%)
1	Banana	Milk mixture, banana, rice, sugar, rice starch and vitamin B	12	6.38	16.11
2	Broccoli-risotto with rabbit	Water, cooked rice, broccoli, rabbit meat and rapeseed oil	12	5.99	7.67
3	Fine vegetables with veal	Water, cooked rice, carrots, cauliflower, veal and rapeseed oil	17	5.91	8.42
4	Apple banana with baby biscuit	Apples, bananas, lemon juice, water, cookies, wheat starch and vitamin C	6.8	4.15	14.99
5	Plums	Plums, water, sugar, rice semolina, rice starch and vitamin C	3.4	3.67	19.89
6	Blueberries with apple	Apples, apple juice, blueberries, rice semolina and vitamin C	5.5	3.50	16.93
7	Fine fruit pap with whole meal cereals	Apple juice, apples, bananas, mango, orange juice, whole wheat and vitamin C	8.6	4.07	15.65
8	Vegetables and noodles with chicken	Tomatoes, carrots, green peas, water, wheat noodles, chicken, wheat flour, rapeseed oil and onion	47	5.33	7.67
9	Fine vegetables with turkey	Water, carrots, corn, cooked rice, turkey meat and rapeseed oil	29	5.78	11.13
10	Rice with carrots and turkey	Water, carrots, boiled rice, turkey meat and rapeseed oil	4.5	5.98	9.31

Table 3.5 continued

Number	Product	Ingredients	Furan (µg kg^{-1})	pH (20°C)	Brix (20°C,%)
11	Peach apricot with curd cream	Peach puree, apricot puree, banana puree, grape juice concentrate, lemon juice, water, milk, skimmed milk curd, cooked rice, starch and vitamin C	10	3.97	18.85
12	Pasta with ham and vegetables	Carrots, tomatoes, corn, onions, water, boiled noodles, ham, boiled rice, rapeseed oil, herbs and spices (cumin)	53	5.40	9.70
13	Carrots and potatoes with lamb	Carrots, potatoes, water, lamb, cooked rice and rapeseed oil	31	5.50	7.51
14	Baby biscuit	Milk, water, milk powder, corn oil, biscuits, water, sugar, rice starch, whole grain cereals and vitamin B_1	17	6.42	14.99
15	Apple and blueberry	Apples, blueberries, fructose and water	7.7	3.38	19.08
16	Apple, pumpkin and plum	Apples, pumpkin, plums, water and fructose	39	3.52	14.51
17	Carrot with sea buckthorn	Carrots, water, fructose and sea buckthorn	28	4.52	13.08
18	Sweet pumpkin	Pumpkin, fructose and water	82	5.30	12.04
19	Vegetables with chicken	Potatoes, water, peas, carrots, tomato puree, chicken, sweet corn, corn starch and rapeseed oil	53	5.84	8.88
20	Steamed salmon with vegetables	Water, gourd, zucchini, potatoes, salmon, meat, wheat starch, canola oil, corn starch and lemon juice	45	5.68	9.20
21	Wild berry puree with yogurt	White grape juice, apples, yogurt, blueberries, raspberries, maize starch, water, rice flour and ascorbic acid	0.69	3.76	15.02
22	Gentle banana dessert with cookies	Bananas, water, apple juice, biscuits, rice flour, lemon juice concentrate and ascorbic acid	8.7	3.86	17.30
23	Vegetable stew with lamb	Water, carrots, potatoes, lamb, apple juice, green peas, millet flour, wheat starch, leek and rapeseed oil	31	5.43	7.92

Table 3.5 continued

Number	Product	Ingredients	Furan (µg kg^{-1})	pH (20°C)	Brix (20°C,%)
24	Cauliflower	Cauliflower	29	5.30	3.98
25	Plums	Plums and ascorbic acid	2.9	3.43	21.40
26	Sweet vegetables with chicken	Pumpkin, carrots, water, milk, potatoes, chicken, wheat starch, onions, canola oil, coriander and ginger root	36	5.78	6.94
27	Beef with potatoes	Potatoes, drinking water, beef, carrots, onions, canola oil, allspice and bay leaves	29	5.52	3.78
28	Potato and flounder puree	Potatoes, drinking water, carrots, flounder, onions, canola oil, rice flour, lemon juice, dill and white pepper	52	5.49	4.17
29	Salmon with vegetables in sweet sauce	Drinking water, celery, carrots, salmon, milk powder, rice, leek, vegetable oil, rice starch, lemon juice concentrate, parsley and white pepper	24	5.55	9.26
30	Blueberry puree	Drinking water, blueberries, sugar, rice starch, gelling agent and lemon juice	0.45	3.30	16.95

The baby food samples were classified depending on their composition in six different groups. Table 3.6 summarizes the mean value per group, as well as the maximum and minimum values found. It is evident that fruit based baby food showed the lowest furan concentrations (0.45 µg kg^{-1}) and the highest values were found in the group of foods containing vegetables (82 µg kg^{-1}).

Table 3.6.

Mean, maximum and minimum values of furan concentration per food group

Baby food group	*n*	Mean (µg kg^{-1})	Min-Max (µg kg^{-1})
Fruits	10	5.8	0.45 – 12
Vegetables	2	56	29 – 82
Mix of fruits and vegetables	2	33	28 – 39
Meat and vegetables	11	31	4.5 – 53
Fish and vegetables	3	40	24 – 52
Cereals (with milk or fruits)	2	13	8.7 – 17

3.3. Correlation of furan content and other food ingredients

Although it is difficult to deduce which ingredients are involved in the formation of furan, as the exact reaction mechanism is not completely understood, it can be seen from the obtained results that fruit based baby foods contain lower concentrations of furan in comparison to the vegetable based foods.

Considering the low amounts of furan in fruit based products, it can be concluded that lower pH may suppress furan formation, but this correlation could in fact be more complicated. Fan (2005) partly investigated the influence of pH on the formation of furan from certain precursors. He used model solutions with pH 3 and 7 and observed higher rates of furan formation at the lower pH for ascorbic acid, sucrose, and fructose, but the opposite for glucose [111]. The influence of pH in baby foods on furan concentration was checked in this study.

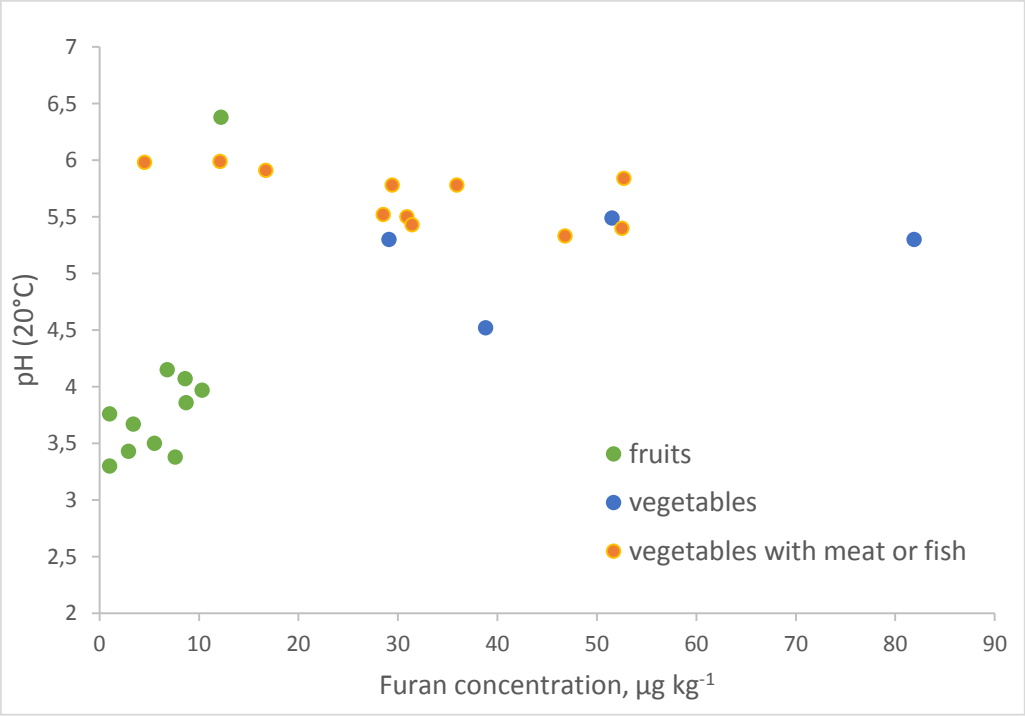

Fig. 3.12. **The relationship between pH value and furan content (µg kg^{-1})**

It can be concluded from Figure 3.12 that at lower pH (3-4) less furan was formed in the fruit samples. It is in a good agreement also with studies by Becalski *et al*. (2005) [6] and Limacher *et al.* (2007) [110], who reported that the formation of furan from sugars or ascorbic acid is less likely

at low pH, and the study by Owczarek-Fendor *et al.* (2012), who concluded that sugars in combination with proteins increase the formation of furan at pH 6 [109].

The impact of sugars, such as fructose, glucose, saccharose, and lactose was evaluated. The Table 3.7 represents the obtained sugar concentrations in baby food samples.

Table 3.7.

Concentrations of sugars in baby food samples

Number of sample	Fructose, %	Glucose, %	Saccharose, %	Lactose, %
1	2.1	< 1.0	< 1.0	1.2
2	< 1.0	< 1.0	< 1.0	< 1.0
3	< 1.0	< 1.0	< 1.0	< 1.0
4	4.1	2.5	2.3	< 1.0
5	2.4	3.5	5.2	< 1.0
6	6.4	3.0	< 1.0	< 1.0
7	4.8	2.7	2.9	< 1.0
8	< 1.0	< 1.0	< 1.0	< 1.0
9	< 1.0	< 1.0	< 1.0	< 1.0
10	< 1.0	< 1.0	1.1	< 1.0
11	4.1	4.3	1.3	< 1.0
12	< 1.0	< 1.0	< 1.0	< 1.0
13	< 1.0	< 1.0	1.7	< 1.0
14	< 1.0	< 1.0	4.0	2.2
15	13	2.2	< 1.0	< 1.0
16	10	2.9	< 1.0	< 1.0
17	6.5	1.7	1.3	< 1.0
18	3.8	1.9	2.0	< 1.0
19	< 1.0	< 1.0	1.6	< 1.0
20	< 1.0	< 1.0	1.1	< 1.0
21	4.4	3.3	< 1.0	1.0
22	3.5	3.0	3.0	< 1.0
23	< 1.0	< 1.0	1.4	< 1.0
24	< 1.0	< 1.0	< 1.0	< 1.0
25	4.8	7.4	< 1.0	< 1.0
26	< 1.0	< 1.0	< 1.0	< 1.0
27	< 1.0	< 1.0	< 1.0	< 1.0
28	< 1.0	< 1.0	1.0	< 1.0
29	< 1.0	< 1.0	< 1.0	< 1.0
30	4.2	4.0	< 1.0	< 1.0

No correlation between the furan content and any of the investigated sugar concentrations was observed. Owczarek-Fendor *et al*. (2012) in their research on furan formation concluded that

the most important carbohydrate causing furan generation at pH 6 was lactose [109]. Unfortunately our study does not contain enough data to assess the impact of lactose on furan concentration. For a further evaluation regarding lactose, baby foods containing dairy products should be selected.

A very slight correlation ($R^2 = 0.39$) between the content of soluble solids (Brix) and furan formation was observed in the current study (see Figure 3.13)

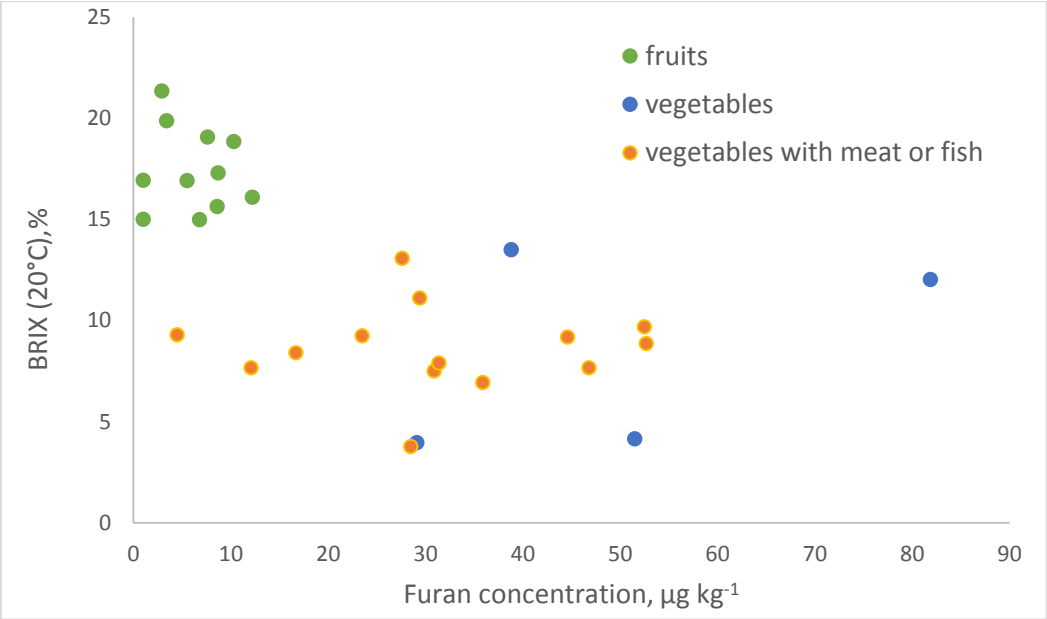

Fig. 3.13. **The relationship between Brix (%) and furan content (µg kg^{-1})**

The highest amount of furan content was found in foods containing such vegetables as carrots, pumpkin, and zucchini. There have been speculations that one of the most likely potential precursors for furan can be carotenoids [60, 107]. In order to verify such origin of furan, analysis for β-carotene was performed, however, no correlation between the concentrations of furan and β-carotene was observed ($R^2 = 0.16$).

3.4. Mitigation strategies to reduce acrylamide content in bread and biscuits

3.4.1. Fermentation with lactic acid bacteria producing bacteriocin-like inhibitory substances in combination with *Aspergillus niger* glucoamylase

The effect of *Lactobacillus* and *Pediococcus* strains in combination with *Aspergillus niger* glucoamylase used for saccharification of extruded rye wholemeal on acrylamide formation in the most popular mixed rye bread of various loaf weight was investigated.

The effect of a single LAB strain used for extruded rye wholemeal fermentation on the acrylamide formation in mixed rye breads of various loaf weight (500 g and 1000 g) was analyzed (Figure 3.14).

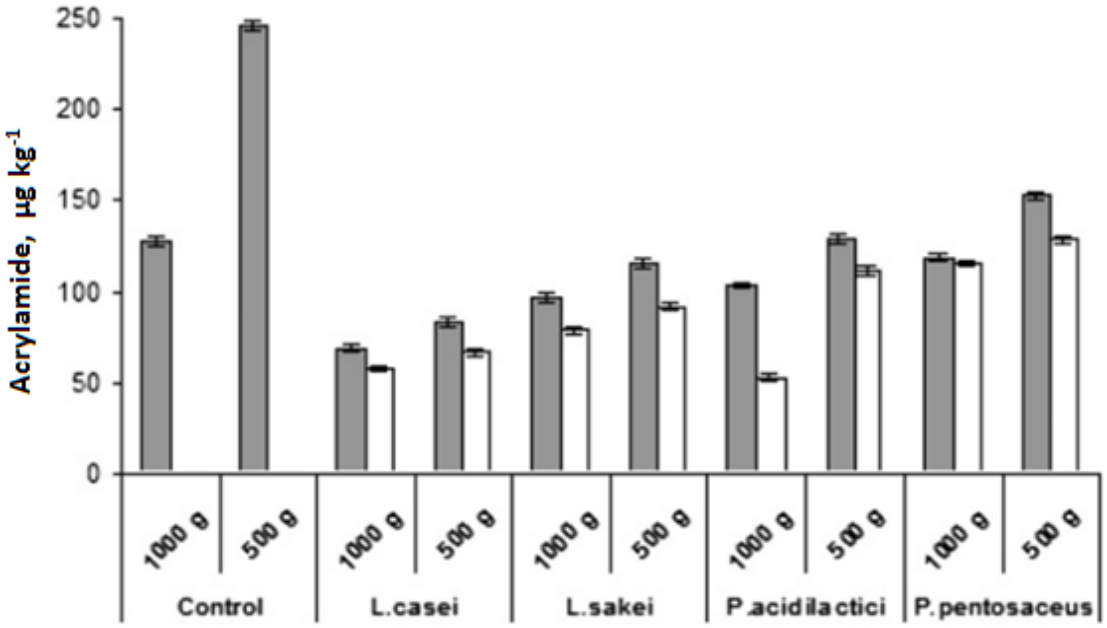

Fig. 3.14. **Acrylamide content in mixed rye breads (500 g and 1000 g loaf weight) made with different LAB strains without (grey) and with (white) the addition of *Aspergillus niger* glucoamylase for scald saccharification**

The results showed that the levels of acrylamide in breads made with LAB starters in all cases were lower than in the control sample. Acrylamide concentrations in bread samples ranged from 58 to 174 µg kg^{-1}. The concentration of acrylamide in control samples, which were prepared without LAB starters and without the addition of *Aspergillus niger* glucoamylase, were 128 and 246 µg kg^{-1} for 1000 g and 500 g loaves of bread, respectively. A difference in acrylamide reduction between the tested LAB strains also was noticed. The acrylamide content in breads made with *L. sakei* fermentation was lower compared to the control samples by 53 and 23 %, followed by *P.*

acidilactici - 48 and 19%, and *P. pentosaceus* – 38 and 7.8% in 500 g and 1000 g loaves of bread, respectively.

It was noticed that the 1000 g loaves of bread contained less acrylamide than the 500 g loaves of bread. The lower concentrations of acrylamide in 1000 g loaves of mixed rye bread versus 500 g loaves may be explained by the prolonged heat treatment and higher moisture content [95]. The acrylamide content was influenced by the product thickness and the changing temperature in different locations inside the loaves [130]. Acrylamide is predominantly generated in the outer crust layer where more than 99% of its content can be found, while only trace amounts are detectable in the crumb [93]. This can be ascribed to the lower baking temperatures of only 100ºC in the inner parts of bread loaves. The higher acrylamide levels in 500 g loaves most probably resulted from the forced loss of humidity, leading to a faster and more intense drying of the bread crust. Since low moisture content enhances acrylamide formation via the Maillard reaction, these effects may, at least partially, be avoided by applying a higher relative humidity during baking [131].

Furthermore, a trend of reduced acrylamide content in bread with decreased pH values during fermentation was found (Figure 3.15).

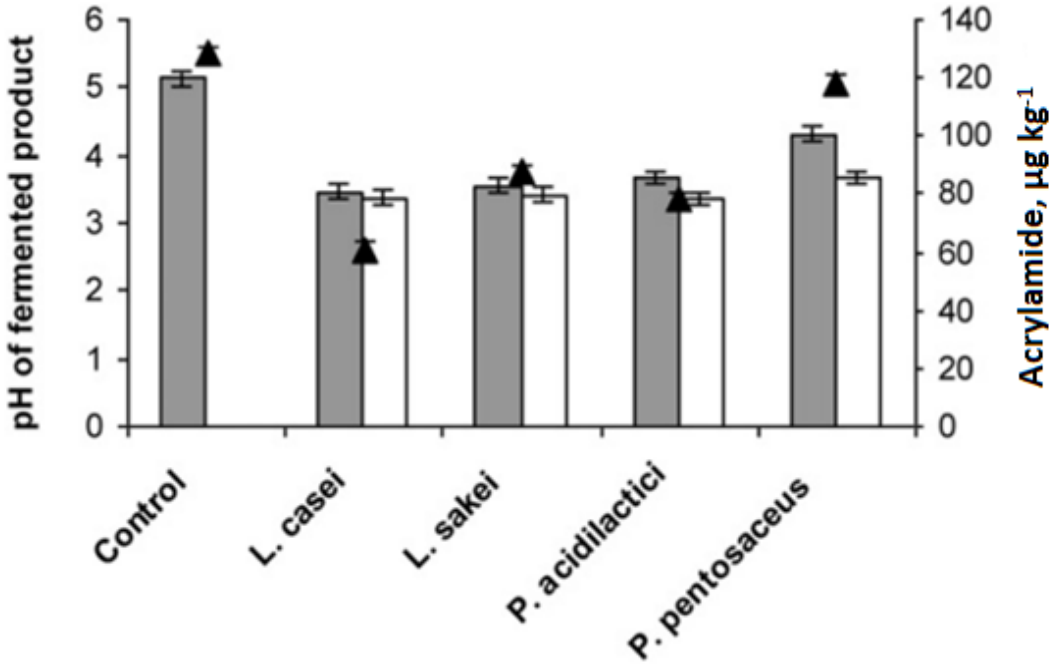

Fig. 3.15. **The effect of pH during fermentation with different LAB strains without (grey) and with (white) treatment with *Aspergillus niger* glucoamylase on acrylamide formation (average values) in 1000 g bread loaves (triangles).**

A significant reduction of acrylamide in all bread samples was achieved by maintaining the pH value of sourdough below 3.7. Saccharification of extruded rye wholemeal with glucoamylase as compared to the control sample was found to have a positive effect on acidification process by lowering the pH values of fermented products by 8.6% on average. Furthermore, the fermentation with *P. acidilactici* in combination with glucoamylase enabled a decrease of pH value from 3.65 to 3.35, associated with acrylamide reduction by 49% compared to the analogous bread without enzyme added (Figure 3.14). In the present study, the pH values of sourdoughs fermented with *L. casei, L. sakei, P. acidilactici,* and *P. pentosaceus* ranged between 3.46 and 4.32 at the end of the fermentation, even though during the initial stage pH was found to be approximately 5.24 (Figure 3.15). The highest acidity was measured for dough fermented with *L. casei* strain (pH 3.46). *L. sakei* and *P. acidilactici* reduced the pH value by 31% on average after 24 h of fermentation, while *P. pentosaceus* was found to produce less acid, decreasing the pH by 18% compared to control sample.

These results are in agreement with other authors, in that low pH values (pH < 5) can be one of the means to inhibit the Maillard reaction, thereby decreasing the acrylamide content in bread. The first step in acrylamide formation (Maillard) reaction is the formation of a Schiff base that can hydrolyze to form 3-aminopropionamide, a potent precursor of acrylamide [132]. The reduction of the pH in the system inhibits the formation of the Schiff base by favoring protonation of the amine group of asparagine. This effect is due to the reduced pH to 3.7 rather than to assimilation of asparagine by the lactic acid bacteria [93]. Hereby, the addition of consumable acids would be a very simple but efficient method to minimize acrylamide content in bakery products [133]. As reported by Baardseth *et al.* (2004) and Blom *et al.* (2009), similar effects could be observed by lacto-fermentation [134, 135]. Rapid acid production is a preferred property of LAB as a starter culture for the fermentation of food products [136]. Furthermore, an acidic flavor of bakery products is only accepted in the case of sourdough, which according to this study, could attribute to improvement of mixed rye bread quality by prevention of acrylamide formation.

3.4.2. The effect of lactic acid fermentation of lupine wholemeal of wheat-lupine bread

Acrylamide formation during the production of wheat bread supplemented with lupine flour, non-fermented and fermented by *Lactobacillus* and *Pedioccocus* strains was investigated. The

effect of supplementing wheat flour with 15% of lupine (*Lupinus angustifolius L.*) wholemeal fermented by various LAB strains on the acrylamide content in bread crumb as well as on bread texture and sensory characteristics was analyzed.

Figure 3.16 shows that the supplementation of wheat flour with untreated lupine flour resulted in a 43% increase of acrylamide content (34 µg kg^{-1} d.w.) compared to control sample (19 µg kg^{-1} d.w.). The main interest in lupine for food is related to its high protein content [137, 138], also lupine does not contain gluten, thus it could be used as a functional ingredient in gluten-free foods [139]. Lupine products are valued for their genetically modified organism - free status, functional food properties, nutritional and health benefits, and seem to be particularly promising as a source of an innovative food ingredient for the food industry in Europe [140, 141]. The new applications of lupine include meat replacement and its use as a component of rational nourishment and food for vegetarians [142], moreover, lupine is used in ‘‘clean-labeled’’ (lactose free) products. The use of wholemeal lupine flour with high protein content for the production of fermented products may cause problems associated with the formation of acrylamide. Protein breakdown products, peptides and amino acids may represent precursors for acrylamide formation during the heating of foods containing reducing sugars [30, 143]. Therefore, the elimination of acrylamide precursors by using biotechnological tools [92] is regarded as an interesting approach to acrylamide reduction.

Figure 3.16 shows the effect of lactobacteria fermentation of lupine flour on the acrylamide content. The acrylamide concentration in tested breads varied from 16 to 34 µg kg^{-1} d.w. The highest acrylamide content (34 µg kg^{-1} d.w.) was found in wheat bread supplemented with untreated lupine flour. The results indicated that acrylamide levels in breads could be lowered by up to 49% using fermentation with *L. sakei*. Lupine sourdoughs prepared with the tested *Pedioccocus (P.p.8 – KTU05-8; P.p.9 – KTU05-9; P.p.10 – KTU05-10)* strains allowed to decrease the acrylamide levels in bread by 19% on average, compared to untreated lupine flour.

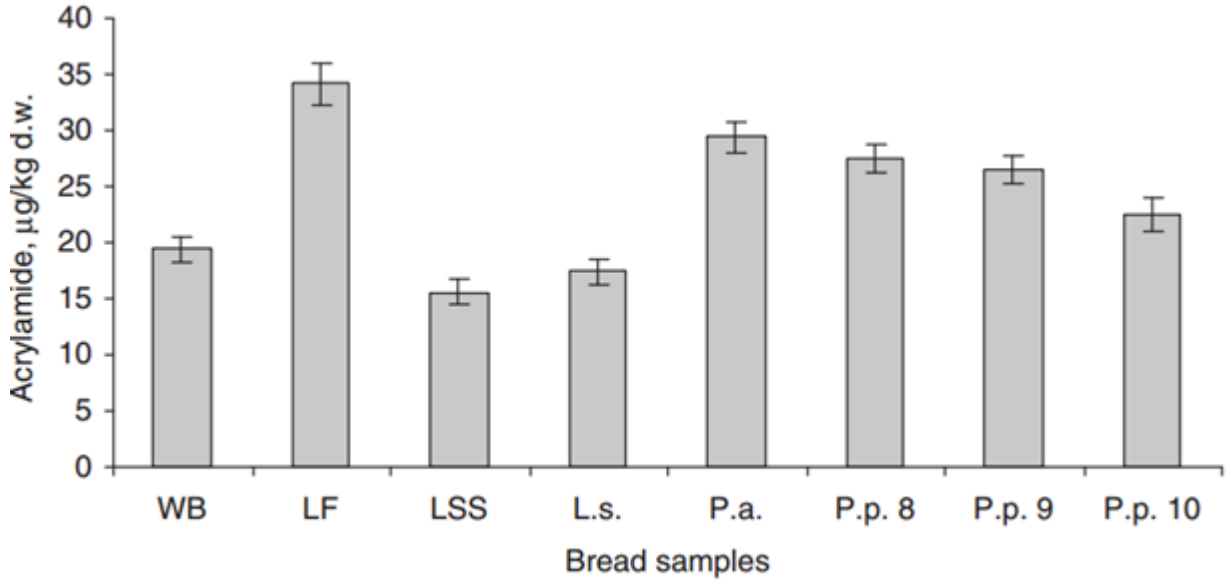

Fig. 3.16. **Acrylamide content in wheat-lupine breads supplemented with various lupine sourdoughs**

Bread samples: WB, wheat bread; LF, with lupine flour; LSS, with lupine spontaneous sourdough; with lupine sourdoughs, fermented by: P.a., *P. acidilactici*, P.p., *P. pentosaceus*, and L.s., *L. sakei* (n = 3).

Spontaneous fermentation of lupine reduced acrylamide contamination in bread by up to 54.4% (sample LSS in Fig. 3.16), but this bread had poor organoleptic properties and was unacceptable to consumers.

The effects of several factors, such as pH, fermentation time and the activity of LAB and/or indigenous cereal enzymes responsible for the proteolytic events during the sourdough fermentation were reported [144]. According to the obtained results, it could be suggested that enrichment of wheat bread with lupine as a high quality protein source should be accompanied by sourdough fermentation with pure LAB cultures, such as *L. sakei KTU05-6* (123 PU g^{-1}) and *P. pentosaceus KTU05-10* (97.0 PU g^{-1}), which provide a high proteolytic activity. The use of highly proteolytic strains for lupine fermentation allowed to reduce the acrylamide content in bread due to hydrolysis of proteins to small peptides and amino acids that may strongly reduce the acrylamide content, probably by promoting competing reactions with the precursors [93]. Increased protease activity of LAB may result in an enhanced release of amino acids from proteins [143]. A cell envelope-associated proteinase may hydrolyse proteins to small peptides and amino acids during sourdough fermentation. Stadler (2006) showed that the addition of other free amino acids from the group of cysteine, lysine, glycine, and histidine strongly reduced the acrylamide content,

probably by promoting competing reactions and/or covalently binding the acrylamide that formed [145].

The most acceptable was bread prepared with 15% of *P. pentosaceus 10* and *L. sakei* lupine sourdough. Overall, there is a growing interest towards industrial exploitation of new protein sources, such as plant proteins, to broaden the range and variety of foods [146]. Thus, in order to reduce the acrylamide levels in baked goods supplemented with protein-rich plant material, highly proteolytic LAB strains could be recommended for lupine flour treatment. Fermentation with pure LAB enables the inclusion of fermented lupine as a high quality protein source for wheat bread without compromising the quality and safety due to acrylamide formation.

3.4.3. The influence of the addition of *Helianthus tuberosus L.* fermented with various lactobacilli on acrylamide content in biscuits

The effects from the addition of JA (*Helianthus tuberosus L.*) fermented with various lactobacilli (*L. sakei KTU05-6, P. acidilactici KTU05-7, and P. pentosaceus KTU05-9*) on the acrylamide content in biscuits was investigated.

The effect of fermented (SMF and SSF) JA additives on asparagine and acrylamide content in biscuits was identified. A significant decrease in asparagine content was found in JA after LAB fermentation, compared to control sample with non-fermented JA (Table 3.8). JA is used as a natural source of inulin in many bakery products such as breads, pastas, biscuits and cakes, and the consumption of the bakery products containing JA may help to increase the daily intake of inulin which has many health benefits. JA contains a limited amount of protein, it is also rich in dietary fiber and carbohydrates, so a successful combination with wheat flour for bread and biscuit production would be nutritionally advantageous [147, 148].

Relative to non-fermented JA samples, the asparagine levels in SSF JA were found to be lower by 7.8% to 8.3%, and in SMF JA lower by 13% to 20%. The reducing sugar content in JA samples increased during the fermentation. The highest reducing sugar content was found in SSF JA fermented with *P. pentosaceus* (4.7 g kg^{-1}). Granvogl & Schieberle (2006) reported that reducing sugars are not always needed for the production of acrylamide [35]. However, the highest amount of asparagine and reducing sugars was found in wheat flour (141 mg kg^{-1} and 38 g kg^{-1}, respectively). The asparagine levels in experimental biscuits with SSF JA additives varied from 87 to 143 mg kg^{-1} d.w. (in biscuit samples with 50 g and 75 g of *P. acidilactici* SSF JA, respectively) (Table 3.8). The acrylamide levels in biscuit samples with 50 g of SSF JA fermented with *P.*

acidilactici and with 75 g of SSF JA fermented with *L. sakei* ranged from <10 μg kg^{-1} to 62 μg kg^{-1}, respectively. The supplementation of biscuits with SSF JA significantly increased the asparagine content compared to control samples without JA additives (86 mg kg^{-1} d.w.). The highest amount of asparagine (149 mg kg^{-1} d.w.) was found in biscuits with non-fermented JA (50 g).

Table 3.8.

The asparagine and acrylamide content in the biscuits

Fermented JA additives, g	Asparagine (mg kg^{-1} d.w.)		Acrylamide (μg kg^{-1} d.w.)	
	SSF	SMF	SSF	SMF
Fermented with *P. pentosaceus*				
25	88	72	12	< 10
50	112	90	17	< 10
75	133	92	23	< 10
Fermented with *P. acidilactici*				
25	91	1.6	11	< 10
50	87	72	< 10	< 10
75	143	93	20	< 10
Fermented with *L. sakei*				
25	87	60	35	< 10
50	101	83	42	< 10
75	127	101	62	< 10
Without JA	86		27	
With non-fermented JA	149		75	

The use of *L. sakei* for SSF of JA gave the higher acrylamide content in all biscuit samples compared to the control sample without JA additives (27 μg kg^{-1} d.w.), but lower than in the samples with non-fermented JA (75 μg kg^{-1} d.w.). In biscuit samples with SSF JA fermented with *P. acidilactici* and *P. pentosaceus*, the acrylamide content was found to be from 1.2- to 2.7-fold lower than in control samples without JA additives. In biscuit samples with SMF JA additives, a lower asparagine content was found compared to SSF treatment of JA. The highest acrylamide content was found in biscuit samples with 75 g of JA treated with *L. sakei* (62 μg kg^{-1} d.w.), the lowest – in biscuit samples with 50 g of JA additive treated with *P. acidilactici* (<10 μg kg^{-1} d.w.). The acrylamide content in all biscuit samples with SMF JA additives was found to be <10 μg kg^{-1} d.w.

The results indicated that the acrylamide levels in biscuits with JA additives could be lowered by applying SMF with the LAB used in these experiments. The success of acrylamide reduction in

biscuits could be influenced by the lower pH of the SMF product, the higher total titratable acidity, and the higher protease and amylase activity, compared to SSF products. It is known that prolonged yeast fermentation of dough was an effective way of reducing the asparagine level, and thus also acrylamide. At moderate fermentation times the fructose levels increased, but the yeast later absorbed this, so the net effect on acrylamide reduction was beneficial [149]. There are many factors that have been shown to be responsible for both acrylamide formation and enzyme activity [150]. In cereal products, acrylamide formation probably depends not only on the available amounts of the precursors - asparagine and reducing saccharides, but also on the enzymatic degradation of starch and proteins during dough preparation [93]. A remarkable decrease of acrylamide content was found with increasing time of dough fermentation, probably due to metabolism of the limiting precursor (asparagine) by the yeast [133]. The results indicated that with selected LAB, fermented JA additives could also be used for acrylamide reduction in biscuits enriched with JA additives, because fermented JA additives are a natural source of organic acids and have amylolytic and proteolytic activities. Thus, there is a market for novel bakery products produced by using alternative ingredients, such as the JA, which contains natural probiotic compounds like inulin, and is highly appreciated and well tolerated by the majority of patients with diabetes.

3.4.4. The supplementation of wheat biscuits with fermented flaxseed and lupine flour

The present study was focused on acrylamide reduction in wheat flour biscuits supplemented with lupine and defatted flaxseed flour treated by SSF and SMF fermentations with *L. sakei, P. pentosaceus,* and *P. acidilactici* strains. The nutritionally most valuable flaxseed product for the preparation of thermally treated food is dried and defatted flaxseed. Defatting is necessary, because during the baking process 50-60% of fatty acids are transformed into trans-isomers.

Table 3.9 represents the effect of fermented flaxseed and lupine flour on the acrylamide content in biscuits.

Table 3.9.

The acrylamide content (μg kg^{-1} d.w.) in biscuits supplemented with flaxseed and lupine flour that was either non-fermented or fermented with various LAB strains

Biscuit samples	Flaxseed		Lupine	
Control	27			
Non-fermented				
25	91		94	
50	103		112	
75	123		124	
	SSF	SMF	SSF	SMF
Fermented with *P. pentosaceus*				
25	81	11	10	17
50	83	18	14	21
75	91	54	26	54
Fermented with *P. acidilactici*				
25	18	< 10	19	11
50	38	23	48	18
75	41	33	78	65
Fermented with *L. sakei*				
25	< 10	22	75	13
50	14	43	79	22
75	30	50	84	22

The data are presented as mean values (n=3). Wheat flour biscuits without other plant additives are used as control. The numbers 25, 50, 75 indicate the amounts of additive (g) in biscuits.

In all of the cases, non-fermented flaxseed and lupine flour increased the acrylamide content in biscuits, and the highest acrylamide content was found in biscuits with a higher amount of plant additives. Flaxseeds were recognized as one of the richest dietary sources of lignans, and the amount of enterolignans formed from flaxseed (10433 nmol/g) is an order of magnitude higher than from cereal products [151]. Flaxseeds may be associated with a decreased risk of breast cancer, and demonstrate antiproliferative effects in breast tissue of women at risk of breast cancer, thus may protect against primary breast cancer [152]. The opposite trends were established in biscuits with fermented plant additives. In most of the cases, lower amounts of acrylamide were found in biscuits with fermented lupine additives, compared to the control samples of biscuits, and compared to the biscuit samples without lupine flour. The use of *P. acidilactici* for SMF of flaxseed and lupine resulted in the most effective acrylamide content reduction in biscuits. The use of pseudo-cereals for wheat products helps to compensate for the deficiency of nutritional value in wheat flour. However, the addition of protein-rich raw materials could increase the acrylamide content in baked products [153]. The major constituents of biscuits are typically wheat flour, sucrose and fat, making such products rather energy rich [154]. Our study indicates that fermented

flaxseed and lupine additives could have a great potential in the development of higher nutritional value bakery products with low acrylamide content.

3.5. Determination of acrylamide levels in selected foods in Latvia

Since a very limited information about the content of acrylamide in food of Latvian origin is available, the main objective of our study was to determine the content of this compound in Latvian foods and to assess the average dietary acrylamide exposure of the Latvian population.

Given that acrylamide is formed in heat-treated potato and cereal products such as bread, breakfast cereals, cookies and biscuits, and during the coffee roasting process, testing was focused on the food products produced in Latvia that might be a significant source of dietary acrylamide intake for the Latvian population. Eight product groups were selected for the study:

i. rye bread, rye bread enriched with seeds, dried fruits or vegetables;
ii. sweet-and-sour bread;
iii. wheat bread;
iv. potato chips;
v. coffee, including instant;
vi. confectionery (cookies, crackers);
vii. gingerbread;
viii. bread products (bagels, crackers, etc.).

A total of 435 samples obtained from all Latvian regions were analyzed for the acrylamide content. The results revealed that the acrylamide content of processed foods varied greatly between various food groups, as well as between brands and within specific brands. The highest concentration of acrylamide was discovered in potato chips and chicory coffee with the average values of 564 µg kg^{-1} and 2790 µg kg^{-1}, respectively. No single bread sample exceeded the European Commission Recommendation 2013/647/EU [129], although the addition of seeds, fruits or vegetables clearly had an increasing effect on acrylamide concentration in bread. The dietary exposure assessment was performed by using analytical results on acrylamide levels in certain food groups and the relevant food consumption data. The calculated data indicates a high dietary intake of acrylamide by certain consumers that may potentially cause adverse health effects. Therefore,

adequate efforts should be made to diminish acrylamide levels in processed foods in order to reduce the health risk to the Latvian population.

Table 3.10

The acrylamide levels in various foods

Product description	N	Mean, μg kg^{-1}	SD, μg kg^{-1}	Median, μg kg^{-1}	Range, μg kg^{-1}
Bread					
Bread, wheat	48	14	6	12	<10-36
Bread, wheat with additives	11	29	16	31	10-54
Bread, rye	77	48	16	47	14-87
Bread, sweet sour	47	28	20	25	<10-133
Bread, rye with additives	33	54	30	52	14-152
Rusk	16	65	27	57	27-93
Toast, rye bread	12	40	17	37	20-79
Snacks					
Crisps	55	564	517	303	42-1570
Bagels	22	162	128	119	39-588
Coffee					
Roasted	13	450	120	450	300-600
Soluble	4	900	110	920	790-980
Chicory	1	2790			2790
Pastry					
Biscuit	53	187	118	91	<10-1060
Biscuit, with nuts	7	97	90	68	<10-279
Biscuit, grain	7	400	165	393	147-606
Biscuit, chocolate	6	141	122	115	23-341
Biscuits, savory	8	125	123	66	<10-322
Butter biscuit, toffee	4	28	9	26	19-41
Gingerbread	2	238	59	238	196-280
Puff pastry	9	102	62	82	20-200

The acrylamide content in 435 samples of food manufactured in Latvia ranged from 8 to 2790 μg kg^{-1}. High standard deviation indicates significant variation of results obtained for individual product groups (Table 3.10).

All food product samples were divided into three groups based on the mean value of acrylamide concentration (<100 μg kg^{-1}, from 100 to 200 μg kg^{-1}, and from 230 to 900 μg kg^{-1}). The highest mean acrylamide content falling into the range from 200 to 900 μg kg^{-1} was obtained

for the following product groups: crisps, grain biscuits, gingerbread, and coffee. The highest variations were observed in the group of crisps - from 42 to 1570 μg kg^{-1}. A relatively higher acrylamide content was found in crisps with added flavor combinations and crisps produced from potato varieties with high sugar content.

The mean acrylamide value in the range from 100 to 200 μg kg^{-1} was obtained for five product groups: bagels, biscuits, chocolate biscuits, savory biscuits, and puff pastry. This group also showed rather high variation of the acrylamide content.

The lowest acrylamide content was found in bread, bread products, biscuits with nuts and toffee. It should be noted that the acrylamide content in bread with added seeds and dried fruit was significantly higher in comparison with the samples of bread without any additives.

3.6. The assessment of Latvian population exposure to acrylamide from food products

The mean daily consumption of seven product groups in which acrylamide content was determined analytically, and the estimated exposure to dietary acrylamide for three age groups (adults 19-35 years, 36-50 years and 51-64 years) is presented in Tables 3.11, 3.12, and 3.13.

Table 3.11.

Estimation of the acrylamide exposure for adults aged 19-35 years

Adults 19-35 years	Consumption (g per person per day)			Acrylamide content (μg g^{-1})			Exposure assessment (μg per person per day)		
Food products	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Rye bread	54.5	0.16	900	0.05	0.01	0.09	2.62	< 0.01	78.3
Rye bread with dried fruits	54.5	0.16	900	0.07	0.01	0.17	3.76	< 0.01	153
Sweet & sour bread	27.1	0.03	375	0.03	0.01	0.13	0.76	< 0.01	49.9
Wheat bread	52.6	0.05	1500	0.02	0.01	0.05	0.89	< 0.01	81.0
Cookies	11.3	0.03	213	0.16	0.01	0.61	1.76	< 0.01	129
Potato chips	9.90	0.01	136	0.56	0.04	1.57	5.58	< 0.01	215
Coffee	6.61	0.00	39.4	0.90	0.45	2.79	5.95	< 0.01	110

Table 3.12.

Estimation of the acrylamide exposure for adults aged 36-50 years

Adults 36-50 years	Consumption (g per person per day)			Acrylamide content (μg g^{-1})			Exposure assessment (μg per person per day)		
Food products	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Rye bread	57.4	0.14	1600	0.05	0.01	0.09	2.75	< 0.01	139
Rye bread with dried fruits	57.4	0.14	1600	0.07	0.01	0.17	3.96	< 0.01	272
Sweet & sour bread	34.9	0.07	625	0.03	0.01	0.13	0.98	< 0.01	83.1
Wheat bread	58.3	0.07	750	0.02	0.01	0.05	0.99	< 0.01	40.5
Cookies	11.6	0.05	300	0.16	0.01	0.61	1.80	< 0.01	181
Potato chips	2.79	0.05	78.4	0.56	0.04	1.57	1.57	< 0.01	123
Coffee	9.06	0.01	232	0.90	0.45	2.79	8.15	< 0.01	647

Table 3.13.

Estimation of the acrylamide exposure for adults aged 51-64 years

Adults 51-64 years	Consumption (g per person per day)			Acrylamide content (μg g^{-1})			Exposure assessment (μg per person per day)		
Food products	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Rye bread	86.9	0.16	810	0.05	0.01	0.09	4.17	< 0.01	70.5
Rye bread with dried fruits	86.9	0.16	810	0.07	0.01	0.17	6.00	< 0.01	137
Sweet & sour bread	41.5	0.08	750	0.03	0.01	0.13	1.16	< 0.01	99.7
Wheat bread	51.4	0.05	470	0.02	0.01	0.05	0.87	< 0.01	25.4
Cookies	11.8	0.05	225	0.16	0.01	0.61	1.85	< 0.01	136
Potato chips	2.11	0.03	58.7	0.56	0.04	1.57	1.19	< 0.01	92.3
Coffee	8.49	0.01	70.5	0.90	0.45	2.79	7.64	< 0.01	196

Based on the results of the actual daily consumption, bread was consumed in the highest quantity out of all the analyzed product groups. In the oldest population group (51-64 years) the consumption of bread was 179 g per person per day. The highest consumption of potato chips was observed for the age group of 19-35 years (9.9 g per person per day). The mean consumption of coffee was equal for all groups of population with high variability between individuals.

The mean daily dietary acrylamide intake ranged from 17 μg in the age group of 19-35 years and 51-64 years to 18.7 μg in the age group of 36-50 years.

The acrylamide intake from food was mainly attributed to three product types. In the Latvian population, bread supplied 31% of the total dietary acrylamide intake in the age group of 19-35 years, 33% in the age group of 36-50 years and even up to 46% in the age group of 51-64 years.

Potato chips on average supplied 16% of acrylamide in the adult population (19-64 years) with a significant difference between age groups (from 32% in the age group of 19-35 years to only 7% in the age group of 51-64 years). A significant source of dietary acrylamide in the Latvian population was also coffee, contributing 40% of the total dietary acrylamide intake in the adult population.

The calculated average dietary acrylamide exposure for the Latvian age group of 19-64 years is 0.26 μg kg^{-1} of body weight per day, and this estimate is in good agreement with the WHO data showing that the average dietary acrylamide exposure for the general population ranges between 0.3 and 0.8 μg kg^{-1} of body weight per day [155]. However, it should be taken into account that not all foodstuffs that might contain acrylamide were regarded in our study, including home-made products that might represent a major source of dietary acrylamide intake. Therefore, the estimated acrylamide intake is probably lower than the actual level, and further research is necessary. Besides that, it is disturbing to note that for the high consumer group the estimated exposure to dietary acrylamide intake exceeds the estimated mean exposure for the total population even ten-fold (up to 3 μg kg^{-1} of body weight per day). This level matches the tolerable daily intake (TDI) estimated in another study [51], and therefore high dietary intake of acrylamide by certain consumers may potentially cause adverse health effects and certain activities are advisable to reduce acrylamide levels in processed foods, in order to minimize the dietary risk to the health of Latvian population.

3.7. Determination of furan levels in selected foods in Latvia

Another part of the study was devoted to investigation of furan levels in canned foods obtained from the Latvian market. For furan analysis the following product groups were chosen:

i. Coffee;
ii. Canned and jarred food;
iii. Baked goods, cereals, and crackers.

A total of 76 samples were analyzed for furan content. The overall median value of furan content was 19 μg kg^{-1}. The highest concentration was found in a coffee sample - 11294 μg kg^{-1}, and the lowest content of furan was found in fruit juice - 0.51 μg kg^{-1} (Table 3.14). Relatively high furan content was discovered in grey beans (with the median value of 48 μg kg^{-1}) and sauces (37 μg kg^{-1}). The lowest furan content was found in canned vegetables and jams. The median values in these products were 5.3 μg kg^{-1} and 3.4 μg kg^{-1}, respectively.

Table 3.14.

Furan concentration in various foods

Sample	N	Mean, μg kg^{-1}	Median, μg kg^{-1}	Range, μg kg^{-1}
Baby food	5	22	9.2	7.1-52
Vegetables	7	7.9	5.3	1.2-21
Jam	8	7.0	3.9	1.8-27
Coffee	12	2808	911	112-11294
Honey	6	11	9.8	5.4-20
Sauce	15	44	37	5.6-122
Backed beans	3	32	28	24-43
Grey beans	5	48	48	18-98
Juice	10	13	10	0.51-54
Soups	4	34	33	8.3-62
Bread	1	3,0	3.0	3.0

The results indicated that the furan content of processed foods varies greatly between different food groups, as well as between brands. For example, the mean concentration of furan in coffee was 2808 μg kg^{-1}, while the lowest result was 112 μg kg^{-1}. It should be noted that the obtained furan concentrations in this study did not differ much from those reported by EFSA [43].

The overall results show that the furan content of most product groups was in the range from 10 to 100 μg kg^{-1}. Higher concentrations were observed only in sauce (13%) and in all coffee samples.

CONCLUSIONS

1. For the first time, a new rapid and reliable HPLC-Q-Orbitrap-HRMS based methodology was developed for the determination of acrylamide in coffee samples. This elaborated analytical method was demonstrated to be an important and powerful tool for the accurate quantification of such small molecules as acrylamide in difficult matrices like coffee. The targeted-MS2 scan mode provided product ion spectra with accurate mass measurement possibilities that allowed an unambiguous confirmation of analyte. The instrument ensured high resolution, very good sensitivity, linear range, and good reproducibility. A comparison of the elaborated method with the triple quadrupole method showed the superiority of the high resolution mass spectrometry for this specific type of analysis.

2. The application of the elaborated method for the analysis of real coffee samples showed that the content of acrylamide in samples varied from 166 to 503 μg kg^{-1} with no specific pattern attributed to the origin or type of coffee. The concentration of acrylamide in some samples exceeded the recommendations issued by the European Commission.

3. HS-GC-MS/MS was found to be a simple, fast, selective, and sensitive method for the analysis of furan in baby foods. The method showed a good linearity, precision, and accuracy. It was found for the first time that the SRM scanning mode provided a much higher selectivity for the determination of furan compared to SIM, and no interference from other matrix components was observed.

4. The present study reports data on the occurrence of furan in baby foods in Latvia. All jarred baby foods showed a furan content between 0.45 to 82 μg kg^{-1}. The higher content of furan was found in vegetables and mixed product groups containing vegetables. It was observed that the pH values and Brix content in the samples may impact the formation of furan, while no correlation was observed between the amounts of β-carotene and sugars and the content of furan in samples.

5. The acrylamide content in 435 food samples of various types manufactured in Latvia ranged from 8 to 2790 μg kg^{-1}. The highest concentration of acrylamide was revealed in potato chips and chicory coffee, with the average concentrations of 564 μg kg^{-1} and 2790 μg kg^{-1}, respectively.

6. A total of 76 different types of samples were analyzed for furan content. The highest concentration was found in a coffee sample (11294 µg kg^{-1}), and the lowest content of furan was found in fruit juice (0.51 µg kg^{-1}).

7. The tendency of the reduction of acrylamide content in bread with decreased pH values in fermented products was found. Saccharification of extruded rye wholemeal with glucoamylase was found to have a positive effect on the acidification process by lowering the pH values of fermented products by 8.6% on average. Fermentation with *P. acidilactici* in combination with glucoamylase enabled a decrease of pH from 3.65 to 3.35, associated with the reduction of acrylamide content in bread by 49%.

8. The supplementation of wheat flour with untreated lupine flour caused a 43% increase in acrylamide content, compared to the control sample. The obtained results indicated that acrylamide levels in bread could be lowered by up to 49%, using fermentation with *L. sakei*.

9. The addition of non-fermented flaxseed increased the acrylamide content in biscuits by up to 78%, compared to control samples. The most effective decrease in acrylamide was achieved in biscuits treated by SSF with *L. sakei* (6 times), by SMF with *P. acidilactici* (6 times), and by SMF with *P. pentosaceus* (8 times).

ACKNOWLEDGMENTS

I would like to express my deep gratitude to my scientific supervisors associate professor, Dr. chem. Vadims Bartkevičs and professor, Dr. chem. Arturs Vīksna for their professional guidance, leadership and valuable support. My special thanks are extended to Dr. Elena Bartkiene from Lithuanian University of Health Sciences (Kaunas, Lithuania) for their technical support. I'm also grateful to all my colleagues of Institute of Food Safety, Animal Health and Environment "BIOR" who have contributed to my work on the doctoral thesis.

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