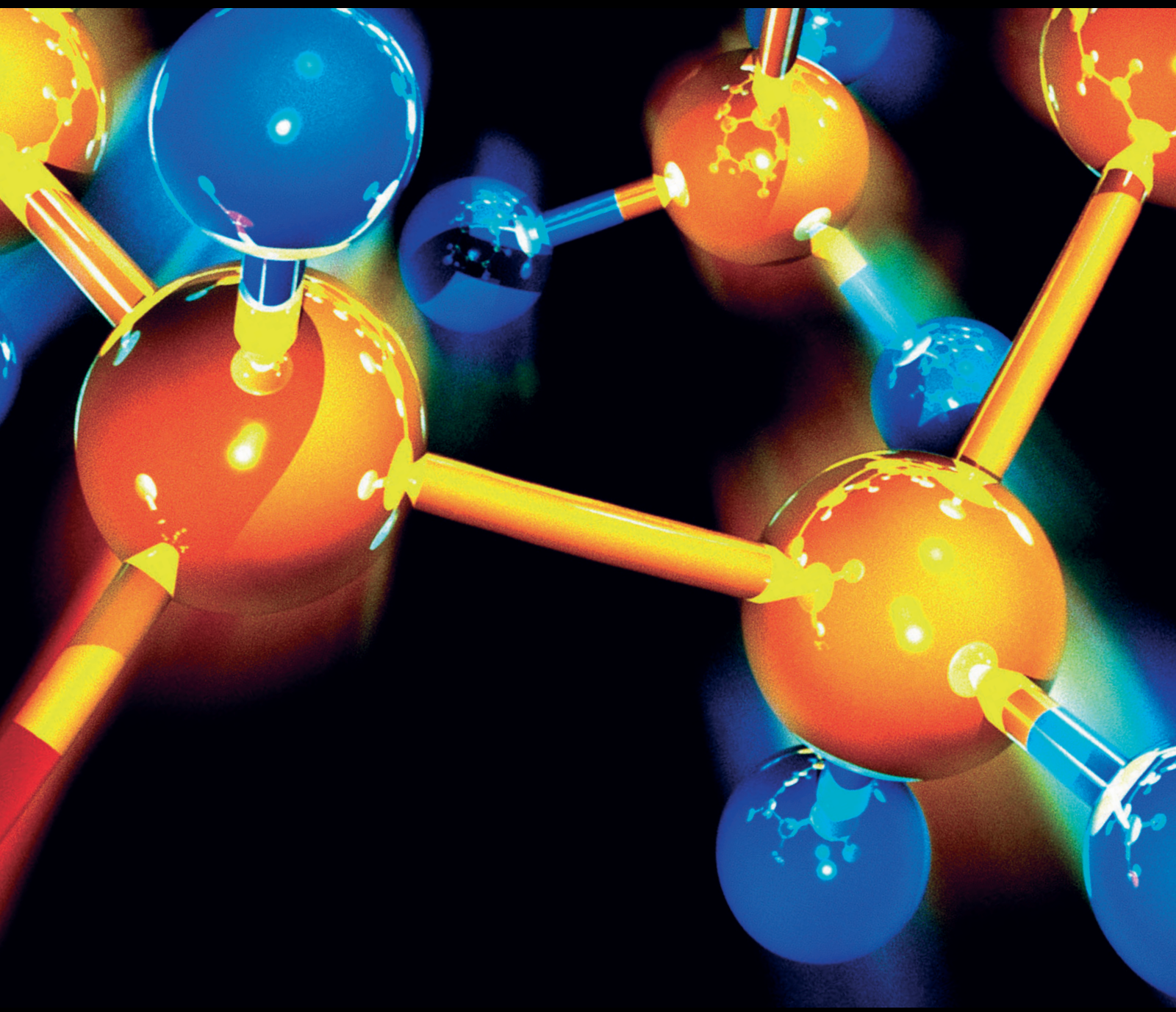
Journal of Chemistry

Food Chemistry: Food Quality and New Analytical Approaches

Guest Editors: Nivia M. M. Coelho, César R. T. Tarley, Claudia Ruiz-Capillas, Luciana M. Coelho, and Miguel de la Guardia



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Contents

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2016, Article ID 7296348, 2 pages
http://dx.doi.org/10.1155/2016/7296348

Editorial

Food Chemistry: Food Quality and New Analytical Approaches

Nivia M. M. Coelho,[1] César R. T. Tarley,[2] Claudia Ruiz-Capillas,[3] Luciana M. Coelho,[4] and Miguel de la Guardia[5]

[1]*Federal University of Uberlândia, 38400902 Uberlândia, MG, Brazil*
[2]*State University of Londrina, 86057970 Londrina, PR, Brazil*
[3]*Instituto de Ciencia y Tecnología de Alimentos y Nutrición (ICTAN), 28040 Madrid, Spain*
[4]*Federal University of Goiás, 75704020 Catalão, GO, Brazil*
[5]*Department of Analytical Chemistry, University of Valencia, 46100 Valencia, Spain*

Correspondence should be addressed to Nivia M. M. Coelho; nmmcoelho@ufu.br

Received 18 March 2016; Accepted 22 March 2016

Foods, their production systems, and quality control are challenging subjects of the today research in an increasing population world and, because of that, the scientific community has made big efforts to improve the analytical tools to effectively control the quality and safety of human foods. That, which is a main topic in the developed countries, merits too special attention in developing areas in order to extend the safety to all the steps of the production of foods and all consumed products all around the world. So, this special issue if devoted to food chemistry: food quality and new analytical approaches.

The continuous efforts of food industries to improve their strategies of production, transformation, and distribution of foods have been focused on the development of new technologies for food processing, which aimed at the enhancement of food properties or to create novel products suitable for covering the needs of consumers and for guaranteeing safety, health, and well accepted standards.

In the aforementioned frame, food industry needs to improve chemical and biological tools suitable for guaranteeing a safe production. To do that, microbiological and chemical methods of analysis have an increasing value as control systems. However, methods available nowadays for a fast and continuous monitoring of the main food components, additives, and contaminants are still scarce and not in all cases are we suitable for solving the problems created by traditional contaminants as heavy metals, pesticides, pharmaceutical residues, and emerging contaminants. In fact there are serious worries in all countries about the presence of pesticides in cereals, vegetables, and milk and dairy products, the contamination of fishes by heavy metals, like mercury, lead, cadmium, or arsenic, between others, or the presence of pharmaceutical residues in farm meat products. So, new approaches are required for food sampling, sample preparation, and analysis of chemical components, their metabolites, and mineral species in foods.

There are inherent difficulties associated with the different types of food samples involved in human nutrition and variations in their composition as a function of the production and industrialization conditions. Additionally, there is a growing interest for the improvement of food properties, food authentication, which is especially concerned with the so-called organic foods and protected designation of origin foods, and/or to produce novel products, as well as for the systematic analysis of contaminants or additives in produced foods to be exported to foreign countries and also to be consumed in the same country and, because of that, not only food producers but also consumers and policy makers are forced to develop accurate, precise, sensitive, and selective tools for a fast control of too many parameters in foods.

This special issue aims to present the most recent advances in the field of food chemistry, which have been achieved in recent years as a consequence of recent progress in food quality requirements and new analytical approaches available in the field.

The topics included in this issue have been properly selected and concern new research challenges in food quality and safety control, general food and beverages analysis, food

forensics, bioactive food constituents and functions determination, chemical changes induced by food processing and storage, recent advances in sample preparation techniques in the food field, and chemical speciation in foods.

Acknowledgments

We are would like to deeply acknowledge the authors, who submitted their excellent contributions, and the editorial staff, who helped us to improve the submitted manuscripts. Thus, based on the aforementioned efforts, we are convinced that the readers will find in this special issue many topics and works of interest for their daily practice and information and we hope that this work could become a reference text for the future research efforts in this field. So, we offer many thanks for reading the enclosed papers and our best wishes for your future research.

Nivia M. M. Coelho
César R. T. Tarley
Claudia Ruiz-Capillas
Luciana M. Coelho
Miguel de la Guardia

Hindawi Publishing Corporation
Journal of Chemistry
Volume 2016, Article ID 1427154, 11 pages
http://dx.doi.org/10.1155/2016/1427154

Review Article

Analytical Strategies for the Determination of Arsenic in Rice

Bruno E. S. Costa,[1] Luciana M. Coelho,[2] Cleide S. T. Araújo,[3] Helen C. Rezende,[4] and Nívia M. M. Coelho[1]

[1]*Institute of Chemistry, Federal University of Uberlândia, Avenida João Naves de Ávila 2121, 38400-902 Uberlândia, MG, Brazil*
[2]*Department of Chemistry, Federal University of Goiás, Avenida Dr. Lamartine Pinto de Avelar 1120, 75704-020 Catalão, GO, Brazil*
[3]*State University of Goiás, BR 153, No. 3105, 75132-400 Anápolis, GO, Brazil*
[4]*Department of Chemistry, Federal University of Goiás, BR 364, km 195, No. 3800, 75801-615 Jataí, GO, Brazil*

Correspondence should be addressed to Nívia M. M. Coelho; nmmcoelho@ufu.br

Received 25 October 2015; Revised 16 February 2016; Accepted 18 February 2016

Academic Editor: Kamlesh Shrivas

Arsenic is an element of concern given its toxicological significance, even at low concentrations. Food is a potential route of exposure to inorganic arsenic and in this regard arsenic in rice is associated with soil contamination, fertilizer application, and the use of arsenic-containing irrigation water. Therefore, there is a need to investigate the regional rice crops with a view to future discussions on the need for possible regulatory measures. Several studies have reported high concentrations of arsenic in rice grown in soils irrigated with contaminated water; however, procedures used, including sample pretreatment and preconcentration steps, have to be followed to ensure sensitivity, accuracy, and reproducibility. Arsenic is a difficult element to measure in complex matrices, such as foods, because the matrix must be destroyed at an elevated temperature without the loss of the analyte or contamination. This review summarizes the major methods for the determination of arsenic in rice samples. The main purpose of this review is to provide an update on the recent literature concerning the strategies for the determination of arsenic and to critically discuss their advantages and weaknesses. These difficulties are described along with recent developments aimed at overcoming these potential issues.

1. Introduction

Arsenic (As) is considered to be one of the most important toxic elements because of its potential risk to human health [1]. It is carcinogenic, the inorganic form being the most harmful, and thus it merits particular attention [2–4].

Sources of arsenic in the environment can be natural or anthropogenic, since this element occurs in trace amounts in most rocks as well as in soil, water, and atmospheric dust. Once released into the environment, arsenic compounds reach water sources, such as rivers and groundwater systems, and subsequently food sources. Arsenic-contaminated soil, sediment, and sludge are the major sources of arsenic in the food chain, surface water, groundwater, and drinking water [5]. Arsenic concentrations in noncontaminated soils are typically below 10 mg/kg while in contaminated soils they can be as high as 30,000 mg/kg [6].

In the soil environment, arsenic is present mostly as the inorganic species (arsenate As(V) and arsenite As(III)). Inorganic arsenic species can be methylated through microbial action to give monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA). Organic and inorganic arsenic species are present in the solution phase of paddy soils and can also be assimilated by plant roots [7]. Under oxidized condition, since As(V) is the predominant form (the lesser toxic form in iAs) and can easily be sequestrated by iron oxyhydroxide, it acts as a favorable process limiting arsenic accumulation during rice cultivation [8]. On the other hand, under highly reduced conditions, As(V) is reduced to As(III), which precipitates out from the solution in sulfur minerals, primarily arsenopyrite. In paddy soils, due to flooding type irrigation, anoxic condition is generated which favors the release of arsenic from soils and sediments and thereby increases the bioavailability to rice plants. Arsenic can be found as a contaminant in drinking water and foods in the form of different

chemical species and this affects the assimilation pathway. Recently, published studies about the arsenic content of different food products (including drinking water), indexed in the ISI Web of Science for the period of 2010 to 2015, show that almost 32% of the papers on arsenic contamination via the diet relate to research on fish samples, while around 29% are associated with drinking water and 39% with other food samples, such as rice (which accounts for 52% of this category). Other foods researched were various cereals and vegetables, edible oils, wine, and beer.

Rice (*Oryza sativa*) is the most important grain crop worldwide, being consumed by half of the world's population. Studies indicate that rice is a major contributor of inorganic arsenic in human diets [9–16]. Although seafood is known to contain high levels of total arsenic, most of it is present as organic arsenic.

The use of arsenic-containing irrigation water can lead to both long-term soil contamination with arsenic and a supply of arsenic to the crop. Rice accumulates a higher amount of arsenic than any other grain crops, largely because of the high availability of arsenic to plants under reduced soil conditions [17]. Arsenic accumulates in different parts of the rice plants and the accumulation rate varies according to the variety. In one study, arsenic concentrations in rice plant parts were found to decrease in the following order: root > straw > husk > whole grain > husked rice [18]. A higher arsenic accumulation in the roots than in other parts of the plant has also been reported by other authors [19, 20]. Some authors have observed the translocation of arsenic in plant systems [21, 22]. The relatively high levels of arsenic in rice are due to several factors including (1) the mobilization and bioavailability of arsenic in the soil after the farmers flood the rice fields and (2) plant uptake of arsenic instead of silicon, which is used by the plant under normal conditions to strengthen the stems and husks. Arsenic and silicon are chemically similar under the soil conditions found in flooded rice paddies and thus arsenic can be transported by the silicon transporters. As the rice plant grows, the plant incorporates arsenic (instead of silicon) into the grain.

Reported levels of As in rice [23–27] are <0.01–2.05 mg kg^{-1} for Bangladesh, 0.31–0.70 mg kg^{-1} for China, 0.03–0.044 mg kg^{-1} for India, <0.10–0.76 mg kg^{-1} for Taiwan, 0.11–0.66 mg kg^{-1} for the US, 0.03–0.47 mg kg^{-1} for Vietnam, and 0.08–0.38 mg kg^{-1} for Italy and Spain.

A survey of the research focused on regions with high levels of arsenic contamination in rice within the period covered by this review (2010–2015) returned 335 articles. The main aspects of this survey are highlighted in Figure 1. Most studies relate to the USA and Bangladesh while the other regions are China, the Indian state of West Bengal, India (as a whole), Mexico, Colombia, and Brazil.

According to WHO, the main sources of human exposure to arsenic are water and food [23]. Inorganic arsenic in water is regulated [24, 25]; however, there is no European Union (EU) or United States of America (USA) standard for inorganic arsenic in food products, despite the fact that food products represent the main route of exposure, especially rice and rice-based products [26, 27]. Recently, the JECFA

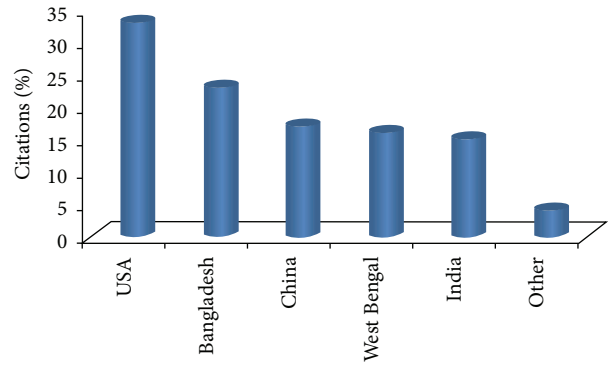

Figure 1: Regions with high levels of arsenic contamination in rice.

proposed a maximum level of 0.2 mg/kg of inorganic arsenic in polished rice [28]. The European Food Safety Authority (EFSA) has reviewed the diet of the European Union population and has recommended that dietary exposure to inorganic arsenic should be reduced. The JECFA also carried out a review and stated that dietary exposure to inorganic arsenic is worthy of considerable attention and should be reduced. Therefore, there is a need to investigate the regional rice crops with a view to future discussions on the need for possible regulatory measures.

Several studies have reported high concentrations of arsenic in rice grown in soils irrigated with contaminated water [29–33]; however, procedures used, including sample pretreatment and preconcentration steps, have to be followed to ensure sensitivity, accuracy, and reproducibility.

Arsenic is a difficult element to measure in complex matrices, such as foods, because the matrix must be destroyed at an elevated temperature without the loss of the analyte or contamination. Determination of As species is generally carried out using hydride-generation (HG) [34, 35], liquid chromatography (LC) [36, 37], gas chromatography (GC) [38], and capillary electrophoresis (CE) [39, 40]. As speciation, particularly in solid samples, requires very careful sample preparation since this element is volatile, and it is important to avoid modification of the form and concentration of the species [41].

This review summarizes the major methods for the determination of arsenic in rice samples. The main purpose of this review is to provide an update on the recent literature concerning the strategies for the determination of arsenic and to critically discuss their advantages and weaknesses compared with the commonly accepted approach of combining nonchromatographic and spectroscopic techniques. The problems focused on involve sample preparation, as well as changes in "species information" that occur during the use of various separation technologies. These difficulties are described along with recent developments aimed at overcoming these potential issues.

2. Analytical Methodology

Analytical procedures for sample preparation are one of the most important steps in analytical methods. At this stage,

Table 1: Sample preparation methods for determination of inorganic arsenic in rice.

Arsenic species	Sample preparation	Arsenic found (mg Kg^{-1})	Detection	Reference
As total	Hot plate (HNO_3)	—	ICP-MS	[44]
As total	Microwave digestion (HNO_3/H_2O_2)	0.046–0.315	ICP-MS	[45]
As inorganic	Microwave digestion (HNO_3)	0.029–0.121	HPLC-ICP-MS	
As total	Ultraclave microwave (trifluoroacetic acid/H_2O_2)	0.036–0.218	HPLC–ICPMS	[46]
As inorganic		0.025–0.171		
As total	—	0.110–0.421	INAA	[47]
As inorganic	Hot plate (water)	0.050–0.172	LC-ICP-MS	
As total	Microwave digestion (HNO_3/H_2O_2)	0.07–0.47	ICP-MS	[48]
As inorganic	Microwave digestion (HNO_3)	0.001–0.17	HPLC-ICP-MS	
As total	Microwave digestion (HNO_3 concentrated)	<0.022–0.271	ETAAS	[49]
As inorganic	Vortexed and ultrasound (HNO_3 solution)	<0.030–0.147		
As(III)	Microwave digestion (HNO_3 solution)	<0.019–0.097		
As(V)	—	<0.030–0.076		
As total	Hot plate (HNO_3)	0.012–0.578	ICP-MS	[50]
As total	Ultrasound (HNO_3)	0.0125–0.1893	ETAAS	[51]
As(V)		0.0433–0.0625		
As total	Microwave digestion (HNO_3/H_2O_2)	0.026–0.464	ICP-MS	[52]
As total	Water bath (HNO_3 solution)	0.199–0.284	HPLC-ICP-MS	[53]
As inorganic	—	0.02–0.18	LC-AFS	[54]
As inorganic	Microwave digestion (HNO_3/H_2O_2)	0.045–0.235	HG-AFS	[55]
As total	Microwave digestion	0.013–0.150	HPLC-ICP-MS	[56]
As(III)	ELL (methanol-water (1 : 1)/HNO_3)	0.0036–0.0311		
As(V)		N.D–0.054		

several variables must be controlled and studied to ensure the quality and reliability of the results. In addition, the determination of arsenic and its species requires very careful sample preparation, since this element is volatile, and it is important to avoid the modification of the chemical form in order to maintain the integrity and concentration of the species of interest during the sample preparation process [42, 43].

Some recent studies reported in the literature in which total arsenic and/or inorganic arsenic species were determined are listed in Table 1 and also detailed in the text.

Dwivedi et al. [44] evaluated the effect of the presence of arsenic on the synthesis of essential and nonessential amino acids in rice grains. Rice seeds were obtained and grown with the cooperation of the Rice Research Station, Chinsurah, West Bengal, India, in a randomized block design, following conventional agronomic practices. Sixteen rice genotypes were selected, based on the overall contrast of the grains, from 90 rice germplasms grown in three As-contaminated areas of West Bengal. All field sites were fertilized with chemical fertilizers (N, P, and K) and a field trial was conducted over two years. The rice grain samples were pulverized and 0.5 g was digested in 3 mL of HNO_3 at 100°C for 2 h and 120°C for 4 h, then filtered in 10 mL of water, and stored at 4°C until the analysis. The total As in the grain samples was quantified using inductively coupled plasma-mass spectrometer (ICP-MS) while the As species were determined according to the protocol described by Zheng et al. [57]. The results of the field tests indicated that the accumulation of As in the soil led to its significant uptake by the rice grains and inhibited the synthesis of amino acids in some genotypes [44].

Carbonell-Barrachina et al. [45] analyzed Spanish rice gluten, cereals with gluten, and baby food and determined the total arsenic (t-As) and inorganic arsenic (i-As) using ICP-MS and high performance liquid chromatography (HPLC) with ICP-MS (HPLC-ICP-MS), respectively. Samples were dried in an oven at 80°C until constant weight and then homogenized by grinding in a ball mill and stored in a desiccator. For the determination of total arsenic, 0.2 g of dried product was placed overnight in contact with 2 mL of concentrated nitric acid. The samples were then digested in a microwave oven with hydrogen peroxide applying a temperature program reaching a maximum of 95°C. A certified reference material (CRM; rice flour NIST SRM 1568a) was used to assess the precision and accuracy of the chemical analysis. Arsenic species were extracted according to conditions previously described for t-As. However, HNO_3 (1% (w/v)) was used and the microwave digestion was conducted without the addition of hydrogen peroxide. The digested samples were centrifuged and 900 μL of the filtered supernatant was mixed with 100 μL of H_2O_2 and left overnight at 4°C. The content of inorganic arsenic was significantly higher in the Spanish rice gluten than in the cereals with gluten, placing children with celiac disease at high risk, since rice gluten is used as a replacement in gluten-free food products (note that rice gluten differs from "true gluten").

Raber et al. [46] proposed an analytical method for quantitatively determining inorganic arsenic in foods (including

rice). Rice samples were ground in a centrifugal mill to a particle size of <0.25 mm. The authors studied different sample preparation methods to improve the extraction efficiency and the compatibility between the extract and the chromatographic separation method. Among the different extractors studied, trifluoroacetic acid was found to be a potential reagent for the extraction of inorganic arsenic (iAs) from rice and no change in the retention time compared with aqueous standard solutions was noted. In the sample preparation procedure a portion of the powdered samples was placed in a quartz tube, to which 5 mL of 0.02 mol L^{-1} trifluoroacetic acid containing 50 μL of a 30% H_2O_2 solution was added and the suspension was sonicated for 15 min. The tubes were transferred to a Teflon rack of the Ultraclave microwave system which was closed. An argon pressure of $4{\cdot}10^6$ Pa was then applied and the mixture was heated to 95°C over a period of 10 min and this temperature was maintained for 60 min. After cooling to room temperature 1 mL aliquots of the extracts were transferred to vials and centrifuged. The supernatant was used directly for the HPLC-ICP-MS analysis.

According to Sun et al. [58], rice can easily accumulate As and the bioavailability of As released from this food matrix was assessed using an *in vitro* gastrointestinal simulator. Water, monosodium arsenate (NaH_2AsO_4), and sodium arsenite ($NaAsO_2$) were used and the samples were taken from the stomach, intestines, and colon. The analysis was carried out by ICP-MS and speciation was performed by HPLC. Procedures such as rice washing and cooking did not affect the result of the rice speciation although the arsenic content decreased in the range of 7.1 to 20.6%.

Brockman and Brown IV [47] extracted arsenic species from infant rice cereals through a hot water extraction procedure. Deionized water was added to each tube and the samples were maintained at 98°C (using a hot block) for 3 h. After cooling to room temperature the samples were centrifuged for 1 h in a centrifuge filtration system. The resultant filtered extract was diluted 1 : 25 with eluent and 100 μL of 30% hydrogen peroxide to oxidize the arsenite. Rice samples were analyzed for total arsenic by instrumental neutron activation analysis (INAA) and inorganic arsenic by liquid chromatography (LC) with ICP-MS (LC-ICP-MS).

The variability observed among rice grains is another dietary research approach to assessing arsenic exposure. Sommella et al. [48] evaluated the total arsenic and iAs in Italian rice grains and found that they varied by geographic origin and type. Concentrated HNO_3 (2.5 mL) was added to the pulverized samples and the mixture was left overnight. In the next step, H_2O_2 was added and the samples were digested in a microwave oven with a specific program. The samples were then cooled to room temperature and diluted to 50 mL with ultrapure deionized water. In the extraction procedure to investigate the speciation, 10 mL of 1% (w/v) HNO_3 was used and the same microwave temperature program applied to determine the total arsenic was employed. At room temperature, the samples were centrifuged and 900 μL of supernatant was mixed with 100 μL of H_2O_2. The samples were left overnight at 4°C before analysis. Inorganic arsenic was quantified by HPLC coupled to ICP-MS.

Pasias et al. [49] developed three different methods for the determination of total arsenic, total inorganic arsenic, and As(III)-As(V) in rice and rice flour food products. For the determination of total arsenic, 0.5 g of the homogenized rice or rice flour samples was digested in a microwave oven with 5 mL of concentrated HNO_3. For the determination of inorganic arsenic, 0.5 g of sample and 5 mL of 1 mol L^{-1} HNO_3 were vortexed and ultrasonicated for 15 min and then centrifuged at 4000 rpm for 15 min. In the next step 15 mL of 0.1% (w/v) EDTA was added to the mixture which was then vortexed again and centrifuged at 4000 rpm for 15 min. The supernatant was analyzed by ETAAS. Finally, for the determination of As(III) and As(V), 0.5 g of the sample was digested in a microwave oven with 5 mL of 1 mol L^{-1} HNO_3 (the highest temperature reached was only 85°C), and the extract was treated with 5 mL 5% EDTA (w/v). As(III) was determined and As(V) was then determined from the difference between the total inorganic As and As(III).

Phan et al. [50] investigated the potential exposure of Cambodian villagers to arsenic from their daily food consumption. Samples of lowland soils, paddy rice (raw and cooked), fish, and vegetables were collected from the Kandal, Kratie, and Kampong Cham provinces in the Mekong River basin in Cambodia. After acid digestion, extracts were analyzed by ICP-MS. The results revealed that the total concentrations of arsenic in lowland soils and paddy rice were significant. The samples were treated with concentrated HNO_3 (65%), the mixture was heated to 96°C and maintained at this temperature for one hour, and after cooling 5 mL of water was added. The extract was centrifuged and filtered and then the total arsenic was quantified by ICP-MS.

An interesting approach to determining total arsenic and As(V) with detection by electrothermal atomic absorption spectrometry after cloud point extraction (ETAAS/CPE), using ultrasound for the sample preparation, was developed by Costa et al. [51]. This procedure is based on the formation of a complex of As(V) ions with molybdate in the presence of sulfuric acid and extraction into the surfactant-rich phase with Triton X-114. The powdered samples were sonicated for 136 min with a 0.5 mol L^{-1} solution of nitric acid. For the As(V) determination an aliquot of the extract was submitted to the CPE methodology and for the total As determination an aliquot of the extract passed through a prereduction step with 1.0 mL of 8×10^{-8} mol L^{-1} $KMnO_4$ and agitation for 30 min was applied before the CPE methodology.

Shraim [52] evaluated the presence of various metals including arsenic in rice sold in Saudi Arabia. The sample preparation consisted of the microwave digestion of samples that were previously milled using an analytical hand mill. Digestion was carried out applying a specific temperature program and the digestion agents used were concentrated nitric acid, water, and hydrogen peroxide. After the solutions had been digested they were diluted with water and a portion was filtered and analyzed by ICP-MS.

A method for the determination of total arsenic and inorganic arsenic in rice using HPLC-ICP-MS has been developed. Prior to analysis the samples are treated with 0.28 mol L^{-1} HNO_3 and heated in a water bath (95°C) for

90 min. After cooling to room temperature, the extracts were centrifuged at 5000 rpm for 35 min at 18°C, filtered, and analyzed [53].

In order to relate the inorganic arsenic (iAs) content in the rice to the As present in the soil, considering properties such as pH, TOC, available P, and available Fe, rice and soil samples were collected in a region of south China (with a typical red soil for which arsenic contamination has not been reported) and analyzed. The rice grain samples were washed with deionized water, air dried at room temperature, and ground into fine particles. The inorganic As in the grains was determined by liquid chromatography-atomic fluorescence spectrometry (LC-AFS). The results showed that the soil pH and available phosphorus were the main factors influencing the uptake of As by rice grains. The presence of phosphorus suppresses As uptake by rice grains and competition between arsenic and phosphorus for transporters affects the transport from the soil to the roots in the rhizosphere [54].

G. Chen and T. Chen [55] carried out a study focused on iAs in rice samples. The samples were processed to a fine powder using a small mill. A microwave reaction system (rated at 1200 W) was used (95°C for 30 min) with an extraction solution comprised of 0.06 mol L^{-1} HNO_3 and 3% (w/v) H_2O_2. After cooling, the solutions were transferred to centrifuge tubes and centrifuged for 15 min at room temperature. During digestion, As(III) was oxidized to As(V) and silica-based strong anion exchange cartridges were used to separate the As(V) from organic As forms. Inorganic As was quantified by hydride-generation atomic fluorescence spectrometry (HG-AFS) after prereduction by iodide [55].

A simple procedure for the extraction of As species from polished rice samples has been described. For arsenic species determination, sample was ground to a fine powder in a homogenizer and 1 g was mixed with 10 mL of a methanol-water (1 : 1) mixture containing 1% HNO_3 in a centrifuge tube and the mixture was sonicated for 30 min. The extracts were centrifuged and stored at −4°C prior to analysis. As species were previously separated by HPLC and measured by ICP-MS [56]. Total arsenic was extracted in a microwave oven with 9 mL of 70% nitric acid and 1 mL of hydrogen peroxide and after the digestion the extracts were diluted with water. The total content of As was measured by ICP-MS [56].

Extraction methods involve the selective separation of a target species from its matrix (e.g., rice). The methodology employed should ensure quantitative and reproducible extraction without altering the species pattern by decomposition, chemical conversion, or insufficient extraction yield. This is especially important due to the complexity of rice sample matrices. In this context, conventional extraction, also known as heating by hot plate, is one of the traditional methods most widely used for sample treatment. Based on this sample preparation strategy, several approaches have been used for arsenic extraction from acidic samples [44, 47, 50]. The concentrated acids are the most used extractant. Water is the extractant most commonly recommended for the more polar or ionic species of arsenic, but few studies are reported in the literature regarding the extraction of these species from water due to the low yields obtained.

In contrast with conventional extraction methods, for example, liquid-liquid extraction (LLE), which are characterized by the use of high volumes of solvents and long extraction times [56], the use of microwave energy results in a significant reduction in the extraction time, because the microwaves accelerate the heating rate [45, 46, 48, 49, 52, 54, 55]. Other advantages of microwave-assisted extraction are high recoveries, good reproducibility, and minimal sample manipulation. The most critical parameters for method optimization using microwave extraction procedures are the extraction medium, applied microwave power, and extraction time [34]. Most of microwave extraction procedures involve the use of nitric acid [46, 59–61]. Nitric acid is a strong oxidizing agent; however, its use in digestion procedures constitutes an important source of interference due to the formation of nitrogen oxides.

Many factors are important in the process of sample preparation, such as the physical state of the sample, the analyte that is determined, and the type of detection. In general, the analysis of solid samples such as rice is more difficult since most detection methods require that the analytes of interest are transferred to a liquid phase. In this context the ultrasound-assisted extraction of the analyte emerged as an efficient alternative which requires a short time and the use of acids in low concentration and operation conditions involving atmospheric pressure and room temperature [51, 62, 63]. The use of ultrasound involves the solid-liquid extraction. Sonication by ultrasound occurs in the acoustic cavitation process resulting in points with extremely high temperature and pressure gradients. This phenomenon occurs near the particle surface or in the surface itself and improves the analyte solubility and diffusivity of solvent inside the solid particles. The chemical effect of ultrasound improves the reactivity of some chemicals, allowing the occurrence and also acceleration of some reactions involved in sample digestion.

3. Arsenic Speciation

Rice is a crop plant which can absorb more arsenic than other cereals, such as barley and wheat, as shown in a study by Meharg and Rahman [64]. Thus, it is important to monitor the contamination of rice with arsenic and in order to estimate the risk to human health variations in the toxicity, mobility and bioavailability, factors which are strongly dependent on the chemical form in which the arsenic is present, must be taken into account.

For the effective diagnosis of the degree of toxicity through contamination by inorganic species of arsenic in rice, it is necessary to develop analytical methodologies to aid the differentiation of these forms. Separation and detection techniques can be used to study the chemical speciation; however, the majority of these techniques are limited because they do not tend to combine an efficient separation capacity with the sensitivity and selectivity required for detection. Hyphenated chromatographic methods, such as HPLC-ICP-MS, have become the preferred and most commonly used approach for this purpose. The main advantages are the high

Table 2: Speciation of arsenic in rice using HPLC-ICP-MS.

Arsenic species	Sample preparation	Figures of merit	Reference
As(III), As(V), DMA, MMA, AsC, and AsB	Microwave digestion with 1% (v/v) HNO_3	LOD: 0.1–0.3 μg/Kg LOQ: 0.5–1.5 μg/Kg Recovery: 91.4–114.3%	[59]
Total As, As(III), As(V), DMA, MMA, and AsB	Pressurized liquid extraction sonication	Recovery: 71.8–104.5%	[62]
As(III), As(V), DMA, and MMA	Microwave digestion with 10 mL of 2% (v/v) HNO_3	LOD: 0.03 μg/L for total As	[60]
As(III), As(V), MMA, and DMA	Sonication by ultrasound probe	LOD: 0.05–0.2 μg/Kg Recovery: 82–99%	[63]
As(III), As(V), MMA, and DMA	Extraction with 1% HNO_3	LOD: 0.01–0.07 μg/L Recovery: 95–100%	[61]
MMA, DMA, As(III), and As(V)	Digestion of the samples using pressurized microwave system	LOD: 0.5–1.0 μg/Kg Recovery: 94–98%	[46]

LOD: limit of detection; LOQ: limit of quantification.

sensitivity, multielement capacity, wide linear range, and possibility for isotope determination.

Studies reported in the literature in which arsenic species were determined mainly by HPLC-ICP-MS are listed in Table 2.

Kim et al. [59] determined As(III), As(V), DMA, MMA, AsB, and AsC in rice grains in samples grown in Korea and USA using HPLC-ICP-MS. As(III) was the species predominantly found in the samples. The results indicated high toxic effect and need for further attention.

Sanz et al. [62] held arsenic speciation analysis in samples of rice, straw, soil, hair, and nail in regions affected by arsenic contamination in the eastern and western plains of the Ganga down and Bangladesh. Arsenic species (As(III), As(V), DMA, MMA, and AsB) were determined by HPLC-ICP-MS. For the samples of rice, the content of inorganic arsenic corresponds to 70–98% of the total arsenic content (up to 636.7 mg/kg in the samples). The authors indicate that the speciation analysis reveals itself as a powerful tool for full analytical assessment in epidemiological studies.

Maher et al. [60] determined As(III), As(V), DMA, and MMA in rice varieties from Australia. Total arsenic was determined by electrothermal atomic absorption spectrometry (ETAAS) after extraction with concentrated nitric acid. Inorganic arsenic and methylated species were determined by HPLC-ICP-MS. The method was validated by comparing the results with X-ray absorption near edge spectroscopy (XANES). The determination by XANES allows direct analysis, dispensing steps prior extraction and preventing problems from changes in arsenic species [60].

Sanz et al. [63] studied a procedure for the extraction of As(III), As(V), MMA, and DMA in rice samples from Spain and India. The speciation was also performed by HPLC-ICP-MS. The total arsenic was determined by ICP-MS after digestion of the rice samples with hydrogen peroxide and concentrated nitric acid assisted by microwave. For speciation analysis, the extraction of arsenic species occurred through the enzymatic action of α-amylase and ultrasonic probe sonication in a short period of time (3 min). The extraction procedure has shown more efficiency than conventional methods, avoiding the use of highly dangerous organic solvents. As(III) was predominantly the arsenic form found in rice samples analyzed. These results show that rice is a bioaccumulative plant for the more toxic form of arsenic.

Sun et al. [61] performed an arsenic speciation study on products derived from rice, such as cereals commonly eaten at breakfast, rice crackers, and condiments used to prepare Japanese rice. Arsenic species (As(III), As(V), MMA, and DMA) were extracted from samples with 1% (v/v) HNO_3 and quantified by HPLC-ICP-MS. The inorganic forms were prevalent (75.2 to 90.1%). The study provided useful information which leads to a better understanding of the distribution of arsenic species in rice products. These are important considerations in the formulation of new rice-based foods.

Hyphenated chromatographic methods contribute significantly to enhancing the study of arsenic speciation, but they are still costly and thus the development of nonchromatographic methods is a more accessible and promising approach. A major challenge in speciation analysis is to maintain the integrity of the chemical species of interest from the sampling to the detection stages. In nonchromatographic methods, selective extraction procedures using a small volume of extractor are often required, based on the analyte partitioning into a phase with compatible polarity. This type of extraction procedure has the advantage of preconcentration and the minimization of matrix effects. However, if the partitioning is not quantitative, low recoveries can be observed.

Nonchromatographic methods offer several advantages over the chromatographic techniques and represent a fast and inexpensive option for application in laboratories, particularly when applied to food and environmental samples. However, many of these methods provide limited information on the samples since most approaches have centered on a single element or a specific type of chemical form (i.e., free ions or organic compounds).

In addition to the challenge of preventing interconversion of species during the extraction steps, most procedures

Table 3: Nonchromatographic methods for speciation of arsenic in rice.

Arsenic species	Sample preparation	Detection	Figures of merit	Reference
As(III) and As(V)	Assisted digestion microwave with 0.14 mol/L HNO_3	HG-AAS	LOD: 1.96–3.85 ng/g	[65]
As(III) and As(V)	Vortex with 25 mL of solution 0.05 mol/L $(NH_4)_2CO_3$	HG-AFS	LOD: 1.3–4.4 ng/g Recovery: 94-95%	[55]
As(III) and As(V)	Cloud point extraction	UV-Vis	LOD: 1.14 μg/L Enrichment factor: 65 Recovery: 95–102%	[66]
Total As and As(V)	Sonication with 0.5 mol/L HNO_3	ETAAS	LOD: 10–33 ng/L Enrichment factor: 78.3 Recovery: 90.8–113.1%	[51]
Inorganic As, MMA, DMA, and AsB	0.01 mol/L TMAH (tetramethylammonium hydroxide)	ETAAS	LOD: 15–50 ng/g	[67]
Total As, As(III), and As(V)	Assisted digestion microwave with 1.0 mol/L HNO_3	ETAAS	LOD: 19–30 μg/Kg LOQ: 57–90.3 μg/Kg Recovery: 92–105%	[49]

LOD: limit of detection; LOQ: limit of quantification.

end up being restricted to inorganic species, and there are few studies using nonchromatographic procedures for determination of organic arsenic species. This challenge makes nonchromatographic speciation procedures more promising, opening an opportunity for research aimed at improving this focus. Table 3 listed some studies reported in the literature using nonchromatographic procedures for arsenic speciation in rice samples.

Speciation is relatively easy to investigate when a property of a particular compound can be measured directly in the sample without interference from the other matrix components. Direct speciation analysis is generally considered to be less challenging although this is not always the case.

Studies on direct and *in situ* analysis are increasing due to interest in simplifying the process of rapid substance identification for monitoring and quality control in the food industry. There are very few methods available for the determination of the speciation in solid food samples through direct analysis. The use of mass spectrometry (MS), with only a few sample preparation steps, allows the speciation of organic arsenic to be determined, with limited analyte loss. In contrast, matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI), and desorption electrospray ionization (DESI), despite being associated with greater losses, have greater tolerance in the presence of impurities, proving to be valuable for metal complexes [68].

Lin et al. [69] demonstrated that DESI-MS can be used as desorption/ionization technique for the determination of arsenic speciation (inorganic arsenic, monomethyl arsenic acid, dimethylarsinic acid, and arsenobetaine) in biological samples under environmental conditions. The results were effective in the identification of inorganic and organic arsenic compounds. Furthermore, the DESI method does not require sample preparation and its use has proved to be promising for *in situ* speciation studies [69].

Finally, more recently, the development of speciation techniques has been highly focused on physical methods, such as X-ray diffraction (XRD), X-ray powder diffraction (XPD), and X-ray absorption spectroscopy (XAS) [70, 71]. However, it is clear that the cost of the instrumentation and the attainable sensitivity are not sufficient for routine speciation analysis of food samples [72]. For speciation, X-ray absorption near edge structure (XANES) spectroscopy is a powerful tool, because it is a direct method by which we can avoid the change of the chemical state of target elements. XANES is a nondestructive technique for identifying inorganic and organic arsenic species in complex environmental samples [73].

Manning described a procedure to evaluate the solid phase oxidation state and mineral surface binding sites in three agricultural soil samples by fitting linear combinations of XANES spectra derived from several synthetic and well characterized As(III)- and As(V)-treated model compounds. The data showed that As(III) is either partially or completely oxidized to As(V) when reacting with soil [74].

There is a trend toward reducing the sample preparation steps in order to limit the interconversion of species, providing more accurate results on the quantity of each species in the sample. However, based on this review it is clear that there are still considerable difficulties associated with reducing the number of sample preparation steps involved in speciation studies. Thus, in this regard, many approaches are focused on total arsenic without considering the specific chemical forms (free ions or organic compounds) and the information provided on the samples is consequently very limited. It is expected that procedures for direct speciation studies or a reduction in the number of preparation steps will be developed in the near future, based on the use of MALDI, ESI, DESI-MS, and X-ray, with chromatographic or nonchromatographic detection techniques. This approach could provide the best aspects of screening methods, particularly in relation to reduced handling, and involves the direct characterization of chemical forms, contributing to improving our understanding of the behavior of the different chemical forms of arsenic in food samples such as rice.

4. Conclusions

High levels of arsenic in rice grains are a potential concern in relation to human health. More information on arsenic speciation in rice and rice-based products for consumption is needed in order to carry out risk assessment studies on inorganic arsenic. The largest gap in our knowledge with regard to assessing inorganic arsenic consumption rates from rice is related to the levels of arsenic species in rice and rice products and appropriate analytical methods for speciation of arsenic ensure the integrity of the species.

A comprehensive risk assessment must be based on information on the dietary status and consumption of rice, along with the calculation of the daily intake of arsenic from the various routes of human exposure. The most commonly reported concern is arsenic entering the food chain, affecting food safety. Management options for health risk prevention and agricultural sustainability should therefore focus on minimizing As inputs to soils and limiting human exposure.

This review is not exhaustive, but it highlights some of the important and unique aspects related to the presence of arsenic in rice, addressed within the context of human nutrition. Due to the increasing consumption of rice, the available analytical methods need to be able to provide more detailed information on the chemical species present, overcoming the barrier created by the complexity of food matrices and the low concentrations of the analyte present. Unfortunately, the procedures for arsenic speciation studies are not yet suitable for routine analysis and clearly the development of such methods offers a great challenge for analysts around the world. Thus, the search for simple strategies suitable for obtaining quantitative information regarding arsenic species should be encouraged. However, these strategies require an interdisciplinary approach in order to cover the various aspects involved and represent a considerable challenge in the areas of toxicology and analytical chemistry.

Abbreviations

As: Arsenic
AsB: Arsenobetaine
AsC: Arsenocholine
As(V): Arsenate
As(III): Arsenite
CE: Capillary electrophoresis
CPE: Cloud point extraction
CRM: Certified reference material
DMA: Dimethylarsinic acid
DESI-MS: Desorption electrospray ionization-mass spectrometry
EDTA: Ethylenediaminetetraacetic acid
EFSA: European Food Safety Authority
ESI-MS: Electrospray-mass spectrometry
ETAAS: Electrothermal atomic absorption spectrometry
EU: European Union
GC: Gas chromatography
HG: Hydride-generation
HG-AFS: Hydride-generation atomic fluorescence spectrometry
HPLC: High performance liquid chromatography
iAs: Inorganic arsenic
INAA: Instrumental neutron activation analysis
ICP-MS: Inductively coupled plasma-mass spectrometer
JECFA: Food and Agriculture Organization/World Health Organization
LC: Liquid chromatography
LC-AFS: Liquid chromatography-atomic fluorescence spectrometry
LC-ICP-MS: Liquid chromatography-inductively coupled plasma-mass spectrometry
MMA: Monomethylarsonic acid
MS: Mass spectrometry
MALDI: Matrix-assisted laser desorption/ionization
USA: United States of America
XRD: X-ray diffraction
XPD: X-ray powder diffraction
XAS: X-ray absorption spectroscopy
WHO: World Health Organization.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

The authors are grateful for financial support from the Brazilian governmental agencies Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), the MG state government agency Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), and the GO state government agency Fundação de Amparo á Pesquisa do Estado de Goiás (FAPEG).

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Hindawi Publishing Corporation
Journal of Chemistry
Volume 2016, Article ID 6898715, 8 pages
http://dx.doi.org/10.1155/2016/6898715

Research Article

Structural Changes in Rice Bran Protein upon Different Extrusion Temperatures: A Raman Spectroscopy Study

Linyi Zhou,[1] Yong Yang,[1,2,3] Haibin Ren,[1] Yan Zhao,[1] Zhongjiang Wang,[1] Fei Wu,[1] and Zhigang Xiao[1,4]

[1]*College of Food Science, Northeast Agricultural University, Harbin, Heilongjiang 150030, China*
[2]*Key Laboratory of Processing Agricultural Products of Heilongjiang Province, College of Food and Bioengineering, Qiqihar University, Qiqihar, Heilongjiang 161006, China*
[3]*College of Food and Biological Engineering, Qiqihar University, Qiqihar, Heilongjiang 161006, China*
[4]*College of Grain Science and Technology, Shenyang Normal University, Shenyang, Liaoning 110034, China*

Correspondence should be addressed to Fei Wu; wufeiname@163.com and Zhigang Xiao; zhigangx@sina.com

Received 12 August 2015; Revised 23 October 2015; Accepted 12 November 2015

Academic Editor: Claudia Ruiz-Capillas

Raman spectroscopy is critically evaluated to establish the limits to which it may be used to detect changes in protein conformation upon extrusion. Rice bran protein (RBP) extruded with different temperatures (100, 120, 140, and 160°C, labeled as ERBP-) was considered. DSC showed that extrusion at 100°C increased T_D of RBP but decreased its ΔH, while, after extrusion treatment at 120°C, RBP completely denatured. A progressive increase in unordered structure and a general decrease in α-helix structure and β-sheet structure of extruded RBP were observed from Raman study. Meanwhile the content of unordered structure increased up to 140°C and then decreased at 160°C, while the trend of α-helix and β-sheet content was opposite, which was contributed to the composite effect of formation of some more protein aggregation and protein denaturation. Extrusion generally induced a significant decrease in Trp band near 760 cm^{-1} but an increase at 160°C. No significant difference was observed in Tyr doublet ratios between controlled RBP samples and extruded RBP below 160°C, whereas Tyr doublet ratios of extruded RBP decreased at 160°C. Intensity of the band assigned to CH_n bending decreased progressively and then increased as extrusion temperature increased, indicating changes in microenvironment and polarity.

1. Introduction

Rice bran, a major coproduct in the rice milling industry, usually contains about 11.3–14.9% protein, 34.0–62.0% carbohydrates (mainly starch), and 15.0–19.7% oil. In spite of being an excellent nutrient source, raw rice bran is not suitable for human consumption due to the rancidity problem caused mainly by lipases. When bran layers are removed from the endosperm during the milling process, the individual cells are disrupted and lipase enzymes come into contact with fat, causing hydrolysis of fat to free fatty acids (FFA) and glycerol [1]. However, stabilization, which is an enzyme inactivation process that extends the shelf life of rice bran, enables incorporation of rice bran back into our diet [2]. Extrusion stabilization is one of the most effect stabilization technologies. Most recent research on extrusion stabilization focuses on the quality and extraction rate of rice bran oil, but the study on structural and functional properties of extruded rice bran protein is rare. It has been suggested that, during extrusion, the combined effect of high temperature, high pressure, and shear forces in the extruder leads soy proteins to form laminated structures, which were easily accessible to enzymes [3]. Product characteristics of extrudes can vary considerably depending on the extrusion processing conditions such as barrel temperature, die geometry, extruder type, feed composition, feed moisture content, feed particle size, feed rate, screw configuration, and screw speed [4]. Moreover, extrusion temperature is one of the most important factors which may affect the quality of extruder. Qi and Onwulata [5] suggested certain functional properties of the extrudates

may be controlled by varying the extrusion temperature. Onwulata et al. [6] reported gel strength of extruded WPI at 35 or 50°C increased but almost was lost at 75 or 100°C. Thus, the effect of extrusion temperature on structure of rice bran protein was studied in this research.

Differential scanning calorimetry (DSC) has gained remarkable popularity in thermal studies of foods and their components following the development of instrumentation of sufficient sensitivity. DSC studies were often used to provide a better insight into the order-disorder transition processes of granular starch and other gelling polysaccharides. DSC also can be used to characterize mixtures of polymorphic forms of fats as well as to evaluate hydrogenation and various tempering regimes for their effectiveness in bringing about desired polymorphic changes. It has been employed to examine the physical state and properties of water in foodstuffs [7]. Above all, it is an excellent method for obtaining thermodynamic data on the unfolding of globular proteins and can provide unique information on the presence and characteristics of stable intermediates [8]. DSC is rapid, facile, and capable of supplying both thermodynamic (heat capacity, enthalpy, and entropy) and kinetic data (reaction rate and activation energy) on protein denaturation. With regard to proteins, denaturation curves result from thermal changes associated with the breakdown of those bonds involved in stabilizing protein structure (e.g., hydrogen bonds, hydrophobic interactions, and electrostatic interactions,) and, in some cases, an additional aggregation process [9].

Various techniques are currently available for obtaining the structural information of proteins, including X-ray diffraction, nuclear magnetic resonance (NMR) spectroscopy, and Fourier transform infrared spectra (FTIR). These methods have limitations. IR spectroscopic techniques could not be applied in solution to provide direct information about the structural changes. X-ray diffraction requires the preparation of perfect single crystal, which can be time-consuming or even impossible. NMR spectroscopy is not easily applied to proteins larger than a few hundred residues [10]. Moreover, studies on protein structure carried out in solution do not necessarily reflect what happens with proteins in the solid state [11]. Raman spectroscopy is a more suitable and direct technique that overcomes most of the above objections and can be used for solid samples and aqueous solutions due to weak background scattering from water, providing information on the peptide backbone structure, the environment of some side chains such as those of tyrosine and tryptophan (hydrophobic groups), and the local conformations of disulphide bonds and methionine residues [12]. In this respect, the usefulness of the Raman spectroscopy for the study of protein structural changes in food in situ during processing and storage has been shown, together with the possibilities of using protein structural changes, to predict protein functional properties and sensory attributes [12]. Raman spectroscopy was therefore used in the present research with the aim of studying the structural changes of rice bran protein (RBP) prepared under different extrusion temperatures. In general, the aim of this research was studying the protein denaturation and structural changes occurred in the rice bran protein subjected to the different extrusion temperatures using DSC and Raman spectroscopy.

2. Materials and Methods

2.1. Raw Materials. Deoiling rice bran was obtained from Orient Group (Harbin, China). All other chemicals were analytical grade products obtained commercially.

2.2. Preparation of Rice Bran Protein (RBP). Deoiled rice bran was ground to pass a 60-mesh screen and 10 g was dispersed in 100 mL distilled water. The protein was solubilized by adjusting the pH to 9.5 with 0.1 M NaOH and shaking at 300 rpm for 2.0 h at 50°C. The mainly nonprotein residue was removed by centrifugation at 3000 ×g for 20 min. The protein in the supernatant was precipitated by adjusting pH to 3.8 with 0.1 M HCl and centrifuged at 3000 ×g for 20 min. The precipitated protein was centrifuged and washed with distilled water two times, and the pH was brought up to 7.0 before freeze-drying. This protein was called RBP. The protein content of RBP was 89.59%.

2.3. Extrusion Treatment of Rice Bran. Deoiled rice bran was subjected to extrusion in a laboratory-scale twin-screw extruder (SPJ-40, Deai Co., Ltd, Shanxi, China) with three individual barrel sections, each with separate temperature control. The temperature of these three barrels was set at 50°C (feed section), 80°C, and 100~160°C, respectively. The rice bran blends were extruded at four different die-exit temperatures: 100, 120, 140, or 160°C. The diameter of the screw was 75 mm, and the length-to-diameter ratio was 28 : 1. The screw elements included kneading blocks and reverse screw elements. Screw speed was operated at 220 rpm. The die was designed with two circular holes at 5 mm diameter.

The moisture content of rice bran was adjusted to 20% specified for the extruder. Moisturized raw material was introduced to the extruder at a rate of 0.2 kg/min. The obtained extrudes of rice bran were ground to pass a 0.2 mm screen and then oven-dried at 50°C to reach a final moisture content of 5.1% (w/w). Ground extruded rice bran was stored in air-tight glass containers. Rice bran protein was prepared as the above-mentioned method and labeled as ERBP-100, ERBP-120, ERBP-140, or ERBP-160, respectively, corresponding to different extrusion temperatures.

2.4. Differential Scanning Calorimetry (DSC). DSC were recorded on a 2920 modulated DSC (TA Instrument, New Castle, DE) with heating rate of 5°C/min and temperature range of 25–120°C. The instrument was calibrated for temperature and enthalpy measurement with indium. Each hermetic aluminum pan was filled with 60 mg of 8% (w/w) rice bran protein dispersions in distilled water and sealed. An empty pan was used as reference. The enthalpy of denaturation (ΔH) and the temperature of denaturation (T_D) were calculated by using the DSC software after manually setting the start and the end points of the endothermic peak.

2.5. Raman Spectroscopic Analysis. The RBPs were dispersed in pH 7.0 phosphate buffers to make 100 mg/mL solutions

for Raman experimentation. Raman spectra were recorded on a Perkin Elmer Raman Station 400F Dispersive Raman Spectrometer equipped with a 785 nm diode laser which was used depending on the fluorescence contribution of the sample. The laser was focused on the samples which were placed on microscope slides. Each spectrum was obtained under the following conditions: 80 mW of laser power; 4 scans; 60 s exposure time; 2 cm^{-1} resolution; and the range of Raman spectra measured was 400–2000 cm^{-1}. Each sample was scanned at least three times, and the Raman spectra of each sample were plotted after calculating the mean. Errors in band position were less than ±3 cm^{-1}.

Spectral data from the scans of samples were smoothed, baseline-corrected, and normalized against the phenylalanine band at 1003 ± 1 cm^{-1} using the Grams 32 Software (Galactic Industries Corporation, Salem, NH, USA). The Phe band located near 1003 cm^{-1} was used as internal standard to normalize the spectra, as it has been reported to be insensitive to the microenvironment [12]. Assignment of the visible bands to vibrational modes of peptide backbone or amino acid side chains was carried out by comparing Raman spectra of model polypeptides or monographs of Raman spectra of proteins with those of in the references [13]. Quantitative estimation of secondary structure of RBP under specific conditions was performed using the Peakfit 4.12 software (Seasolve Software, Framingham, MA). Raman spectra (400–2000 cm^{-1}) were plotted as relative intensity (arbitrary units) against Raman shift in wavenumber (cm^{-1}). Raman spectra of each sample were collected in triplicate and the results were reported as the averages of these replicates.

2.6. Statistical Analysis. All experiments were performed in triplicate. Statistical analysis was performed using Statistical Analysis System (SAS 8.12, SAS Institute Inc., Cary, NC). Analysis of variance (one-way ANOVA) was employed taking relative intensity of each band as variables to determine the significance of different extrusion temperatures. The normal distribution of samples was checked using the Shapiro-Wilks test. The Kruskal-Wallis test was used to test samples that did not fit the normal distribution. Significant differences ($P < 0.05$) between means were identified using Duncan's multiple range test.

3. Results and Discussion

3.1. Thermal Characteristics of RBP Determined by DSC. Differential scanning calorimetry (DSC) can reveal structural and conformational changes of proteins. The denaturation temperatures (T_D, peak of the denaturation curve) and ΔH (enthalpy of the denaturation) both can be determined from the thermograms. Denaturation temperatures indicate protein thermostability, while ΔH is an indication of hydrophobic/hydrophilic interactions and compactness of the proteins.

RBP measured by DSC had a denaturation temperature of 79.9°C with endotherm of 1.70 J/g, which was consistent with pervious works. Pervious research reported that RBPI had denaturation temperature of 83.4°C with low endotherm (0.96 J/g of protein) [14]. Tang et al. [15] reported that denaturation temperatures of freeze-dried and spray-dried rice bran proteins were 84.1 and 84.6°C and enthalpies of them were 2.5 and 2.37 J/g, respectively.

Table 1: Differential scanning calorimetric (DSC) characteristics of rice bran protein (RBP) and extruded rice bran protein (ERBP) at different extrusion temperatures.

Sample	T_D (°C)	ΔH^{***} (J/g)
RBP*	79.9 ± 0.1[a**]	1.70 ± 0.10[b]
ERBP-100	82.3 ± 0.3[b]	1.54 ± 0.08[a]
ERBP-120	—	—
ERBP-140	—	—
ERBP-160	—	—

*RBP: rice bran protein; ERBP-100: extruded rice bran protein at 100°C temperature; ERBP-120: extruded rice bran protein at 120°C temperature; ERBP-140: extruded rice bran protein at 140°C temperature; ERBP-160: extruded rice bran protein at 160°C temperature.
**Different superscript letters in the same column indicate significant differences ($P < 0.05$).
***T_D means peak of the denaturation curve and ΔH means enthalpy of the denaturation.

It was observed from Table 1 that T_D of RBP increased from 79.9 to 82.3°C with an extrusion treatment at 100°C and ΔH was decreased from 1.70 to 1.54 J/g. Those observed results suggest that extrusion may result in a part denaturation of rice bran protein and partial exposure of hydrophobic cores initially buried in the interior; as a result the partially dissociated RBPs are refolded to form more stable aggregates with higher T_D, while, after extrusion treatment at 120°C, the endothermic peak of RBP disappeared, indicating complete denaturation of RBP.

3.2. Raman Spectroscopic Analysis. The Raman spectra of the controlled RBP and extrusion pretreated RBP at different temperatures are shown in Figure 1(a), respectively, and data for selected bands are shown in Table 2. Figure 1(b) showed the Raman band attributed to Trp and Tyr residues of RBP to give a clearly visible spectrum of these bands. The assignments of some major bands (Table 1) were made base on previous works [10]. The frequency and intensity changes in the Raman bands were mainly indicative of changes in the secondary structure and variations in local environments of RBP.

3.3. Amide Conformations Region. The conformations of RBP were mainly determined by the Raman characteristic bands of amide I band and amide III band. The Raman characteristic bands of amide I band were located as follows: α-helix, 1645–1660 cm^{-1}; β-sheet, 1665–1680 cm^{-1}; β-turn, 1680–1690 cm^{-1}; random coil, 1660–1670 cm^{-1} [10]. The Raman characteristic bands of amide III band were located as follows: α-helix, 1265–1300 cm^{-1}; β-sheet, 1230–1240 cm^{-1}; β-turn, 1305 cm^{-1}; random coil, 1240–1260 cm^{-1} [16]. The quantitative calculation of the secondary structures of the RBP Raman spectra was performed using the amide I band with Peakfit 4.12 software. Amide III bands were not used for quantifying because vibrational spectroscopy of proteins

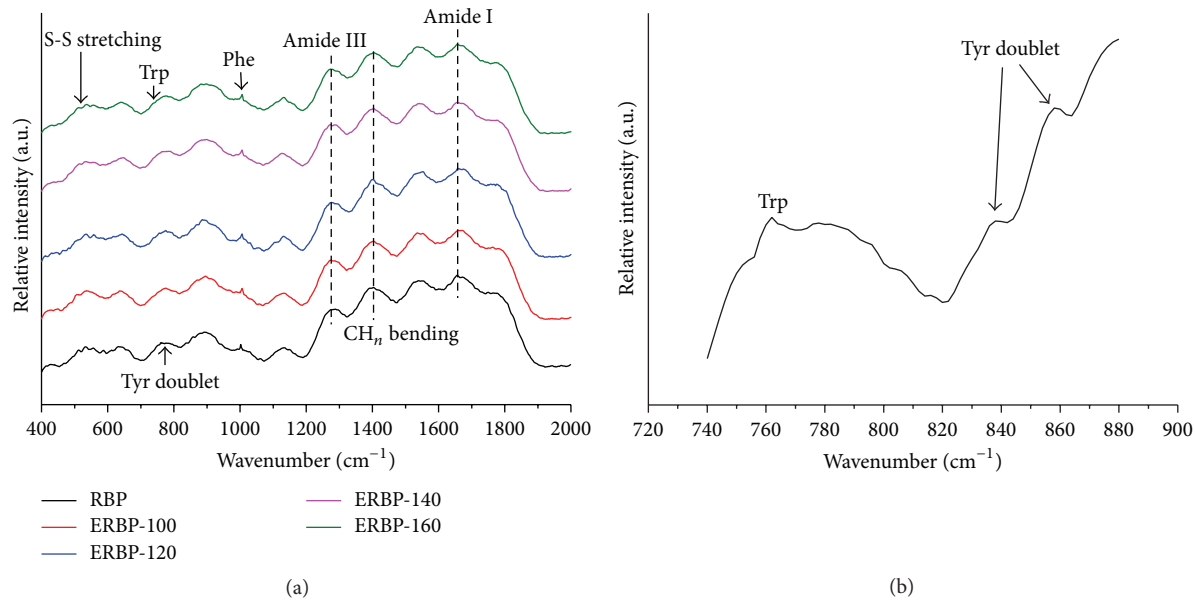

Figure 1: Raman spectrum of rice bran protein (RBP) and extruded rice bran protein (ERBP) at 100°C, 120°C, 140°C, and 160°C (a) and detailed spectrum of rice bran protein in 740–880 cm^{-1} region which include Trp band and Tyr doublet (b).

Table 2: Tentative assignment of some bands in the Raman spectrum of rice bran protein (RBP).

Frequency (cm^{-1})	Assignment
514	*νS-S *gauche-gauche-gauche* conformation
530	νS-S *gauche-gauche-trans* conformation
547	νS-S *trans-gauche-trans* conformation
620–640	Phenylalanine (Phe)**
644	Tyrosine (Tyr)
760	Tryptophan (Trp)
830	Tyr ν-ring
850	Tyr ν-ring
940	νC-C (α-helix)
1003	Phe ν-ring
1250	Amide III bands (β-sheet, random coil)
1273	Amide III bands (α-helix)
1309	Amide III bands (α-helix)
1321	Trp ν-ring
1340	δCH
1360	Trp ν-ring
1450	$\delta_{vs}CH_3$, δCH_2, δCH
1645–1690	Amide I bands

*ν, stretching vibrations; δ, bending vibrations; vs, very strong.
**Phe, phenylalanine; Tyr, tyrosine; Trp, tryptophan; α-helix, β-sheet, β-turn, and unordered structure are secondary structure elements of protein.

produces a complex pattern of bands in this range (located in the 1200–1300 cm^{-1}) [16].

Raman bands corresponding to amide I and amide III can be used to characterize protein backbone conformation. Amide I band region is mainly due to the stretching vibrations of the carbonyl groups and is representative of the secondary structure of the proteins [17]. These absorption bands allow the assignment of secondary structure of proteins. The strong Raman band centered at 1665–1675 cm^{-1} has been assigned unambiguously to the amide I vibrational mode, which involves mainly C=O stretching vibrations and partly N-H in-plane bending of peptide groups [12]. The strongest Raman band at 1665–1670 cm^{-1} demonstrated the predominance of β-sheet in RBP. The spectral profile of the amide I band is used for quantifying the secondary structure of proteins [18]. Table 3 lists the secondary structure percentages from samples analyzed. Results showed that nontreated RBP contained 17.43% α-helix, 41.14% β-sheet, 19.67% turns, and 19.76% unordered structures (Table 3). Adebiyi et al. [19] reported the secondary structure of rice bran globulin may be summarized to be an unordered, random coil, and antiparallel chain of intramolecular β-sheet structure, while rice bran albumin may be composed of α-helix conformation and ordered structure with intermolecular β-sheet. The high content of β-sheet structure of RBP in this study may be attributed to the high percentage of rice bran albumin and globulin. Ma et al. [20] suggested the overall secondary structures of RBPF were very similar to those of plant proteins having low α-helix, large contents of β-sheet.

The estimated secondary structure results from Table 3 showed a progressive increase in unordered structure and a general decrease in α-helix structure and β-sheet structure of extruded RBP in comparison with controlled RBP sample. Qi and Onwulata [5] reported that extrusion at or above 75°C leads to a uniform densely packed polymeric product with no secondary structural elements (mostly α-helix) remaining. Qi and Onwulata [21] reported that protein denaturation may decrease its β-sheet content.

Table 3: Percentages of protein secondary structure: α-helix, β-sheet, β-turn and unordered structure of rice bran protein (RBP) and extruded rice bran protein (ERBP) at different extrusion temperature.

Sample	Percentage of secondary structure elements (%)			
	α-helix structure	β-sheet structure	β-turn structure	Unordered structure***
RBP*	17.43 ± 0.01c**	41.14 ± 0.04e	19.67 ± 0.02c	19.76 ± 0.01a
ERBP-100	17.83 ± 0.02d	38.00 ± 0.03d	19.67 ± 0.04c	23.50 ± 0.02b
ERBP-120	17.08 ± 0.03b	34.51 ± 0.02b	19.33 ± 0.03a	29.08 ± 0.01d
ERBP-140	17.01 ± 0.02a	31.83 ± 0.04a	19.32 ± 0.00a	31.83 ± 0.03e
ERBP-160	18.31 ± 0.01e	36.31 ± 0.02c	19.59 ± 0.01b	25.78 ± 0.03c

*RBP: rice bran protein; ERBP-100: extruded rice bran protein at 100°C temperature; ERBP-120: extruded rice bran protein at 120°C temperature; ERBP-140: extruded rice bran protein at 140°C temperature; ERBP-160: extruded rice bran protein at 160°C temperature.
**Different superscript letters in the same column indicate significant differences ($P < 0.05$).
***α-helix, β-sheet, β-turn, and unordered structure are secondary structure elements of protein.

It was observed that the content of unordered structure increased up to 140°C and then decreased at 160°C, while the trend of α-helix and β-sheet content was opposite. Heat and shear alter the conformation of proteins through partial denaturation of the protein molecules, exposing groups that are normally concealed in the folded native protein, resulting in an increase of unordered structure [22]. Those observed Raman results were consistent with DSC study which suggest that extrusion may result in a denaturation of rice bran protein and partial exposure of hydrophobic cores initially buried in the interior. Proteins texturized at the higher temperature showed an increase in surface hydrophobicity and unfolding of protein structure [22], which may result in a progressively increase in unordered structure. Qi and Onwulata [5] indicated that extrusion results in a loss of secondary structure of around 15%, total loss of globular structure at 78°C, and conversion to a random coil at 100°C.

The decreased unordered structure and increased β-sheet structure at 160°C extrusion temperature may be related to the formation of more or new protein aggregation. Chen et al. [23] reported that increasing moisture content from 28% to 60% or decreasing cooking temperature from 160 to 140°C all could reduce the degree of aggregation in extruded soybean protein. Choi and Ma pointed that protein aggregation may increase its antiparallel β-sheet conformation. Lee et al. [24] also reported that β-sheets played a role in the aggregate and network formation. As previously reported by Simmons et al. [25], heating at different temperatures and in the presence of shear resulted in two types of aggregates. At lower temperatures, small and weakly bonded aggregates were formed, due to weak van der Waals bonding, while rigid and dense aggregates were observed after heating at higher temperatures. In the present study, it could be assumed that the protein aggregates formed in ERBP-160 are rigid-textured aggregates, which may result in a decrease in unordered structure and an increase in β-sheet structure. However, the correctness of this speculation still needs to be further confirmed.

3.4. Local Environments of Proteins

3.4.1. Tryptophan Residues. Tryptophan (Trp) residues show several characteristic Raman bands, some of which are useful to monitor the polarity of the microenvironment, or involvement in hydrogen bonding. Li-Chan [26] reported that tryptophan residues from a buried, hydrophobic microenvironment become exposed to the polar aqueous solvent; there may be a decrease in the intensity of a band near 760 cm^{-1} region. Pervious works often reported a decrease in intensity of Trp band of denatured proteins due to more exposed Trp residues caused by destruction and unfolding of protein structure [27].

However, in this study, extrusion induced a significant decrease in the stretching vibration of the tryptophan residues ring, which indicated that tryptophan residues tended to an exposed, hydrophilic microenvironment. Taylor et al. [28] reported that extrusion process frequently results in realignment of disulfide bonds and breakage of intramolecular bonds, while disulfide bonds stabilize the tertiary structure of protein and may limit protein unfolding during extrusion. The increase in normalized intensity of Trp band of RBP extruded at 160°C may contribute to the formation of aggregation or new more ordered structure [29]. These observed results are consistent with changes found in secondary structure analysis and DSC analysis, while the decreased ΔH of ERBP in DSC confirmed those observed results.

3.4.2. Tyrosine Doublet Ratio. The tyrosyl (Tyr) doublet ratio (I_{850}/I_{830}) can be useful in monitoring the microenvironment around tyrosyl residues. In fact, the I_{850}/I_{830} ratio achieved its minimum value of about 0.3 when tyrosine residues were buried and the phenolic OH group acted as a strong hydrogen bond donor to an electronegative acceptor, such as carboxyl oxygen [30]. When tyrosines were exposed at the surface of the proteins, the phenolic OH acted as both a donor and an acceptor of moderate hydrogen bonds, and the I_{850}/I_{830} was approximately 1.25 [30]. The ratio ranged from 0.95 to 1.01 in this experiment, which suggested that the tyrosine residues of ERBP were exposed to the aqueous or polar microenvironment or act as simultaneous acceptor and donor of moderate to weak hydrogen bonds. From Table 4, there was no significant difference in Tyr doublet ratios between controlled RBP and extruded RBP below 160°C, which indicated that natural, exposed Tyr residues to the aqueous or polar microenvironment had remained

Table 4: Normalized intensities of the tryptophan stretching band (759 cm^{-1}), tyrosyl doublet stretching ring (850/830 cm^{-1}), CH bending vibration band (1450 cm^{-1}) of rice bran protein (RBP), and extruded rice bran protein (ERBP) at different extrusion temperature.

Sample*	Trp band (I_{760}/I_{1003} cm^{-1}**)	Tyr doublet (I_{850}/I_{830} cm^{-1})	CH band (I_{1450}/I_{1003} cm^{-1})
RBP	$1.03 \pm 0.01^{d***}$	1.01 ± 0.01^{b}	1.11 ± 0.01^{e}
ERBP-100	1.00 ± 0.01^{c}	1.00 ± 0.00^{b}	0.95 ± 0.00^{c}
ERBP-120	0.97 ± 0.00^{b}	1.01 ± 0.01^{b}	0.92 ± 0.01^{b}
ERBP-140	0.94 ± 0.01^{a}	1.00 ± 0.00^{b}	0.88 ± 0.00^{a}
ERBP-160	0.98 ± 0.01^{b}	0.95 ± 0.01^{a}	0.98 ± 0.01^{d}

*RBP: rice bran protein; ERBP-100: extruded rice bran protein at 100°C temperature; ERBP-120: extruded rice bran protein at 120°C temperature; ERBP-140: extruded rice bran protein at 140°C temperature; ERBP-160: extruded rice bran protein at 160°C temperature.
**Normalized intensities are the ratio of relative intensity of each Raman band and intensity of phenylalanine band at 1003 cm^{-1}.
***Different superscript letters in the same column indicate significant differences ($P < 0.05$).

during extrusion process. Extruded RBP at 160°C decreased its tyrosine doublet $I_{850/830}$ ratios, which could be contributed to formation of more protein aggregation. Ikeda and Li-Chan [31] reported that protein denaturation makes a protein molecule to partially unfold, followed by aggregation; in this case, a more compact protein was formed and the initially exposed tyrosine residues were buried into the intermolecular interface. Herrero et al. [16] also reported that heated SPI reveal either increasing buriedness or involvement of Tyr residues as strong hydrogen bond donors when comparing refrigerated and heated samples.

3.5. Aliphatic C-H Bending Vibration. The band assigned to the CH_2 and CH_3 bending vibrations is observed near 1450 cm^{-1}. In the 2800–3050 cm^{-1} region of the Raman spectrum, aliphatic amino acids, peptides, and proteins exhibit C-H stretching vibrations. Only the CH_2 and CH_3 bending vibrations (1450 cm^{-1}) band was studied, because no changes were observed in the intensity of the 2930 cm^{-1} band which increased with increasing polarity of the environment aliphatic hydrophobic side chains of proteins.

A decrease in the intensity of these bands indicates exposure of aliphatic residues, while an increase indicates buried residues [12]. However, some researchers have argued that an increase in C-H bending intensity suggests exposure of hydrophobic groups to a more polar environment [32]. The indicators of changes in C-H bending vibration are controversial, but it is commonly thought that changes in C-H bending vibration relate to changes in tertiary structure of proteins. An overall tendency of intensity decreasing of 1450 cm^{-1} band was observed from Table 4 in comparison of controlled samples; it can be ascribed to the fact that tryptophan residues tend to expose to a hydrophilic microenvironment. Moreover, the intensity of 1450 cm^{-1} band decreased progressively and then increased as extrusion temperature increased, indicating changes in microenvironment and polarity. Pervious works reported these results can be attributed to decreased interior hydrophobic interactions and increased exposure of hydrophobic groups to a more polar environment due to unfolding of protein during denaturation [27]. The decreased intensity of this band may be also attributed to denaturation of extruded rice bran protein as determined in DSC study.

4. Conclusions

Raman spectroscopy was used to determine structural changes in RBP during extrusion process. Raman study observed extrusion progressively increased unordered structure but general decrease in α-helix structure and β-sheet structure of RBP. In conclusion, thermal denaturation in extrusion process generally increased the content of unordered structure and exposed more hydrophobic Trp residues, while aggregation formation when extruding RBP at 160°C increased α-helix and β-sheet content and buried more hydrophobic Trp and Tyr residues. Intensity of the band assigned to CH_n bending decreased progressively and then increased as extrusion temperature increased, indicating changes in microenvironment and polarity. Though mechanism of denaturation and aggregation process of RBP remains to be clearly defined, Raman spectroscopy provides a feasible tool to study the structural changes of RBP prepared under different extrusion temperatures. The greater exposure of aromatic hydrophobic residues could improve the emulsifying and foaming properties of extruded RBP (especially at 140°C). Thus, the RBP extruded at different temperatures can be advantageously applied for various product applications.

Besides, frequently used spectra such as CD and NMR spectrum could not be applied in detecting RBP's structure for its low solubility, while FT-IR spectrum could hardly study the structure of protein in aqueous solution. Raman spectroscopy would be the most convenient analytical method that could be used for proteins in solution with low solubility and provide a direct, nondestructive, and faster determination of the structure of proteins and give a new perspective to elucidation of protein structure.

Conflict of Interests

All of the authors have no conflict of interests.

Authors' Contribution

Dr. Linyi Zhou and Dr. Yong Yang contributed equally to this research.

Acknowledgments

The authors acknowledge the financial support from National Key Technologies R&D Program of China during the 12th Five-Year Plan Period (2012BAD34B0204), NNSF of China (Serial number: 31301501 and Serial number: 31101387), and the Northeast Agricultural University (2012RCB12) for funding this work.

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Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 185967, 6 pages
http://dx.doi.org/10.1155/2015/185967

Research Article

Simultaneous Determination of Lactulose and Lactose in Conserved Milk by HPLC-RID

Michelle Fernandes Silveira,[1] Lourdes Maria Pessôa Masson,[1] José Francisco Pereira Martins,[2] Thiago da Silveira Álvares,[3] Vânia Margaret Flosi Paschoalin,[3] César Lázaro de la Torre,[4,5] and Carlos Adam Conte-Junior[3,5]

[1]*Federal Institute of Rio de Janeiro, No. 88, Pereira de Almeida Street, 20260-100 Praça da Bandeira, RJ, Brazil*
[2]*Department of Food Technology, Federal Rural University of Rio de Janeiro, 23890-000 Seropédica, RJ, Brazil*
[3]*Institute of Nutrition, Federal University of Rio de Janeiro, Macaé Campus, Imburo Road, Ajuda, 27979-000 Macaé, RJ, Brazil*
[4]*Laboratory of Pharmacology and Toxicology, Faculty of Veterinary Medicine, Major National University of San Marcos, Circunvalación Avenue, San Borja, Lima 41, Peru*
[5]*Department of Food Technology, Fluminense Federal University, No. 64, 24230-340 Niteroi, RJ, Brazil*

Correspondence should be addressed to Carlos Adam Conte-Junior; carlosconte@id.uff.br

Received 31 July 2015; Accepted 10 September 2015

Academic Editor: Miguel de la Guardia

Heat treatment is applied to dairy products to ensure microbiological quality and increase the shelf life. However, a suitable control of this process is necessary to guarantee nutritional and sensory quality. The aim of this study is to adapt the high performance liquid chromatography (HPLC) method for determination of lactulose and lactose content in commercial samples of UHT and sweetened condensed milk. The HPLC method used showed a good resolution of the analytes evaluated. The analyzed UHT milk samples presented levels for lactulose in accordance with the limit recommended by the International Dairy Federation. There was no significant variation in lactulose concentration for sweetened condensed milk samples. However, one sweetened condensed milk sample showed lactose level lower than the established values (10–12%).

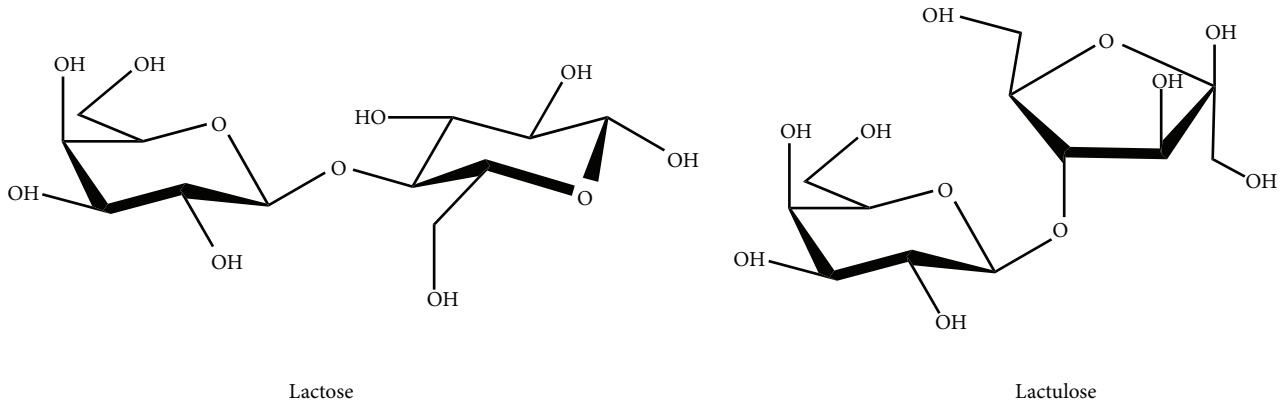
This paper is dedicated to the memory of Professor Joab Trajano Silva, Ph.D.

1. Introduction

According to Brazilian legislation, milk is a product obtained from full and uninterrupted milking, in conditions of hygienic, healthy, well-fed, and rested cows [1]. It is a highly nutritious food to ensure the supply of essential nutrients, especially for children. Since the most remote times, dairy animals have been domesticated for this purpose [2]. According to national and international organizations, Brazil is the fourth largest milk producer in the world, with production of 32,091 billion liters in 2011, staying behind only the United States, India, and China [3, 4].

One of the challenges of the milk industry is the conservation. Nowadays many technologies to achieve this purpose exist. However, the ultrahigh temperature (UHT) treatment is applied in most dairy products to guarantee their microbiological safety and increase the shelf life [5]. Another method commonly used is the concentration; in this method, the milk is preserved with a considerable reduction of water content by evaporation. If a large quantity of sucrose is added, the product is called sweetened condensed milk [6, 7].

A proper control of the thermal process in both methods is important to ensure their nutritional and sensory quality since their effect on milk constituents is fundamental in



Lactose Lactulose

Figure 1: Basic structure of lactose and lactulose.

the characterization of the final product [8, 9]. The severity of thermal treatment can lead to degradation of milk constituents like proteins, enzymes, and vitamins; some of these substances could be indicators to assess the thermal damage in milk [8, 10].

Among the carbohydrates, lactose, originating from the blood glucose in the mammary gland, is one of the main constituents of milk [11]. However, when lactose is subjected to moderate heating, its isomerization can occur with the lactulose formation (glucose/fructose) via Lobry de Bruyn-Alberda van Ekenstein reaction, through the intermediate compound 1,2-enediol [12–14]. Consequently, the quantity of lactulose is directly proportional to the intensity of the heat treatment applied [15] and could be useful such as indicators of the quality of milk processing [8]. Lactose and lactulose chemical structures are shown in Figure 1. According to the International Dairy Federation (IDF), UHT milk contains less than 0.06 g/mL of lactulose, while the hydrostatically sterilized milk presents level of lactulose higher than this value [16]. A precise analysis of lactose and lactulose in food is interesting because both substances exert dose-dependent effects upon intake (lactose maldigestion and prebiotic or laxative action of lactulose) [17].

Early methods of lactose analyses such as polarimetric, gravimetric, Lane-Eynon, and Chloramine-T methods are no more useful because all of them assumed that lactose is the only carbohydrate present in milk and this is a problem in dairy products (fermented milk, cheese, etc.) with appreciable amounts of monosaccharides [18]. More recently, infrared spectroscopic [19], chromatography [20, 21], enzymatic colorimetric [22–24], and capillary electrophoresis [25] have been used for the detection and quantification of lactose and lactulose. From these, high performance liquid chromatography (HPLC) is a very promising technique and considerable research was carried out on the quantitative determination of lactulose [26]. Over all detectors coupled to HPLC, the refractive index detector (RID) is the most widely used for sugars because no fluorophore (fluorescence detector) or chromophore (UV detector) is necessary; in other words, no derivatization is required. RID operates under the principle that the refractive index changes depending upon the refracting or light bending properties of liquids [27]. The aim of this paper was the development of a modified HPLC-RID method for simultaneous determination and quantification of lactulose and lactose in commercial UHT and sweetened condensed milk.

2. Materials and Methods

2.1. Sample Collection. Commercial brands of skimmed UHT milk (UHT) ($n = 5$) and sweetened condensed milk (COND) ($n = 5$) were obtained in markets located in the city of Rio de Janeiro, Brazil. Samples were transported to the laboratory in insulated polystyrene boxes on ice during 1 h.

2.2. Chemicals and Reagents. The standard of lactose and lactulose (both with 98% of purity) was purchased from Sigma-Aldrich (Sao Paulo, Brazil). The HPLC grade reagents used were acetonitrile (Tedia, RJ, Brazil) and all other reagents like methanol (Tedia, RJ, Brazil), zinc sulphate solution (Carrez I), and potassium hexacyanoferrate solution (Carrez II) were of analytical grade. Ultrapure water was obtained from Simplicity System (Millipore, Molsheim, France).

2.3. Standard Preparation and Calibration Curve. Linearity of UHT milk was performed injected six lactose (range of 0.625 to 20 mg/mL) and six lactulose standards (range from 0.0625 to 1 mg/mL). On the other hand, the linearity of sweetened condensed milk was performed with six levels of lactulose standard (range of 1 to 100 mg/mL) and the same range for lactose. The standard solutions were dissolved in ethanol : water (1 : 1) and filtered on PTFE membrane with pore size of 0.45 μm and Ø = 13 mm (Millipore, USA). Solutions were kept at 4°C until the injection in the chromatography system.

2.4. Milk Sample Preparation. The methods proposed by Chávez-Servín et al. [28] and Schuster-Wolff-Bühring et al. [17] were adapted for our experiment. In brief, a protein precipitation was achieved with a sequential mixture of 1.5 mL of the skimmed UHT milk, 30 μL Carrez I solution, and the same volume of Carrez II solution. The resulting volume was centrifuged (Hermle Z 360 K, Germany) at 12000 RPM for 4°C/30 min. Afterwards, the solution was

filtered in the sterile polyethersulfone membrane with pore size of 0.22 μm and Ø = 33 mm (Millipore, USA). Before the chromatograph injection, the extract obtained was diluted in ethanol : water (1 : 1). Sweetened condensed milk (0.60 g) was weighted and diluted with 1.5 mL of ultrapure water. The solution was stirred in mechanical agitator (Biotech International, Melsungen, Germany), during 30 min. After dilution of the sample, the resulting solution followed the same procedure described for the skimmed UHT milk.

2.5. Chromatographic Conditions. Milk samples and standard solution of lactose and lactulose were analyzed from the HPLC system (Shimadzu) provided by degasser (DGU-20A3), binary pump (LC-20AD), automatic injector sample (SIL-20AC), column oven (CTO-20A), and refractive index detector (RID 10A). An isocratic HPLC method involves a mobile phase of acetonitrile : water (75 : 25, v/v), a Prevail Carbohydrate ES precolumn (5 μm, 7.5 mm × 4.6 mm), and a Prevail Carbohydrate ES column (5 μm, 250 mm × 4.6 mm). The system used a flow of 1.1 mL/min, an oven temperature of 30°C, an injection volume of 20 μL, and a running time of 20 min. Lactose and lactulose were identified by retention time and were quantified by peak area.

2.6. Experimental Design and Statistical Analyses. Data collected in this study were analyzed using GraphPad Prism 5.00 package [29] for Windows by one-way ANOVA, and the means were compared with Tukey's test ($P < 0.05$).

3. Results and Discussion

3.1. Validation Parameters. A linearity of the chromatography method was verified with the coefficient of determination (R^2). According to the results, values of R^2 for lactose and lactulose (UHT and sweetened condensed milk) were superior to 0.99 (Figure 2). These results were possible due to the use of six different levels for the construction of the calibration curve, which showed a best-fit linear regression model. The legislation of the European Community recommends at least five concentration levels for the construction of calibration curves [30]. Our results are consistent with Brazilian legislation. Health Surveillance Agency (ANVISA) and National Institute of Metrology, Quality and Technology (INMETRO) consider 0.99 and 0.90 excellent R^2 values, respectively [31, 32].

A separation performance of lactose and lactulose is represented in Figure 3. A standard of (I) lactulose and (II) lactose showed a retention time of 13.6 and 17.2 min, respectively. The same retention time was observed in milk samples. This retention time was similar to that reported by Corzo et al. [18] who used similar chromatography conditions to ours except for flow rate (0.9 mL/min). These authors remarked the advantage of Prevail column in terms of clear peak resolution and short time analysis for lactose and lactulose. Our result showed that a highest content of lactose in UHT milk and of lactulose in condensed milk has been verified. Food samples may contain components that interfere with performance measurement and may increase or decrease the signal detector [33]. It is important to remark that HPLC method coupled to refractive index detector (RID) is preferred when lactose and lactulose are determinate simultaneously [14, 26]. The HPLC is one of the most extensively used techniques employed for the separation of a large variety of carbohydrates in foods [18], as it is particularly advantageous in terms of speed, simplicity of sample preparation (without a prior derivatization), and obtaining a high-resolved chromatogram in a short period of time (20 min).

Table 1: Concentrations of lactose and lactulose found in five different commercial brands of UHT milk.

Brand codification	Concentration in UHT milk (g/100 mL)	
	Lactose	Lactulose
UHT_1	5.66 ± 0.01^a	0.04 ± 0.00^a
UHT_2	5.50 ± 0.51^a	0.02 ± 0.00^b
UHT_3	6.64 ± 0.35^a	0.05 ± 0.00^c
UHT_4	5.57 ± 0.21^a	0.02 ± 0.00^b
UHT_5	5.47 ± 0.11^a	0.06 ± 0.00^c

UHT_n: ultrahigh temperature milk brand.
Values with the same letter in the column indicate that there is no statistically significant difference between the samples to the level of 5%.
Values represent the mean ± SEM.

3.2. Commercial Milks. The results obtained for quantification of lactose and lactulose in UHT milk are shown in Table 1. Brands did not present a significant difference ($P > 0.05$) for lactose values varied from 5.47 to 6.64 g/100 mL. Our results are similar to those reported by Walstra et al. [34]; milk presents the levels of 3.8 to 5.3 g/100 mL of lactose and these values may vary with environmental and biological conditions. On the other hand, concentrations of lactulose showed a significant difference among brands ($P < 0.05$) with values ranging from 0.02 to 0.06 g/100 mL.

When milk is heated, lactose may isomerize into lactulose. According to the IDF and the European Union (EU), the quantification of lactulose allows distinguishing the milk submitted to different thermal processes and can be used as an indicator of the intensity of the heat treatment [28, 35, 36]. Walstra et al. [34] recommend that UHT milk must contain less than 0.06 g/100 mL of lactulose but these values can vary according to the thermal process. The UHT milk obtained by direct system (injection and infusion) is heated by direct contact with the steam, keeping the high temperature for a short period of time, causing less damage to the product (lactulose values range of 0.005 to 0.011 g/100 mL). However, the indirect system is characterized by slow heating of the product in tubes or plates (lactulose values range of 0.010 to 0.065 g/100 mL) [37]. Although the UHT system was not showed in any label, the levels of lactulose were within the limit values and suggested that there was not an additional heating to UHT process. Our results are according to Morales et al. [8], Elliott et al. [38], Feinberg et al. [39], and Sakkas et al. [40] who found values of lactulose between 0.012 and 0.046 g/100 mL, 0.013 and 0.024 g/100 mL, 0.014 and 0.040 g/100 mL, 0.082 g/100 mL, respectively.

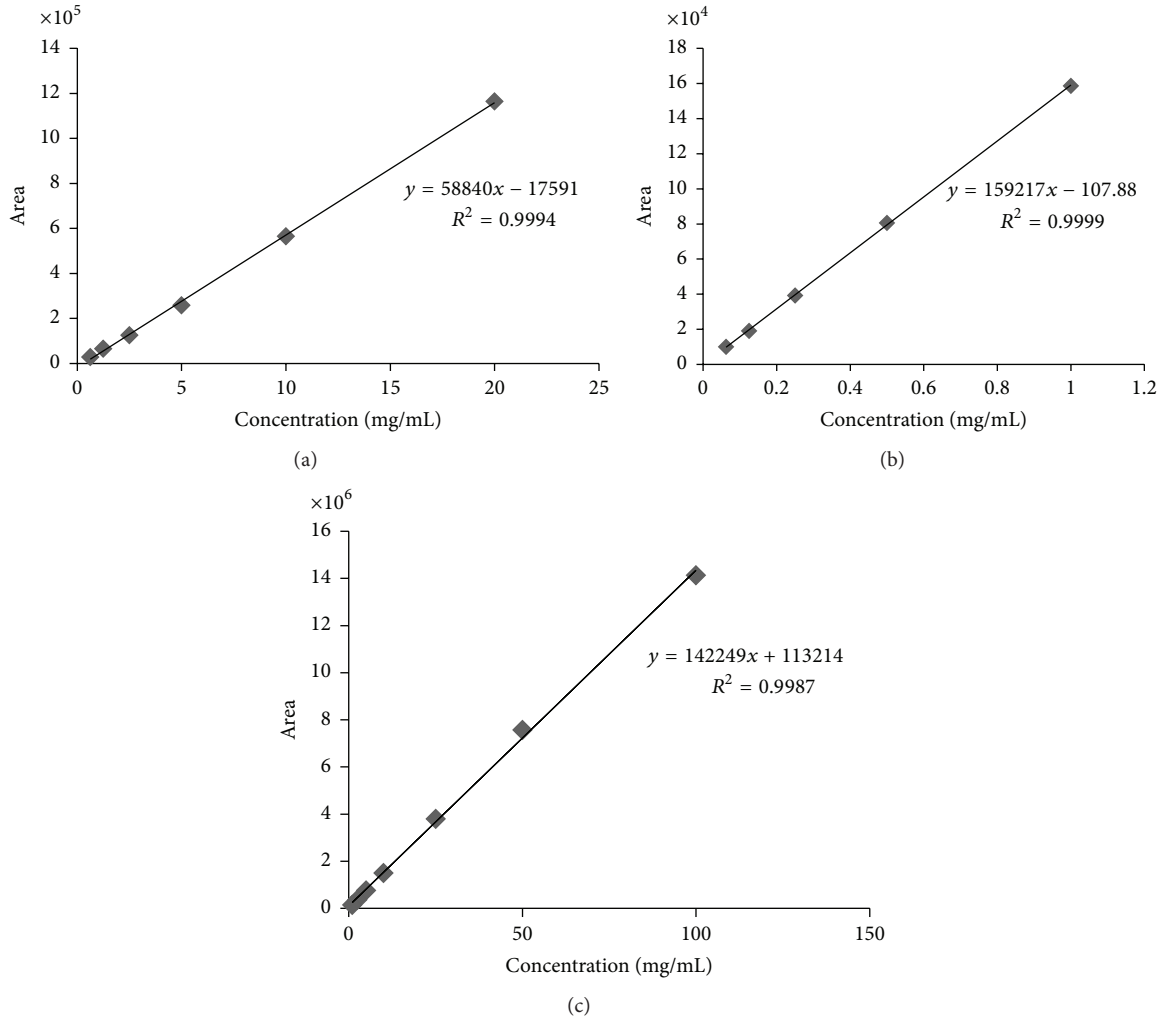


Figure 2: (a) Linearity of lactose by HPLC-RID for both samples. (b) Linearity of lactulose by HPLC-RID for UHT milk samples. (c) Linearity of lactulose by HPLC-RID for condensed milk.

Table 2 shows the values of lactulose and lactose in sweetened condensed milk samples. Lactulose levels ranging from 43.56 to 48.97 g/100 mL were similar in all brands ($P > 0.05$). These values were higher in contrast to UHT milk; this finding was expected because in the condensed process 60% of water is removed from the milk and all solid components increase including lactulose. However, lactose levels showed values between 9.96 and 13.86 g/100 mL. These values are similar to those reported by Muehlhoff et al. [2] who found lactose values between 10 and 14 g/100 mL in different sweetened condensed milks. However, these values were lowest compared with the range from 38 to 45 g lactose per 100 g water reported by Walstra et al. [41]. Our results showed slight variations in lactose values in all brands since sweetened condensed milk is elaborated following different methods and the process did not applied high temperature compared with the UHT milk. The determination of lactulose and lactose values in this milk product is not an important indicator for heat treatment.

Table 2: Concentrations of lactose and lactulose found in five different commercial brands of condensed milk.

Brand codification	Concentration in condensed milk (g/100 mL)	
	Lactose	Lactulose
$COND_1$	13.86 ± 1.15^a	43.56 ± 3.28^a
$COND_2$	10.63 ± 0.22^b	44.31 ± 1.75^a
$COND_3$	9.96 ± 0.01^b	48.97 ± 1.22^a
$COND_4$	13.60 ± 0.56^a	44.62 ± 1.18^a
$COND_5$	$12.36 \pm 0.03^{a,b}$	43.72 ± 0.02^a

$COND_n$: condensed milk brand.
Values with the same letter in the column indicate that there is no statistically significant difference between the samples to the level of 5%.
Values represent the mean ± SEM.

Although the heat treatment in UHT milk interferes with the levels of lactose and lactulose, other chemical indicators such as furosine, 5-hydroxymethylfurfural, galactosyl-β-pyranona, and lysinoalanine can be used to evaluate changes

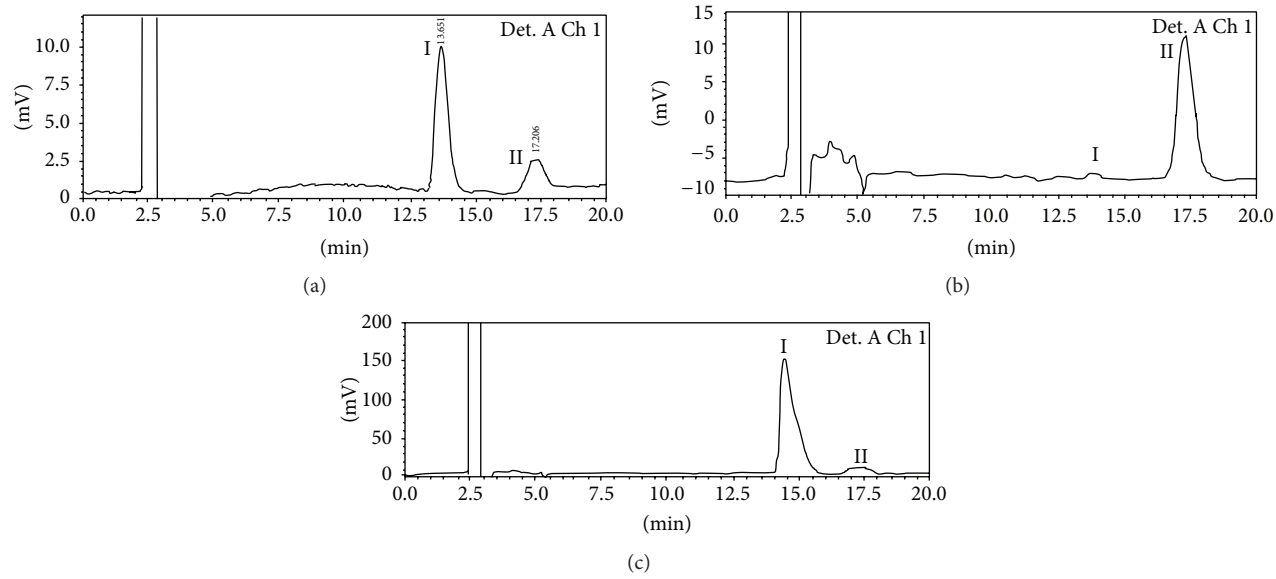


Figure 3: Chromatogram by HPLC-RID: (a) standard lactose-lactulose; (b) UHT milk sample; (c) condensed milk sample. I: lactulose (13.6 min); II: lactose (17.2 min).

in the milk submitted to heat processing [38]. From both, lactulose is a better indicator for heat process but it does not represent itself as a distinguishing criterion. In fact, the combination of different indicators would be a better characterization [42].

4. Conclusion

The modified HPLC-RID method is useful in the detection and quantification of lactulose and lactose in dairy products simultaneously. Values of lactose were normal in both milk products. Although UHT milk included high thermal processing, lactulose values remained within the limit values. On the other hand, values of lactulose were high in sweetened condensed milk but this is not an important indicator because the process does not use high temperature. Lactulose is an important indicator of heat treatment; however, the correlation with other substances formed in the milk products after the heat process to define their application in the dairy industry is suggested.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (Grant no. E-26/103.003/2012, FAPERJ, Brasil) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico (Grant nos. 311361/2013-7, 313917/2013-2, and 401922/2013-8, CNPq, Brasil) for the financial support.

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Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 696303, 7 pages
http://dx.doi.org/10.1155/2015/696303

Research Article

Female *Leuciscus lepidus* May Be a New Alternative for Fish Oil Supplements

Ozlem Cakmak,[1] Aynur Altuntas,[2] Veli Ugurcu,[3] Hacı Kemal Erdemli,[4] and Sumeyya Akyol[5]

[1]*Department of Biology Education, Faculty of Education, Gazi University, 06560 Ankara, Turkey*
[2]*Department of Chemistry, Ankara Regional Office of Council of Forensic Medicine, 06300 Ankara, Turkey*
[3]*Department of Medical Biochemistry, Faculty of Medicine, Dumlupınar University, 43050 Kütahya, Turkey*
[4]*Department of Biochemistry Laboratory, Çorum Training and Research Hospital, 19000 Çorum, Turkey*
[5]*Department of Medical Biology, Faculty of Medicine, Turgut Özal University, 06010 Ankara, Turkey*

Correspondence should be addressed to Ozlem Cakmak; ocakmak21@gmail.com

Received 5 May 2015; Accepted 3 August 2015

Academic Editor: Nivia Coelho

The proximate composition of male and female *Leuciscus lepidus* in Beyşehir Lake was investigated. The fatty acid profiles of total lipid, phospholipid, and triacylglycerol in muscle and liver of male and female *L. lepidus* were evaluated by gas chromatography. Proximate analyses showed that meat of male and female *L. lepidus* had 15.13 ± 0.04 and 18.75 ± 0.11% fat, 20.42 ± 0.45 and 22.21 ± 0.56% protein, 65.47 ± 1.37 and 61.28 ± 1.03% moisture, and 1.51 ± 0.05 and 1.50 ± 0.03% ash, respectively. The percentage of total saturated fatty acids was higher in liver than in muscle, whereas the total polyunsaturated fatty acid (PUFA) content was the lowest in all fatty acid profiles. The phospholipids contained more PUFAs than triacylglycerol. Analysis of variance indicated significant differences ($P < 0.05$) between male (47.51%) and female (49.98%) muscle PUFAs in total lipid. The proportion of omega 3 ($\omega 3$) to omega 6 ($\omega 6$) fatty acids of total lipid was 3.15 in male and 3.68 in female. The ratio is an important indicator for comparing the value of fish oil. Therefore, it was concluded that *L. lepidus* was considered to be a high quality product for healthy food choice. Additionally, female *L. lepidus* may especially be used to produce fish oil supplements from freshwater fish combined with vegetable oils.

1. Introduction

Human body cannot synthesize omega 3 ($\omega 3$) polyunsaturated fatty acids (PUFAs) [1]; for this reason, it has to be taken with nutrition [2]. Fish oil is especially rich in $\omega 3$ PUFAs. Two of the most important $\omega 3$ PUFAs contained in high percentage in fish oil are eicosapentaenoic acid (C20:5$\omega 3$, EPA) and docosahexaenoic acid (C22:6$\omega 3$, DHA) [3]. EPA and DHA are characteristic PUFAs of all kinds of fishes. These are either not present or found in very small amounts in foods except for water products. Fish species vary in their capacity to biosynthesize $\omega 3$ long-chain PUFAs, EPA and DHA, which are crucial to the health of higher vertebrates. The synthesis of long-chain PUFAs involves enzyme-mediated fatty acyl desaturation and elongation. Studies show strong evidence that EPA and DHA fatty acids from fish oil can reduce the incidence of blood pressure, triglyceride, cancer, allergy, cardiovascular diseases, depression, Alzheimer's disease, dyslipidemia, insulin resistance, adiposity, psoriasis, inflammatory bowel diseases, and eye diseases [4–7]. There are also good lines of evidence that diets rich in $\omega 3$ PUFAs help with rheumatoid arthritis and have positive effects on bone formation [8], treatment of autoimmune diseases [9], and attention deficit/hyperactivity disorders [10]. The other studies suggested that $\omega 3$ PUFAs reduce the risk of heart attacks, strokes, and death from heart disease [11].

Fish oil supplements are used for a wide range of conditions, and they are usually extracted from marine fish such as white tuna, mackerel, sardines, herring, halibut, salmon, cod liver, or blubber [12]. Fatty acid composition of marine and freshwater fishes is significantly different. That is, marine fish generally contain higher levels of $\omega 3$ PUFAs than freshwater fish. However, freshwater fish have a greater capacity than marine fish to elongate and desaturate the shorter fatty acid

(synthesized by algae or plants) into longer EPA and DHA. In other words, they convert food of poor nutritional value into food of rich nutritional value [13]. In addition, freshwater fish serve as a major source of protein and lipid for a large portion of the world population who live around the rivers and lakes and do not have access to marine fish.

On the whole, the fatty acid composition of fish lipids is affected by age, seasonal change, nutrition, sex, reproductive cycle, salinity, and geographical location [14–20].

Fatty acid profiles of phospholipids are different than that of triacylglycerol, which contains PUFA. The lipid content of diet affects PUFA absorption [21]. In particular, the fatty acid composition of the triacylglycerol strongly reflects that of the diet, implying that triacylglycerol acts as a nutritional storage site in the fish body [22]. Hence, it is necessary to figure out the fatty acid composition of phospholipids and triacylglycerol in order to estimate the nutritive value of fish.

Beyşehir Lake is the third largest lake in Turkey. It is a freshwater lake spanning over the borders of Isparta and Konya provinces in the southwestern part of Turkey. It is located at around $37^{\circ}47'0''$N, $31^{\circ}33'0''$E. It has an area of 650.00 km^2 and is 45 km long and 20 km wide. It carries the same name as the principal urban centre of its region, Beyşehir. The water level in the lake often fluctuates by year and by season of the year. The maximum depth is 10 meters. Beyşehir Lake is mostly harnessed for watering. Besides, it is also a national park. There are thirty-two islets in varying sizes on the lake. The lake has various water sources. Among the major ones are the streams and creeks of the Sultan and Animas mountains. Besides, precipitation is another contributing water source to the lake. In total, 27 streams feed the lake. The most important fish populations of the lake are *Tinca tinca*, *Sander lucioperca*, *Cyprinus carpio*, *L. lepidus*, and *Carassius carassius* [23].

Leuciscus lepidus differs from all other *Leuciscus* species in its elongated, long, rather pointed head with projecting lower jaw [24]. This species has great economic value and people who live in southwestern Anatolia consume this fish abundantly [25].

Research on *Leuciscus lepidus* (Cypriniformes: Cyprinidae) (Heckel, 1843) in Beyşehir Lake has focused on its growth features [25], but no research is available on the nutritional quality of *L. lepidus*. Therefore, the goal of this study is to investigate the proximate composition and fatty acid profiles of total lipid, phospholipid, and triacylglycerol in male and female *L. lepidus*. We expect that the determination of the composition and fatty acid profiles of these species will provide significant information on the nutrient value of this food for consumers.

2. Material and Methods

2.1. Sample Preparation. *L. lepidus* were caught from Beyşehir Lake, in September 2014. The samples were kept in ice after capturing and transported to the laboratory immediately. Male ($n = 3$) and female ($n = 3$) fishes of the same age were used for each analysis. The total length and weight of all samples were measured. The average fork lengths and weights of male and female individuals were 32.4 ± 4.2 cm and 528 ± 81.90 g, respectively.

2.2. Biochemical Analysis. Proximate composition analysis of lipid content was carried out by Bligh and Dyer [26] method. Protein content was determined by Kjeldahl method [27]. Moisture content was determined by oven drying at 105°C to constant weight [28]. Ash content was determined by combustion at 550°C for 3–5 hours [28].

2.3. Fatty Acids Analysis. The fishes were processed for lipid extraction and analysis following the methods described in Bligh and Dyer [26]. For analysis, three groups of three male and female *L. lepidus* were used and each sample was analyzed 3 times. Tissues of fishes (approximately 3 g) were homogenized in glass tubes and extracted three times with chloroform/methanol (2 : 1, v/v). Autoxidation of unsaturated components was minimized by adding 50 μL of 2% butylated hydroxytoluene in chloroform to each sample during the extraction process.

The total lipid extracts were dried under a stream of N_2 and then phospholipid and triacylglycerol fractions were isolated by thin-layer chromatography (TLC), using Silica Gel TLC plates (20 by 20 cm, 0.25 mm thick). After applying the total lipid extracts, the TLC plates were developed in petroleum ether : diethyl ether : acetic acid (80 : 20 : 1, v/v). Lipid fractions were made visible by spraying the TLC plates with 2′,7′-dichlorofluorescein (Supelco, Supelco Park, PA, USA), and phospholipid and triacylglycerol fractions were identified by corresponding standards.

The phospholipid and triacylglycerol fractions were scraped into reaction vials, and the associated fatty acids were transmethylated by refluxing the fractions in acidified methanol for 90 min at 85°C. The fatty acid methyl esters (FAMEs) were extracted from the reaction vials three times with hexane and then concentrated [29].

2.4. Gas Chromatography. The FAMEs were analyzed by gas chromatography using an Ati Unicam 610 gas chromatograph equipped with SP-2330 capillary column (30 m by 0.25 mm i.d., 0.2 μm film thickness, Supelco), a flame ionization detector, and Unicam 4815 recording integrator. A split injection of 0.5 μL was used. The temperature condition detector was set at 250°C. The oven temperature was set at 180°C for 5 min and then reached 200°C with a ramp rate of 2°C/min and then was held for 15 min. The carrier gas was nitrogen (flow rate 1 mL/min) and split ratio was 40 : 1. FAMEs were identified by comparisons of retention times with authentic standards (Sigma Chemical Co., St. Louis, MO, USA). Individual FAMEs were identified by comparisons with the chromatographic behaviors of authentic standards.

2.5. Gas Chromatography-Chemical Ionization Mass Spectrometry. The chemical structures of the FAMEs were confirmed by capillary gas chromatography-mass spectrometry (GC-MS) (HP 5890-E series GC-System, *Hewlett-Packard*, Palo Alto, CA, USA) with mass-selective detection. An *Innowax column* (30 m by 0.25 mm i.d., 0.25 μm film thickness) was used and the temperature was programmed from

Table 1: Proximate composition (%) of males and females of *Leuciscus lepidus*.

Chemical composition	Male	Female
Fat content (%)	15.13 ± 0.04a	18.75 ± 0.11b
Protein content (%)	20.42 ± 0.45a	22.21 ± 0.56b
Moisture content (%)	65.47 ± 1.37a	61.28 ± 1.03b
Ash content (%)	1.51 ± 0.05a	1.50 ± 0.03a

The values are shown as mean ± SD. Mean values are averages of three replicates.
Different letters in the same row represent significant statistical differences, $P < 0.05$.

150 to 230°C at a 2°C/min increase with an initial hold of 36 min. The carrier gas was helium (1 mL/min) and the split ratio was 1 : 50. The injection port and the detector temperatures were 250°C and 300°C, respectively. The mass spectrometer was operated in the electron impact ionization mode (70 eV). Chemical structures of the FAMEs were determined by comparison of the spectra with the Wiley 275 and NIST 98 databank and by comparing obtained spectra with that of authentic standards. The presence of mono- and polyunsaturated fatty acids containing 20 carbon atoms was illuminated using GC-MS. Analysis was carried out at TUBITAK Ankara Test and Analysis Laboratory (ATAL).

2.6. Statistical Analysis. The statistical analysis was performed using a commercial statistical program (SPSS 13.0). Statistical analysis of percentages of fatty acid was tested by *t*-test. Differences between mean values were evaluated as significant when $P < 0.05$. The results were shown as mean ± standard deviation.

3. Results and Discussion

The results of proximate composition of male and female *L. lepidus* are given in Table 1. The contents of fat, protein, moisture, and ash of male *L. lepidus* were 15.13, 20.42, 65.47, and 1.51%, respectively; and those values in female were 18.75, 22.21, 61.28, and 1.50%, respectively. Statistical analysis results showed that significant differences were observed between males and females in terms of fat, protein, and moisture. Proximate composition of females was found to have higher quality content than males. Arslan [30] reported that fat content in fillets of *Cyprinus carpio* is 2.60% in males and 2.20% in females. In females, fat quantity is higher than those of males as shown in a study by Karaton and Gürel İnanlı [31] which reported that fat, protein, moisture, and ash in male *Squalius cephalus* were 12.65, 20.87, 64.18, and 1.51%, respectively, while in females those values were 15.82, 19.40, 62.53, and 1.54%, respectively.

A comparison of males and females in terms of muscle and liver fatty acid composition of total lipid in *L. lepidus* is presented in Table 2. Twenty different fatty acids were detected. The major fatty acids identified in both sexes were palmitic (C16:0), palmitoleic (C16:1*ω*-7), stearic (C18:0), oleic (C18:1*ω*-9), linoleic (C18:2*ω*-6), arachidonic (C20:4*ω*-6, AA), eicosapentaenoic (C20:5*ω*-3), docosapentaenoic (C22:5*ω*-3, DPA), and docosahexanoic (C22:6-*ω*3) acids. The total

Table 2: Comparison of males and females in terms of muscle and liver fatty acid composition of total lipid in *L. lepidus*.

Fatty acids	Muscle		Liver	
	Male	Female	Male	Female
C12:0	0.02 ± 0.01a	0.12 ± 0.02b	0.15 ± 0.04y	0.10 ± 0.02z
C13:0	0.82 ± 0.07a	1.02 ± 0.09a	0.32 ± 0.05y	0.08 ± 0.01z
C14:0	1.30 ± 0.02a	1.32 ± 0.02a	1.38 ± 0.13y	1.12 ± 0.17z
C15:0	0.40 ± 0.05a	0.53 ± 0.06b	0.28 ± 0.06y	0.87 ± 0.08z
C16:0	14.84 ± 0.30a	10.42 ± 0.41b	15.30 ± 0.39y	13.20 ± 0.75z
C17:0	1.30 ± 0.08a	0.32 ± 0.01b	0.80 ± 0.07y	0.82 ± 0.08y
C18:0	6.15 ± 0.13a	8.06 ± 0.21b	9.01 ± 0.31y	11.21 ± 0.43z
ΣSFA	24.83 ± 0.92a	21.79 ± 1.02b	27.24 ± 1.65y	27.40 ± 1.85y
C16:1*ω*-7	7.26 ± 0.25a	6.07 ± 0.11a	7.87 ± 0.09y	5.63 ± 0.28z
C18:1*ω*-9	17.86 ± 0.76a	19.22 ± 0.99b	16.72 ± 0.42y	18.03 ± 0.72z
C20:1*ω*-9	2.54 ± 0.49a	2.94 ± 0.45a	3.89 ± 0.53y	3.14 ± 0.48y
ΣMUFA	27.66 ± 1.13a	28.23 ± 1.83a	28.48 ± 1.91y	26.80 ± 1.77z
C18:2*ω*-6	4.65 ± 0.19a	4.18 ± 0.14a	5.39 ± 0.87y	6.95 ± 0.74z
C18:3*ω*-6	1.02 ± 0.11a	1.50 ± 0.18b	0.70 ± 0.13y	0.81 ± 0.10y
C18:3*ω*-3	0.82 ± 0.11a	0.53 ± 0.18b	2.70 ± 0.31y	2.83 ± 0.35y
C20:2*ω*-6	0.27 ± 0.17a	0.51 ± 0.20b	0.42 ± 0.08y	0.56 ± 0.15z
C20:3*ω*-6	2.42 ± 0.13a	2.40 ± 0.10a	0.20 ± 0.12y	0.24 ± 0.20y
C20:4*ω*-6	3.07 ± 0.03a	2.10 ± 0.07b	6.14 ± 0.16y	7.14 ± 0.11z
C20:5*ω*-3	8.16 ± 0.48a	8.06 ± 0.65a	5.10 ± 0.15y	5.07 ± 0.10y
C22:3*ω*-3	0.06 ± 0.03a	0.12 ± 0.05b	0.10 ± 0.04y	0.04 ± 0.04z
C22:5*ω*-3	4.02 ± 0.11a	4.57 ± 0.24b	2.70 ± 0.31y	2.02 ± 0.33z
C22:6*ω*-3	23.02 ± 1.14a	26.01 ± 1.25b	20.83 ± 1.10y	20.14 ± 1.28y
ΣPUFA	47.51 ± 1.68a	49.98 ± 1.71b	44.28 ± 0.68y	45.80 ± 0.68y
Σ*ω*3	36.08	39.29	31.43	30.10
Σ*ω*6	11.43	10.69	12.85	15.70
Σ*ω*3/*ω*6	3.15	3.68	2.44	1.91

The values are shown as mean ± SD. Mean values are averages of three replicates.
Different letters in the same row and values in male and female represent significant statistical differences, $P < 0.05$.
SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

saturated fatty acids (SFA) were measured as 24.83% in male and 21.79% in female muscle. The percentages of total monounsaturated fatty acids (MUFA) compared to total lipid extracted from muscle males and females were 27.66% and 28.23%, respectively. C18:1*ω*-9 was a predominant fatty acid to take the form of MUFA and the acid is attributed as healthy due to the monounsaturated status [32]. Analysis of variance indicated significant differences ($P < 0.05$) between male (47.51%) and female (49.98%) muscle PUFA compositions where EPA and DHA acids had the highest levels among others. Similar results have also been reported for other freshwater fish [19, 22, 33], except for *ω*3/*ω*6 ratio. In this study, the first major finding is the observation of high level *ω*3/*ω*6 ratio (i.e., 3.15 in male and 3.68 in female muscle total lipid). The ratio of *ω*3/*ω*6 is an important indicator for comparing the value of fish oil [34]. The UK Department of Health advises an ideal ratio of *ω*3/*ω*6 of 4.0 at maximum

[35]. This ratio is very important in order to reduce coronary heart diseases, plasma lipid levels, and risk of cancer [36].

Cengiz et al. [37] investigated total fatty acids in the muscle of female *L. lepidus* from the Tigris river (Turkey), in January. The ratio of $\omega 3/\omega 6$ was detected as 2.31 by Cengiz et al. A similar study was carried out by Özparlak [38] to measure total fatty acids in female *L. lepidus* muscle in Apa Dam Lake (Turkey), in January. He reported the ratio of $\omega 3/\omega 6$ as 2.08. We also carried out our studies with the same fish and in the same month as previous studies, but we observed a higher ratio (3.68 in female) during our study. Hence, in this study, the second major finding is the effect of regional differences on fatty acid composition. The reason for this difference might be the feeding properties of Beyşehir Lake by 27 streams, which may have led to highly nutritious freshwater habitat.

Analysis of variance showed significant differences between total fatty acid compositions in the liver of males and females. The percentage of ΣMUFA was significantly ($P < 0.05$) higher in males (28.48%) than in females (26.89%). However, in the liver, total SFA level (27.24% in males and 27.40% in females) was higher and total PUFA level (44.28% in males and 45.80% in females) was lower than muscle (47.51% in males and 49.98% in females) fatty acid composition (Table 2). Similar results for *Salmo trutta macrostigma* [39] and *Vimba vimba* [40] were also reported in the literature. The most dramatic change in fatty acid composition of fish is observed during their reproduction period. Most of the lipid that would be mobilized for later use in the formation and growth of gonads is stored in muscle, liver, and abdominal regions before the reproduction period [41]. More specifically, the liver stores lipids that will be used to produce the energy required for growth of gonads and gamete. On the other hand, the energy required for reproductive activity is provided by the lipids stored in muscle [42].

A comparison of males and females in terms of muscle and liver fatty acid composition of phospholipid in *L. lepidus* is shown in Table 3. A similar comparison in terms of triacylglycerols is shown in Table 4.

In particular, phospholipids in muscle and liver of *L. lepidus* had higher rate of C20:4ω-6 and C22:6ω-3, but lower rate of C18:1ω-9 compared to triacylglycerol. In addition, phospholipids differed from total fatty acid and triacylglycerol with higher proportion of ΣPUFA and lower proportions of ΣSFA and ΣMUFA. In triacylglycerol fraction, C16:0, C16:1, and C18:1 had the highest and C20:4ω-6 and C22:6ω-3 had the lowest level. The accumulation of ΣSFA and ΣMUFA in triacylglycerol fraction is probably related to energy requirement and the aim of this accumulation may be storing energy-rich material for subsequent consumption during metabolic activity and hard conditions.

Phospholipids function as structural components of membranes, being incorporated to a larger extent into the membrane structure of cellular and subcellular particles. Triacylglycerols are often considered as storage lipids and reflect the fatty acid composition of the food to a greater extent than phospholipids do.

Table 3: Comparison of males and females in terms of muscle and liver fatty acid composition of phospholipid in *L. lepidus*.

Fatty acids	Muscle		Liver	
	Male	Female	Male	Female
C12:0	0.18 ± 0.03a	0.13 ± 0.02b	0.85 ± 0.07y	0.93 ± 0.02z
C13:0	1.02 ± 0.07a	0.42 ± 0.07b	0.35 ± 0.05y	0.08 ± 0.01z
C14:0	1.32 ± 0.09a	1.30 ± 0.03a	1.43 ± 0.13y	1.12 ± 0.17z
C15:0	0.53 ± 0.05a	0.40 ± 0.05b	0.42 ± 0.06y	0.87 ± 0.08z
C16:0	11.42 ± 1.80a	12.68 ± 1.74a	13.11 ± 1.15y	12.20 ± 0.25z
C17:0	1.32 ± 0.08a	1.02 ± 0.05b	1.89 ± 0.07y	0.82 ± 0.08y
C18:0	4.78 ± 0.23a	3.12 ± 0.20b	7.20 ± 0.31y	6.38 ± 0.43z
ΣSFA	20.57 ± 1.22a	19.07 ± 1.62a	25.25 ± 1.65y	24.40 ± 1.85y
C16:1ω-7	2.34 ± 0.22a	1.80 ± 0.13b	3.88 ± 0.09y	3.69 ± 0.28z
C18:1ω-9	14.01 ± 0.96a	12.86 ± 1.04b	14.70 ± 1.42y	14.07 ± 0.72z
C20:1ω-9	2.14 ± 0.45a	1.12 ± 0.05a	0.87 ± 0.53y	1.03 ± 0.48y
ΣMUFA	18.49 ± 1.13a	15.78 ± 1.83b	19.45 ± 1.91y	18.79 ± 1.77z
C18:2ω-6	2.35 ± 0.13a	2.15 ± 0.14a	3.31 ± 0.42y	4.99 ± 0.24z
C18:3ω-6	1.55 ± 0.10a	1.25 ± 0.09b	1.78 ± 0.11y	1.82 ± 0.10y
C18:3ω-3	1.45 ± 0.11a	1.82 ± 0.17b	2.74 ± 0.28y	2.82 ± 0.33z
C20:2ω-6	0.22 ± 0.07a	0.14 ± 0.08b	0.41 ± 0.08y	0.59 ± 0.15y
C20:3ω-6	1.07 ± 0.09a	0.48 ± 0.11b	1.23 ± 0.10y	1.28 ± 0.05y
C20:4ω-6	11.10 ± 0.73a	11.07 ± 0.68a	10.14 ± 0.14y	11.03 ± 0.16y
C20:5ω-3	7.02 ± 0.43a	13.10 ± 0.75b	7.06 ± 0.11y	8.08 ± 0.13z
C22:3ω-3	0.12 ± 0.03a	1.06 ± 0.05b	0.16 ± 0.06y	0.08 ± 0.05z
C22:5ω-3	4.15 ± 0.31a	5.14 ± 0.44b	3.73 ± 0.21y	2.03 ± 0.18z
C22:6ω-3	31.91 ± 1.84a	28.94 ± 1.25b	24.74 ± 1.19y	24.09 ± 1.22y
ΣPUFA	60.94 ± 1.93a	65.15 ± 2.11b	55.30 ± 1.46y	56.81 ± 1.57y
Σω3	44.65	50.06	38.43	37.10
Σω6	16.29	15.09	16.87	19.71
Σ$\omega 3/\omega 6$	2.74	3.31	2.27	1.88

The values are shown as mean ± SD. Mean values are averages of three replicates.
Different letters in the same row and values in male and female represent significant statistical differences, $P < 0.05$.
SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

Logue et al. [43] found increased proportions of C20:4ω-6 and C22:6ω-3 as a result of cold adaptation while the proportion of saturated fatty acids fell in phospholipid. The fatty acid composition correlates well with the temperature-adaptive interspecies differences in membrane physical structure. The increases in the PUFA content of phospholipids may be due to cold weather. Phospholipids, particularly their fatty acid composition, are the principle factors that define the physical properties of the membrane. Fluidity of the membrane is essential to establish an appropriate setting for the membrane to perform its functions. The fluidity is in part dependent on the degree of unsaturation of fatty acids. Furthermore, the adaptation capabilities of fish and other poikilotherms to various environmental temperatures are to a large extent shaped by the same factor, that is, the degree of unsaturation of fatty acids [44]. The ratio of unsaturated fatty acids changes depends on seasonal temperature changes. For instance, as the weather gets colder in winter, an increase in unsaturation

Table 4: Comparison of males and females in terms of muscle and liver fatty acid composition of triacylglycerol in *L. lepidus*.

Fatty acids	Muscle		Liver	
	Male	Female	Male	Female
C12:0	0.04 ± 0.01a	0.18 ± 0.03b	0.22 ± 0.04y	0.19 ± 0.02z
C13:0	1.42 ± 0.06a	1.83 ± 0.10a	1.68 ± 0.05y	1.96 ± 0.01z
C14:0	1.68 ± 0.05a	1.44 ± 0.02a	2.44 ± 0.13y	2.42 ± 0.17z
C15:0	0.45 ± 0.05a	0.57 ± 0.06b	1.25 ± 0.06y	1.22 ± 0.08z
C16:0	20.41 ± 0.30a	17.65 ± 0.41b	22.15 ± 0.39y	23.47 ± 0.25z
C17:0	1.33 ± 0.08a	1.47 ± 0.01b	1.43 ± 0.07y	1.42 ± 0.08y
C18:0	7.15 ± 0.13a	8.43 ± 0.21b	13.01 ± 0.31y	13.05 ± 0.43z
ΣSFA	32.48 ± 0.92a	31.57 ± 1.02b	42.18 ± 1.65y	43.73 ± 1.85y
C16:1ω-7	13.26 ± 0.25a	13.06 ± 0.11a	11.23 ± 0.09y	10.45 ± 0.28z
C18:1ω-9	23.12 ± 0.76a	23.04 ± 0.99b	20.11 ± 0.42y	21.03 ± 0.72z
C20:1ω-9	3.12 ± 0.49a	3.15 ± 0.45a	3.03 ± 0.53y	3.02 ± 0.48y
ΣMUFA	39.50 ± 1.13a	39.25 ± 1.83a	34.37 ± 1.91y	34.50 ± 1.77z
C18:2ω-6	4.89 ± 0.19a	4.49 ± 0.14a	3.39 ± 0.87y	2.51 ± 0.74z
C18:3ω-6	1.24 ± 0.11a	1.59 ± 0.18b	0.81 ± 0.13y	0.88 ± 0.10y
C18:3ω-3	0.85 ± 0.11a	0.64 ± 0.18b	1.24 ± 0.31y	1.13 ± 0.35z
C20:2ω-6	0.25 ± 0.17a	0.47 ± 0.20b	0.38 ± 0.08y	0.44 ± 0.15z
C20:3ω-6	2.10 ± 0.13a	2.12 ± 0.10a	0.46 ± 0.12y	0.34 ± 0.20y
C20:4ω-6	1.14 ± 0.03a	1.16 ± 0.07a	2.61 ± 0.16y	2.13 ± 0.11z
C20:5ω-3	6.93 ± 0.48a	6.14 ± 0.65a	4.83 ± 0.15y	5.05 ± 0.011y
C22:3ω-3	0.11 ± 0.03a	0.19 ± 0.05b	0.09 ± 0.04y	0.02 ± 0.04z
C22:5ω-3	2.02 ± 0.11a	2.14 ± 0.24a	1.51 ± 0.31y	1.17 ± 0.33z
C22:6ω-3	8.49 ± 1.14a	10.24 ± 1.25b	8.13 ± 1.10y	8.10 ± 1.28y
ΣPUFA	28.02 ± 1.68a	29.18 ± 1.71b	23.45 ± 1.12y	21.77 ± 1.03z
Σω3	18.40	19.35	15.80	15.47
Σω6	9.62	9.83	7.65	6.30
Σω3/ω6	1.91	1.96	2.06	2.45

The values are shown as mean ± SD. Mean values are averages of three replicates.
Different letters in the same row and values in male and female represent significant statistical differences, $P < 0.05$.
SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid.

degree in body fat is often observed. A common way that fish uses to achieve such increase is through the conversion of saturated fatty acids of phospholipids in biological membrane into their corresponding mono- and dienic fatty acids. This mechanism is vital for fish to survive. Through this mechanism, regardless of the environmental temperature, biological membranes can consistently carry out their regular functions [20].

Fish oil can be obtained from eating fish or by taking supplements. Fish oil supplements are usually made from marine fishes. This is because marine fish are especially richer in omega 3 than those of freshwater. On the other hand, Ahlgren et al. [45] reported high ω3/ω6 ratio (5.83%) in the freshwater whitefish (*Coregonus* spp.). Our study suggests that female *L. lepidus* especially may also be used to produce fish oil supplements from freshwater fish combined with vegetable oils (e.g., sunflower, soybean, canola, walnut, flaxseed, and olive) and food sources (e.g., walnuts, flaxseeds, and olives) that are rich in C18:3ω-3 (α-linolenic acid) in a healthy diet for the general population [46]. In addition, many marine fish types require 20:5ω-3 and 22:6ω-3 as part of their diet, as they do not have Δ^5 desaturase enzyme and cannot perform de novo 20:5ω-3 and 22:6ω-3 synthesis [44]. Therefore, fatty acids such as AA, EPA, and DHA are essential in diets of marine fish in order to establish a healthy development and growth [47]. In comparison, freshwater fish are superior to marine fish, as they have Δ^5 desaturase enzyme and are capable of de novo 20:5ω-3 and 22:6ω-3 synthesis. These results showed that fatty acid profiles of some freshwater fish are basically comparable to marine water fish in terms of sources of PUFAs.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Hindawi Publishing Corporation
Journal of Chemistry
Volume 2015, Article ID 243574, 7 pages
http://dx.doi.org/10.1155/2015/243574

Research Article

Detection of Residual Levels and Associated Health Risk of Seven Pesticides in Fresh Eggplant and Tomato Samples from Narayanganj District, Bangladesh

Md. Nur Alam,[1,2] M. Alamgir Zaman Chowdhury,[1] M. Sabir Hossain,[2] Mohammad Mijanur Rahman,[2] M. Abdur Rahman,[1] Siew Hua Gan,[3] and Md. Ibrahim Khalil[2,3]

[1] *Agrochemical and Environmental Research Division, Institute of Food and Radiation Biology, Atomic Energy Research Establishment, Savar, Dhaka 1344, Bangladesh*
[2] *Department of Biochemistry and Molecular Biology, Jahangirnagar University, Dhaka 1342, Bangladesh*
[3] *Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia*

Correspondence should be addressed to Md. Ibrahim Khalil; drmikhalil@yahoo.com

Received 12 February 2015; Revised 29 June 2015; Accepted 1 July 2015

Academic Editor: Nivia Coelho

Residual levels of seven frequently used pesticides were investigated in 140 samples of two common vegetables, eggplants and tomatoes, from agricultural fields in the Narayanganj district of Bangladesh. The analysis of pesticide residues was performed by high-performance liquid chromatography with photodiode array detection. A large percentage of the eggplants (50%) and tomatoes (60%) from the Narayanganj district were contaminated with pesticides, and all of the levels were above the *maximum residual limit* (MRL) proposed by the EC regulation. Diazinon was the most common (35%) pesticide detected in the vegetable samples at a concentration of 45–450 times higher than the MRL. The health risk index for diazinon was highest for both eggplant and tomato samples, which may be due to its physiochemical properties. Fenitrothion and linuron are the two second most common types of pesticides detected in the vegetable samples. Regular monitoring of the use of common pesticides on vegetables should be conducted.

1. Introduction

The contamination of food by chemicals is a public health concern worldwide, and pesticides are a chemical hazard associated with food contamination [1]. Pesticide refers to any substance or mixture of substances in the food of humans or animals, including any specified derivatives such as degradation and conversion products, metabolites, reaction products, and impurities, of toxicological significance [2]. Pesticide residues cause both short- and long-term toxic effects that are hazardous to health, especially at higher levels that can lead to toxicity. Headaches, nausea, irritation, vomiting, diarrhea, abdominal pain, and hypersensitivity are repeatedly reported impacts of acute pesticide exposure. Additionally, chronic pesticide exposure increases the risk of reproductive defects, neurodegenerative disorders, organ damage (kidney/liver), mutagenic and carcinogenic transformation, and endocrine disruption [3–5]. Children are more susceptible due to their small body size, immature immune systems, and rapid growth cycles, especially in the brain and nervous systems [6].

Pesticide exposure is increasing in Bangladesh due to the acreage of irrigated agriculture. Approximately 80 types of registered pesticides with 170 different trade names are commonly used in agriculture and public health sectors in Bangladesh [7]. Of this number, organophosphates comprise 60.4%, carbamates comprise 28.60%, organochlorines comprise 7.60%, and others comprise 3.4% of the total pesticides used [8]. The problem is compounded when the indiscriminate use of pesticides by farmers is made worse due to illiteracy and low levels of awareness of the hazardous effect of pesticides to human health [8, 9].

Several studies have demonstrated that vegetables sprayed with pesticides will absorb them internally, which can create adverse effects [10–12] when they are consumed by humans and animals. Therefore, the identification and quantification of pesticides in food are of increasing public interest. However, in Bangladesh very little information is available on pesticide levels in vegetables [13], although they are consumed almost daily by the general public. In this study, we investigated the health risk and residue contamination levels of five organophosphates (diazinon, dimethoate, fenitrothion, parathion, and phosphamidon), one carbamate (carbofuran), and one phenyl urea herbicide (linuron) in two common vegetables (eggplant and tomato) collected from agricultural fields in the Narayanganj district of Bangladesh.

2. Experiments

2.1. Collection of Vegetable Samples. The vegetables were collected between June 2011 and December and March 2011 from the agricultural fields of three districts from Narayanganj (Rupgonj, Sonargaon, and Arihajar Upazilas), which are industrial areas located close to Dhaka City. Ten samples each of eggplants and tomatoes were collected directly from selected fields in the sampling area. The samples were placed in sterile polyethylene bags and transported to the laboratory on ice. They were stored at 4°C until analysis (within 24 hours).

2.2. Sample Preparation. Each vegetable sample (10 g) was homogenized with an Ultra-Turrax macerator (IKA-Labortechnik, Janke & Kunkel GMBH & CO.KG, Germany) at high speed for 3 min using a 40 mL solvent mixture that consisted of double-distilled hexane : acetone (3 : 1) at room temperature. Anhydrous sodium sulfate (20.0 g) was added to remove any remaining moisture. The samples were then centrifuged for 5 min at 3000 rpm, and the supernatant was transferred to a clean, graduated cylinder for volume measurement. The organic extract was concentrated to 5 mL on a vacuum rotary evaporator (Buchi, Switzerland) using a water bath at 45°C and 3.63 psi. The extract was cleaned up in a heat-activated (150°C for 4 h) charcoal-silica gel-alumina (0.1 : 5.0 : 5.0) column followed by elution with a solvent mixture of dichloromethane (2%) in double-distilled hexane. The elute was concentrated to 1 mL in a rotary evaporator, followed by evaporation to dryness under a gentle nitrogen stream. The dried sample was reconstituted in acetonitrile (1 mL) prior to injection into high-performance liquid chromatography (HPLC).

2.3. Chromatographic Analysis. Analysis was conducted by a HPLC (Shimadzu, Japan) LC-10ADvp, equipped with an SPD-M 10 Avp attached to a photodiode array detector (Shimadzu SPD-M 10 Avp, 200–800 nm). A C18 Reverse Phase Alltech (250 × 4.6 mm, 5 μm) was used as the analytical column, and the column temperature was maintained at 30°C. Acetonitrile in distilled water (70 : 30) was used as the mobile phase at a flow rate of 1.0 mL/min. Prior to HPLC analysis, the samples were filtered through 0.45 μm nylon (Alltech Associates, IL, USA) syringe filters. The chromatograms were obtained following manual injection (20 μL) of both standard and sample solution (Figure 2). The suspected pesticides were identified based on the retention times of the respective standard pesticide preparation.

For preparation of the calibration curve, equal volumes of several different concentrations of standard solutions were injected into the HPLC machine. Quantification was performed according to the calibration method described by Bhattacharjee et al. [13]. Pesticide residue levels were determined by the following equation:

$$R = \frac{(H_A \times V_{\text{END}} \times W_{\text{ST}})}{(H_{\text{ST}} \times V_I \times G)}, \quad (1)$$

where R is mg/kg for vegetable samples, G is sample weight (kg), V_{END} is terminal volume of the sample solution (mL), V_I is volume, V_{END} is volume injected into the HPLC (μL), W_{ST} is amount of standard pesticide injected with standard solvent (μg), H_A is peak area obtained from V_I (mm^2), and H_{ST} is peak area obtained from W_{ST} (mm^2).

2.4. Chromatographic Method Validation Protocol and Evaluation. The validation of the analytical method was performed according to the European Commission (EC) guidelines in terms of the accuracy, precision, and limit of quantification (LOQ) [14]. Accuracy was calculated by analyzing the samples of known concentration ($n = 3$) and comparing the estimated values with the actual values. Within our experimental limit, mean recovery for accuracy should be within 70–120%. For accuracy experiments, tomato and eggplant (20 g) were utilized as matrix after homogenization and addition of appropriate amount of pesticides standards at two different fortification levels (0.05 and 0.50 μg/mL). Control samples were processed along with spiked ones. Both sample and standard preparation were stood for one hour to permit equilibration. Equilibration was followed by extraction and cleanup process as described above. Percentage recovery was calculated by following equation:

$$\text{Percentage recovery} = \left[\frac{\text{CE}}{\text{CM}} \times 100\right], \quad (2)$$

where CE is the experimental concentration determined from the calibration curve and CM is the spiked concentration.

The precision was estimated by monitoring the repeated ($n = 6$) peak response and expressed by relative standard deviation (RSD). The acceptance criterion for precision is RSD ≤ 20%. The LOQ was evaluated as signal-to-noise ratios of 10 : 1 and was obtained by analyzing unspiked samples ($n = 10$). LOQ must be lower than reporting limit and MRL with mean recoveries within 70–120% and RSD ≤ 20%.

The average percentage recoveries ranged from 80.33% to 99.00% while precision ranged from 3.14% to 13.25%. In the present study, the LOQ was lower than reporting limit and MRL and ranged from 0.0036 to 0.0045 mg/kg for tomato and 0.0030 to 0.0037 for eggplant.

2.5. Health Risk Index (HRI). The health risk indices associated with pesticide residues were estimated from food consumption. The health risk index was calculated by the following equation [15]:

$$\text{HRI} = \left(\frac{\text{Estimated daily intake}}{\text{Acceptable daily intake}} \right). \quad (3)$$

The estimated daily intake was determined by multiplying the residual pesticide concentration (mg/kg) by the food consumption rate (kg/day) and dividing this number by a body weight of 60 kg for an average adult.

3. Results and Discussion

Large percentages of the eggplants (50%) and tomatoes (60%) were contaminated with pesticides (Table 1). This result is alarming because all of the contamination levels were above the maximum residual limit (MRL) proposed by the EC regulation [14].

The HRI in the eggplant samples was highest for diazinon eggplant, followed by dimethoate, phosphamidon, and fenitrothion (Table 2). The calculated HRI in eggplant for linuron eggplant was less than 1, which is considered safe for human health [16].

The HRI in the tomato samples was also highest for diazinon (Table 3), followed by phosphamidon and carbofuran. The calculated HRIs for fenitrothion and linuron in the tomato samples were less than 1 and were therefore considered safe for human health [16].

Approximately 23.53% of the contaminated samples contained multiple residues. Overall, only fenitrothion contamination in both eggplants and tomatoes caused no health risk, as indicated by the HRI.

We investigated the pesticide residue contamination levels of five organophosphates (diazinon, dimethoate, fenitrothion, parathion, and phosphamidon), one carbamate (carbofuran), and one phenyl urea herbicide (linuron) in two common vegetables, eggplants and tomatoes, from agricultural fields in the Narayanganj district of Bangladesh. Large percentages of the eggplants (50%) and tomatoes (60%) were contaminated with pesticides at levels exceeding the maximum residual limit (MRL) set by the EC regulation.

None of the eggplant samples were contaminated with carbofuran. However, carbofuran was detected in a single tomato sample at a level 33 times above the MRL. The carbofuran level of market vegetables in Pakistan, including eggplants and tomatoes, has been recorded as 0.00–0.39 mg/kg, 0.0–19.5 times higher than the MRL [17, 18]. However, the residual carbofuran level of market eggplant in Haryana, India, was within the MRL (0.009–0.020 mg/kg) [12].

Diazinon was the most common (35%) pesticide detected in the vegetable samples (Figure 1). The highest concentration for diazinon was 4.514 mg/kg in an eggplant sample. This level is approximately 450 times higher than the MRL, which is alarming. The highest diazinon level detected in tomatoes was 3.612 mg/kg, which is approximately 360 times higher than the MRL. This result is also alarming because tomatoes are usually consumed raw in salads. The residual diazinon level of tomatoes was 10 times higher than the MRL in Spain and 0.0–1.3 times higher than the MRL in Ghana [19, 20].

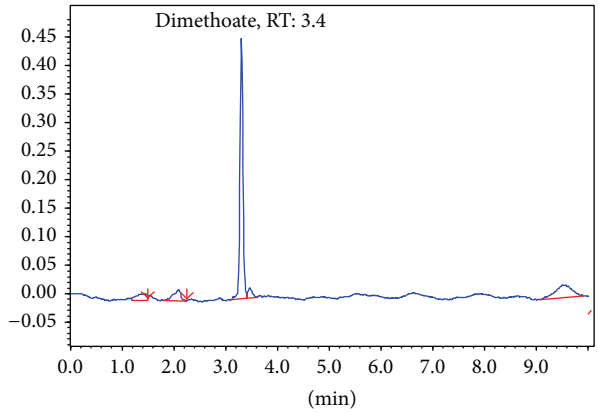

Figure 1: Typical chromatogram of a dimethoate standard (5 mg/kg) (retention time = 3.4 min).

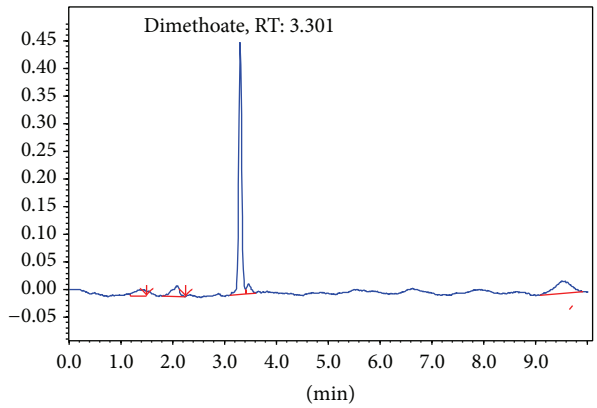

Figure 2: Chromatogram of VS-9 showing the presence of dimethoate (RT: 3.301 min).

Dimethoate was detected in only a single eggplant sample at 1.806 mg/kg but was not found in the tomato samples. The detected level was 90 times higher than the MRL established by the European Union [15] but only 3.6 times higher than the MRL limit (0.5 mg/kg) set by the Codex Alimentarius Commission [21]. Bempah et al. reported a residual dimethoate level of 0.007–0.019 mg/kg in Ghanaian tomatoes [22], while the residual level in Indian eggplants was 0.001–0.002 mg/kg [12].

Fenitrothion and linuron are the next two most common types of pesticides detected in the vegetable samples after diazinon. The highest fenitrothion level (1.88 mg/kg) was detected in a tomato sample, which was approximately 188 times higher than the MRL. The highest linuron level in an eggplant sample (1.07 mg/kg) was approximately 21 times higher than the MRL. In India, the fenitrothion residual level for both tomato and eggplant was reported to exceed the MRL by 33 times [23]. In Ghana, the residual fenitrothion level of cabbage was also 16.50 times higher than the MRL set by the Codex Alimentarius Commission [24].

Table 1: Residual levels of carbofuran, diazinon, dimethoate, fenitrothion, linuron, parathion, and phosphamidon in eggplant and tomato samples.

Sample	Concentration (mg/kg)						
	Carbofuran	Diazinon	Dimethoate	Fenitrothion	Linuron	Parathion	Phosphamidon
Standard	5	25	5	5	25	5	5
MRL	0.020	0.010	0.020	0.010	0.050	0.050	0.010
B-1	BDL	0.453 ± 0.033	BDL	BDL	BDL	BDL	BDL
B-2	BDL	BDL	BDL	BDL	BDL	BDL	BDL
B-3	BDL	BDL	BDL	BDL	BDL	BDL	BDL
B-4	BDL	4.514 ± 0.397	BDL	BDL	1.073 ± 0.054	BDL	1.416 ± 0.141
B-5	BDL	BDL	BDL	BDL	0.657 ± 0.059	BDL	BDL
B-6	BDL	BDL	BDL	BDL	BDL	BDL	BDL
B-7	BDL	BDL	BDL	BDL	BDL	BDL	BDL
B-8	BDL	BDL	BDL	BDL	BDL	BDL	BDL
B-9	BDL	BDL	1.806 ± 0.148	BDL	BDL	BDL	BDL
B-10	BDL	BDL	BDL	0.674 ± 0.038	BDL	BDL	BDL
T-1	BDL	1.888 ± 0.204	BDL	BDL	BDL	0.116 ± 0.003	BDL
T-2	BDL	2.611 ± 0.111	BDL	BDL	BDL	BDL	BDL
T-3	BDL	ND	BDL	BDL	BDL	BDL	BDL
T-4	0.673 ± 0.032	3.451 ± 0.417	BDL	BDL	BDL	BDL	BDL
T-5	BDL	BDL	BDL	0.657 ± 0.059	BDL	BDL	BDL
T-6	BDL	BDL	BDL	BDL	BDL	BDL	BDL
T-7	BDL	BDL	BDL	BDL	BDL	BDL	BDL
T-8	BDL	3.612 ± 0.169	BDL	BDL	0.540 ± 0.020	BDL	0.693 ± 0.062
T-9	BDL	BDL	BDL	1.888 ± 0.204	BDL	BDL	BDL
T-10	BDL	BDL	BDL	BDL	BDL	BDL	BDL

The results are expressed as the mean ± SD (standard deviation) of triplicates measurements. Here, B = eggplant; T = tomato; BDL = below the detection limit (0.001 ppm); MRL = maximum residual limit determined by EC regulation 396/2005 (from EU pesticide database) [15].

Table 2: Percentage contamination and HRI of the investigated pesticides for eggplant.

Pesticide	Contamination rate (%)	MRL (mg/kg)	ADI (mg/kg/day)	EDI (mg/kg/day)	HRI	Health risk
Carbofuran	0	0.02	0.003	—	—	—
Diazinon	20	0.01	0.001	0.0143	14.3	Yes
Dimethoate	10	0.02	0.001	0.0104	10.4	Yes
Fenitrothion	10	0.01	0.002	0.0039	1.9	Yes
Linuron	20	0.05	0.010	0.0050	0.5	No
Parathion	0	0.05	0.005	—	—	—
Phosphamidon	10	0.01	0.001	0.0081	8.1	Yes

MRL = maximum residual limit; HRI = human risk index; ADI = acceptable dietary intake; EDI = estimated dietary intake. The HRI was calculated by considering an average daily vegetable intake for a 60 kg adult as 0.345 kg/person/day [16, 36] and using the MRL determined by EC regulation 396/2005 (from the EU pesticide database) [15].

Table 3: Percentage contamination and HRIs of the investigated pesticides for tomato.

Pesticide	Contamination rate (%)	MRL (mg/kg)	ADI (mg/kg/day)	EDI (mg/kg/day)	HRI	Health risk
Carbofuran	10	0.02	0.003	0.0039	1.3	Yes
Diazinon	40	0.01	0.001	0.0166	16.6	Yes
Dimethoate	0	0.02	0.001	—	—	—
Fenitrothion	20	0.01	0.002	0.0073	0.7	No
Linuron	10	0.05	0.010	0.0031	0.3	No
Parathion	10	0.05	0.005	—	—	—
Phosphamidon	10	0.01	0.001	0.0040	4.0	Yes

MRL= maximum residual limit; HRI = human risk index; ADI = acceptable dietary intake; EDI = estimated dietary intake. The HRI was calculated by considering an average daily vegetable intake for an adult (60 kg) as 0.345 kg/person/day [15, 16] and using the MRL determined by EC regulation 396/2005 (from the EU pesticide database) [15].

Table 4: Physiochemical properties of the investigated pesticides [37, 38].

Name	Molecular formula	Molecular weight	Soil half-life (days)	Water solubility (mg/L)
Carbofuran	$C_{12}H_{15}NO_3$	221.30	50	351
Diazinon	$C_{12}H_{21}N_2O_3PS$	304.35	40	60
Dimethoate	$C_5H_{12}NO_3PS_2$	229.26	7	39,800
Fenitrothion	$C_9H_{12}NO_5PS$	277.24	4	30
Linuron	$C_9H_{10}Cl_2N_2O_2$	249.10	60	75
Parathion	$C_{10}H_{14}NO_5PS$	291.26	14	24
Phosphamidon	$C_5H_{12}NO_3PS_2$	229.26	17	1,000,000

Parathion was detected in only a single vegetable sample (tomato) at 0.116 mg/kg, approximately 2.3 times higher than the MRL. A similar multiclass pesticide residue analysis showed that parathion levels in market eggplants and tomatoes in Bangladesh exceeded the MRL by 0–6.4 and 0–4.6 times, respectively [25]. In Rajasthan, India, the residual parathion level was 1.2–3.6 times higher than the MRL (0.06–0.18 mg/kg) for tomatoes and 0–28.4 times higher than the MRL (0–1.42 mg/kg) for eggplants [26].

Phosphamidon was detected in a single sample for each of eggplant and tomato, with the highest level in an eggplant sample at 1.146 mg/kg. This level is approximately 140 times higher than the MRL, which is alarming. In several field vegetables in India, the residual phosphamidon level exceeded the MRL (0.51 mg/kg) by 51.0 times [27].

Several factors may contribute to the high occurrence of pesticide residues detected in the vegetable samples from the Narayanganj district. Bangladesh frequently has a weak control mechanism, and rampant pesticide overdosing is practiced to increase crop productivity [28]. Furthermore, the majority of farmers lack sufficient perception and knowledge about the nature of chemical pesticides and their effects on health. Therefore, consumers may unknowingly use vegetables and fruits containing uncontrolled levels of pesticide residues [8, 9, 29].

Fortunately, the soil degradation times of some pesticides, such as fenitrothion (4 days) and dimethoate (7 days), are relatively short, and it is possible that both pesticide levels would have dramatically decreased by the time the vegetable was consumed by the general public. This fact could also contribute to the relatively lower pesticide levels of these two types of pesticides seen in the analyzed samples. The differences in the soil half-life indicate that the natural decontamination of a pesticide also depends on its physiochemical properties. This may also explain the highest contamination seen with diazinon, which could actually be due to its relatively longer soil degradation time (40 days) and low water solubility (60 mg/L) (Table 4). The water solubility of phosphamidon is very high, indicating that proper washing of vegetables before consumption may be crucial for its removal.

The HRI is the ratio of the estimated dietary intake (EDI) to the accepted daily intake (ADI) and indicates whether the calculated amount of the pesticide residue exceeds the amount of the pesticide that can be consumed every day for a lifetime. Thus, an HRI value greater than 1 indicates that the estimated dietary intake exceeds the ADI and is considered unsafe for human health [16]. The HRI for diazinon was the highest in both eggplant and tomato samples, which may be due to its physiochemical property.

Not only were the levels very high, but some of the vegetable samples also contained multiresidues of different types of pesticides. Exposure to pesticides through contaminated food leads to a spectrum of adverse health effects that depend on the nature of the pesticide and on the amount and duration of exposure [30]. Symptoms of exposure to organophosphates such as diazinon, dimethoate, fenitrothion, parathion, and phosphamidon include miosis, urination, diarrhea, diaphoresis, lacrimation, excitation, and salivation [31]. Anxiety, depression, coma, and convulsions are acute psychological and behavioral effects of organophosphorus pesticide exposure, while chronic exposure leads to cognitive and emotional deficits. Because organophosphates act directly on the nervous system by inhibiting the enzyme acetylcholinesterase (AChE) [32], severe organophosphate exposure is clinically manifested by marked miosis and loss of the pupillary light reflex, fasciculations, flaccid paralysis, pulmonary rales, respiratory distress, and cyanosis with less than 10% of the normal value of serum cholinesterase [33]. Exposure to carbofuran, an anticholinesterase carbamate, leads to overstimulation of the nervous system due to direct inhibition of acetylcholinesterase. Symptoms of carbofuran overexposure in humans include headache, weakness, abdominal cramping, nausea, blurred vision, convulsions, tremor, and coma [34]. Linuron exhibits relatively low acute toxicity but is classified as an unquantifiable Group C carcinogen and shows some evidence of developmental and reproductive toxicity. In low concentrations, linuron exposure antiandrogenically alters sexual differentiation [35].

This is a small pilot study that successfully pasteurized the residual pesticide levels of seven commonly used pesticides in two field vegetables before they were transported to market. The actual levels of exposure to consumers may thus be different after they reach their homes and following proper washing. More comprehensive studies should be undertaken to determine the levels of different pesticide residues on other vegetables and fruits originating from different regions in Bangladesh and at several different sampling intervals. Regular monitoring of the use of common pesticides on vegetables should also be undertaken as this study indicated the presence of high residual pesticide levels that may pose a health hazard. The adoption of effective legislation for properly regulating pesticide use and increasing awareness and technical know-how in the farming community should be incorporated in Bangladesh.

4. Conclusion

Large percentages of the eggplants (50%) and tomatoes (60%) from the Narayanganj district were contaminated with pesticides, and all of the levels were above the MRL proposed by the EC regulation. Diazinon was the most common (35%) pesticide detected in the vegetable samples. The HRI for diazinon was the highest for both eggplant and tomato samples, which may be due to its physiochemical property. Fenitrothion and linuron are the two second most common types of pesticides detected in the vegetable samples. Regular monitoring of the use of common pesticides on vegetables should be undertaken.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment

This study was financially supported by Universiti Sains Malaysia RU Grants (1001/PPSP/815073).

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