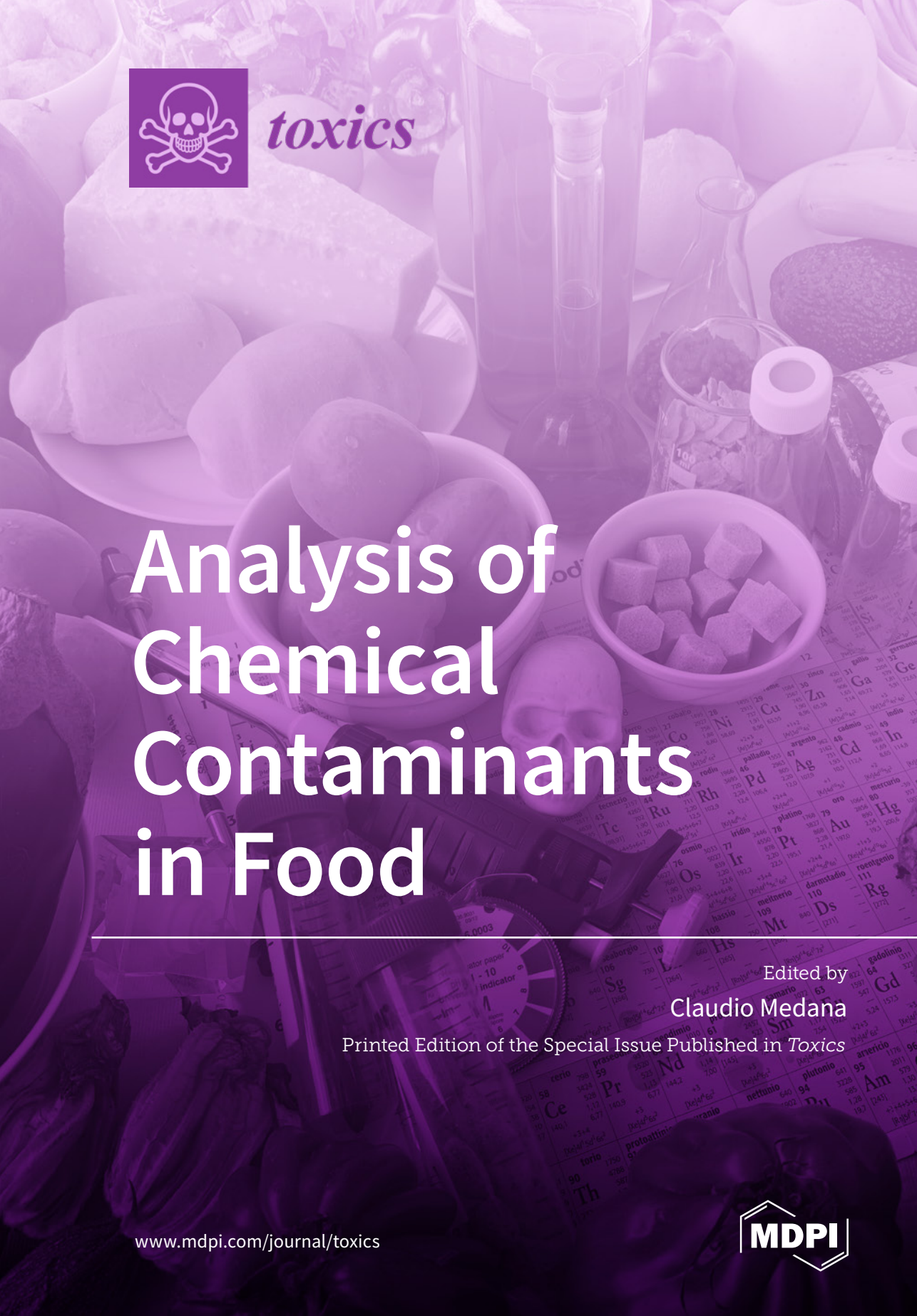

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Analysis of Chemical Contaminants in Food

Edited by
Claudio Medana

Printed Edition of the Special Issue Published in *Toxics*

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Analysis of Chemical Contaminants in Food

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Special Issue Editor

Claudio Medana

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Special Issue Editor
Claudio Medana
Department of Molecular
Biotechnology and Health
Sciences, University of Torino
Italy

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

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About the Special Issue Editor

Claudio Medana is Full Professor of the Analytical Chemistry, Molecular Biotechnology, and Health Sciences Department, University of Torino, Italy. He is in charge of the Unit of Mass Spectrometry of the department and coordinator of the MS facility (open access lab). His recent research activity is focused on the development of analytical mass spectrometry methods for the identification, structure determination, and quantification of bioactive molecules. His past activity was focused on synthesis and analytical characterization of new potential drugs. He serves as mentor and tutor of Master's students in Chemistry, Pharmacy, and Biotechnology and PhD students in Chemical and Materials Sciences. The most recent research topics are:

- Assessment of the transformation of bioactive molecules by HPLC coupled to high-resolution multistage mass spectrometry to study drug metabolism and the environmental fate of drugs and toxics.
- Studies about MS fragmentation pathways and application to structural determination of synthetic intermediates or unknown molecules.
- Targeted metabolomics determination of biochemicals, including the endogenous toxins formed by interaction of sugars and proteins, the enzymatic products of drugs and lipid molecules, and the bioactive components of food.
- Determination of phytochemical profiles to characterize bioactive principles of plant preparations.

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Editorial

Analysis of Chemical Contaminants in Food

Claudio Medana

Molecular Biotechnology and Health Sciences Department, Università degli Studi di Torino, Via Pietro Giuria 5, 10125 Torino, Italy; claudio.medana@unito.it

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Food chemical analysis is recognized as a unique tool for the characterization of nutritional value, quality and safety of foods and feeds. It is of growing importance to have an accurate knowledge of the global chemical composition of food and in particular of the chemical entities known as molecular (bio)markers. Quantitative determination of chemical markers is needed for risk assessment both in food and in environmental research after toxicological characterization of natural and synthetic chemicals. The potential adverse effects of chemical species, i.e., their hazard, can be classified as acute, subacute, subchronic and chronic. However, in all cases, the risk management does refine the toxicological evaluation by the chemical quantitation and the toxicokinetic assessment.

On these bases, we proposed this special issue, in order to have a view on emerging analytical methodologies to perform toxicology marker determination.

The drive from the great development of methods in the industrial, pharmaceutical and environmental fields has extended the impact of chromatographic, spectrometric and spectroscopic techniques to the study of composition and contamination of foods. The interdisciplinary nature of analytical laboratories has allowed the extension of existing applications to the targeted and untargeted measurement of minor food components. These molecules are useful as toxicity biomarkers and in describing contamination in general.

This special issue contains a limited anthology of these kind of methods, but is highly representative of a broad worldwide overview, by collecting authors from ten different countries and four continents. Very different analytes are described, from volatile compounds to heavy metals and from highly polar substances to classical heterocyclic and organic aromatic contaminants. A large variety of analytical techniques is represented, including sample preparation and clean-up methodologies; the main current chromatographic-hyphenated spectroscopies together with mass spectrometry are more frequently reported. Finally, a differentiated variety of foods was the subject of the presented works: meat, fishery products, fruits and miscellaneous beverages are included in the studied matrices. Some applications of foods that require special care, such as infant formulae and human breast milk, are also presented.

Summarizing the highlights of this special issue:

- Okaru and Lachenmeier focused the attention on potential human carcinogenic compounds occurring in beverages, both newly formed by food processing, such as furfuryl alcohol, and naturally present, such as myrcene, a well-known plant metabolite [1]. NMR spectroscopy was employed for quantitative evaluation and obtained data were compared with a review of the literature on the topic.
- The work of Zeiner and Juranović Cindrić is centered on the determination of metal contaminants in Croatian wild fruits, such as lingonberries, blueberries and rosehips. Microwave digestion of samples and subsequent ICP-AES (Inductively coupled plasma–atomic emission spectroscopy) determination of metal species are discussed. Aluminum, nickel and lead levels were contextualized by reporting the results obtained for analyzed samples and wild fruit consumption as a function of acceptable intake [2].

- Fattore et al. applied complementary mass spectrometry-based methods (HPLC-MS/MS and HRGC-HRMS) to investigate polyhalogenated contaminants of Mediterranean marine fish and seafood of commercial interest at different trophic levels of the food chain. In particular, the authors searched for perfluorinated acids and brominated dioxins and furans as persistent organic pollutants, showing a low but noteworthy concentration of these molecules [3].
- Nuñez et al. proposed a useful spectral discrimination of isomeric food pollutants in order to distinguish compounds with high structural similarity. Isomeric compounds share elemental composition and mass value and are not distinguishable by first generation mass spectrometry, even if high resolving power instruments are used. By means of tandem mass spectrometry (MS/MS), the studied isomer pairs are clearly differentiated on the basis of the different fragmentation pathways of the corresponding ions formed in MS ionization [4].
- Raina-Fulton and Mohamad faced the problem of challenging food matrices as nutraceutical products. The necessity to overcome the presence of large number/concentrations of interferents leading to a severe matrix effect in HPLC-MS encouraged the authors to develop a complete analysis procedure. Applying these concepts to conazole fungicides as contaminants, a complete pathway was developed. At first the preanalytical procedure (pressurized extraction) was studied to optimize clean-up, then a HPLC-MS method was developed to identify conazole fungicide residues in Matcha tea [5].
- In their paper, Tran-Lam et al. applied a central composite design (CCD) to select the most valued operating condition to perform the extraction and gascromatographic-mass spectrometric determination of plasticizers in non-alcoholic beverages in Vietnam. The method was successfully applied to the determination of ten phthalate esters, obtaining satisfactory recoveries and limits of determination [6].
- The work of Lachenmeier et al. exploited analytical chemistry to monitor the relative concentration of four heat-induced coffee contaminants; acrylamide, furfuryl alcohol (FA), furan and 5-hydroxymethylfurfural (HMF). Different roasting modes were studied showing a direct/inverse relationship between roasting degree and analyte concentration depending on the molecule [7].
- The modulation of extraction parameters, the use of demulsifiers and the validation of the analytical performances were applied by Vichapong et al. to the analysis of polycyclic aromatic hydrocarbon residues (potential carcinogens formed by cooking) in grilled pork samples from Thailand [8].
- Vella and Attard described a multi-analyte and multi-technique study to characterize infant foods. Markers of toxicity may be neo-formed contaminants or derived from raw materials or processing and were quantified using various techniques. UV and ICP-AES spectroscopy applications were proposed and applied to evaluate both metal and HMF contaminants in infant foods and formulae sold in Malta [9].
- The paper of Smadi et al. reports the result of a study aimed to detect toxic residues in breast milk of Syrian refugee lactating mothers in Lebanon. The aim of the project was the potential identification of a relationship between antibiotic and pesticide residues and socio-demographic characteristics, dietary and smoking habits and medical history of participating lactating mothers. The results considered the breast milk collected from Syrian refugee lactating mothers as safe from chemical contamination [10].
- Finally, Dal Bello et al. reported the development of mass spectrometry methods (HPLC-MS and GC-MS) to detect illicit treatment of fishery products with hydrogen peroxide. Quantitation of residues of hydrogen peroxide was indirectly achieved by GC-MS monitoring of the reaction product between H_2O_2 and anisole to afford guaiacol. Biogenic amines, such as trimethylamine-N-oxide (TMAO), trimethylamine (TMA), dimethylamine (DMA), and cadaverine (CAD), were detected and measured by ion pair HPLC-MS. The developed analytical methods

were validated and shown to be suitable to detect the illicit management of fishery products with hydrogen peroxide [11].

In summary, this collection of research articles provides a valuable selection of tools for food investigators to compare analytical methodologies and applications useful in the evaluation of toxicity markers.

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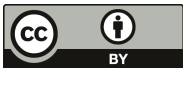

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Communication

The Food and Beverage Occurrence of Furfuryl Alcohol and Myrcene—Two Emerging Potential Human Carcinogens?

Alex O. Okaru [1,2] and Dirk W. Lachenmeier [2,*]

[1] Department of Pharmaceutical Chemistry, University of Nairobi, P.O. Box 19676-00202, Nairobi, Kenya; alex.okaru@gmail.com

[2] Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weissenburger Strasse 3, 76187 Karlsruhe, Germany

* Correspondence: lachenmeier@web.de; Tel.: +49-721-926-5434; Fax: +49-721-926-5539

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Abstract: For decades, compounds present in foods and beverages have been implicated in the etiology of human cancers. The World Health Organization (WHO) International Agency for Research on Cancer (IARC) continues to classify such agents regarding their potential carcinogenicity in humans based on new evidence from animal and human studies. Furfuryl alcohol and β-myrcene are potential human carcinogens due to be evaluated. The major source of furfuryl alcohol in foods is thermal processing and ageing of alcoholic beverages, while β-myrcene occurs naturally as a constituent of the essential oils of plants such as hops, lemongrass, and derived products. This study aimed to summarize the occurrence of furfuryl alcohol and β-myrcene in foods and beverages using literature review data. Additionally, results of furfuryl alcohol occurrence from our own nuclear magnetic resonance (NMR) analysis are included. The highest content of furfuryl alcohol was found in coffee beans (>100 mg/kg) and in some fish products (about 10 mg/kg), while among beverages, wines contained between 1 and 10 mg/L, with 8 mg/L in pineapple juice. The content of β-myrcene was highest in hops. In conclusion, the data about the occurrence of the two agents is currently judged as insufficient for exposure and risk assessment. The results of this study point out the food and beverage groups that may be considered for future monitoring of furfuryl alcohol and β-myrcene.

Keywords: furfuryl alcohol; β-myrcene; carcinogens; occurrence

1. Introduction

The production and processing of foods and beverages may invariably lead to significant changes in the chemical composition of the products. The Maillard reaction—which yields furanic compounds such as furfural and 5-hydroxymethylfurfural (HMF) and furfuryl alcohol, among other products—is common during processes that involve heating or roasting [1–5]. Furfuryl alcohol is a food contaminant which occurs in significant amounts in thermally processed foods such as coffee, fruit juices, baked foods; in cask-stored alcoholic beverages such as wines, wine-derived spirits such as brandy, and whiskies as a result of enzymatic or chemical reduction of furfural [6–8]; and in butter and butterscotch when furfuryl alcohol is used as a flavouring agent [9]. Furfuryl alcohol may also be formed from quinic acid or 1,2-enediols as precursors during the heating of foods such as coffee beans [5] . In acidic conditions, furfuryl alcohol polymerizes to aliphatic polymers that give a brown colouration to foods [5].

Myrcene is a terpenoid compound that exists in two forms—β and α, with the former occurring naturally in essential oils of plants such as hops, bay, lemongrass [10,11], and orange juice [12],

and is permitted for use as a flavouring additive of food both by the United States Food and Drug Administration (FDA) since 1965 and by the European Council since 1974. β-Myrcene is also an ingredient in the preparation of olefinic scents such as menthol, and the alcohols linalool, nerol, and geraniol [13], found in household items.

Analysis of furfuryl alcohol can be done by either gas and liquid chromatography with UV, biosensor, or fluorescence detection [5,6,14–17], while β-myrcene is typically determined using gas chromatography with mass spectrometry or flame ionization detection [18–21].

Diet is considered to be the greatest source of human exposure to furfuryl alcohol and β-myrcene. However, unlike the furanic compounds furan, 5-hydroxymethylfurfural (HMF), and furfural, and other food and beverage constituents such as ethanol, ethyl carbamate, or polycyclic aromatic hydrocarbons for which extensive occurrence data is available [22–26], there is a paucity of information on human dietary exposure to furfuryl alcohol and β-myrcene. The two agents are due for assessment as to their carcinogenicity by the International Agency for Research in Cancer (IARC) expert working group in their meeting to be held in June 2017. This study aims to provide an overview of the occurrence of furfuryl alcohol and β-myrcene in foods and beverages.

2. Materials and Methods

Occurrence data on furfuryl alcohol and β-myrcene were searched in the following databases: PubMed, Toxnet and ChemIDplus (U.S. National Library of Medicine, Bethesda, MD, USA), Web of Science (Clarivate Analytics, Philadelphia, PA, USA), and IPCS/INCHEM (International Programme on Chemical Safety/Chemical Safety Information from Intergovernmental Organizations, WHO, Geneva, Switzerland). Reference lists of all articles were hand-searched for relevant studies not included in the original search results. The literature sources (including abstracts) were evaluated using Mendeley (Mendeley Inc., New York, NY, USA). By manual screening, relevant articles were identified and ordered in full-text. No unpublished study was identified.

Additional data on the occurrence of furfuryl alcohol was also obtained from in-house analysis of 30 coffee (roasted coffee as beans, powder, or pods), 15 bread, 20 wine, and 50 alcoholic spirit samples (whiskey, brandy, and rum) submitted to our laboratory in the context of official control using nuclear magnetic resonance spectroscopy (NMR) [27]. For this, spectra previously acquired for other purposes were re-quantified for furfuryl alcohol. The coffee samples were analysed according to Monakhova et al. [28]. Quantification was conducted using the integral of the CH group at the C5 resonance of furfuryl alcohol (δ 7.47–7.35 ppm) in relation to the internal standard 1,2,4,5-tetrachloro-3-nitrobenzene (δ 7.75–7.72 ppm). Quantification was conducted using TopSpin 3.2 (BrukerBioSpin GmbH, Rheinstetten, Germany) and Mestrenova V. 11.0.2 (Mestrelab Research, Santiago de Compostela, Spain) [29]. For evaluation of spirits, the NMR method of Monakhova et al. [27] was applied. The NMR methods achieved a limit of detection (LOD) of 3.2 mg/L and limit of quantification (LOQ) of 8.6 mg/L. The results of NMR must be interpreted as semi-quantitative, because only one single non-overlapped signal of furfuryl alcohol was available for quantification. Identity was confirmed by spiking with pure furfuryl alcohol to authentic samples, but co-occurrence of compounds with a similar chemical shift cannot be completely excluded. The statistical parameters of mean, median, and percentiles (90th, 95th, 97.5th, and 99th) were used to describe the occurrence data. Similar NMR analysis of β-myrcene (e.g., in hops) was not possible due to considerable matrix interferences of all relevant signals. The concentration of β-myrcene in compounded products such as beer was below the detection limit of NMR.

3. Results

This study summarizes the occurrence of furfuryl alcohol and β-myrcene in various foods and beverages. Limited studies on β-myrcene (7) were observed compared to 19 studies for furfuryl alcohol. Meta-analysis was not possible due to the sparsity of studies for each type of food and beverage. The occurrence of furfuryl alcohol was recorded in many foods and beverages that had

been subjected to thermal processing. The literature studies summarized in Table 1 were extended by inclusion of original results from our own analyses on furfuryl alcohol in 30 coffee, 15 bread, 20 wine, and 50 aged alcoholic spirit samples. From these, only coffee samples were positive (average furfuryl alcohol content of 251 mg/kg), while all other samples were below the detection limit of the method. A typical spectrum of a coffee sample is shown in Figure 1.

Out of the seven studies on β-myrcene, four were in hops and related products, while two were in beer, and the final reference reported about general use levels in various foods/beverages. Chewing gum, gelatin, beer, and hops were suggested as products with high concentration of β-myrcene. The studies are summarized in Table 2.

Table 1. Furfuryl alcohol content in various foods and beverages.

Category [Reference]	N	Furfuryl Alcohol Concentration							Units [a]
		Mean	Median	P90	P95	P97.5	P99	Maximum	
Roasted coffee/This study	30	251	243	342	392	402	406	408	mg/kg
Bread/This study	15	<LOD [b]	-	-	-	-	-	-	mg/kg
Wine/This study	20	<LOD [b]	-	-	-	-	-	-	mg/L
Spirits/This study	50	<LOD [b]	-	-	-	-	-	-	mg/L
Sweet potatoes [4]	1	0.014	-	-	-	-	-	-	mg/kg
Wine [7]	8	3.4	2.9	7.3	8.5	9.0	9.4	9.6	mg/L
Baked goods [9] [d]	-	110	-	-	-	-	-	-	ppm
Spirits [9] [d]	-	10	-	-	-	-	-	-	ppm
Candy [9] [d]	-	59	-	-	-	-	-	-	ppm
Ice cream/ices [9] [d]	-	88	-	-	-	-	-	-	ppm
Beverages [9] [d]	-	19	-	-	-	-	-	-	ppm
Honey [14]	1	1.6	-	-	-	-	-	-	mg/kg
Popcorns [15]	6	0.064	0.067	0.081	0.081	0.082	0.082	0.082	mg/kg
Fried fish [16]	1	10.5	-	-	-	-	-	-	mg/kg
Breaded fish products [17]	4	10.3	8.8	16	18	18	19	19	mg/kg
Wine [30]	6	1.51	0.89	1.57	1.60	1.62	1.63	1.64	mg/L
Vinegar [31] [c]	27	0.35	0.28	0.58	0.59	0.59	0.59	0.59	mg/L
Vinegar [32] [c]	9	0.34	0.28	0.58	0.59	0.59	0.59	0.59	mg/L
Coffee [33]	7	49	49	64	67	68	69	70	mg/kg
Instant coffee [34]	1	267	-	-	-	-	-	-	mg/kg
Roasted coffee [34]	1	564	-	-	-	-	-	-	mg/kg
Pineapple juice [34]	1	8.3	-	-	-	-	-	-	mg/L
Rice cakes [35]	2	2, 2.3	-	-	-	-	-	2.3	mg/kg
Bread [36]	1	187	-	-	-	-	-	-	mg/kg
Toasted almonds [37]	3	6.4	6.0	8.3	8.6	8.7	8.8	8.9	mg/kg
Non-fat dried milk [38]	1	15	-	-	-	-	-	-	mg/kg
Corn tortilla chips [39]	1	0.54	-	-	-	-	-	-	mg/kg
Cocoa powder [40]	1	0.02	-	-	-	-	-	-	mg/kg
Palm sugar [41]	1	0.14, 0.52	-	-	-	-	-	-	mg/kg

[a] The ambiguous unit ppm was interpreted as mg/L for liquids/beverages and as mg/kg for solid foods. [b] All samples evaluated (spirits types whiskey, rum, brandy as well as various wines and breads) were below the limit of detection (LOD; 3.2 mg/L). [c] Studies from the same research group with probably overlapping data. [d] Number of samples not provided. The data are suggested as being "usual concentrations" found in these food/beverage types.

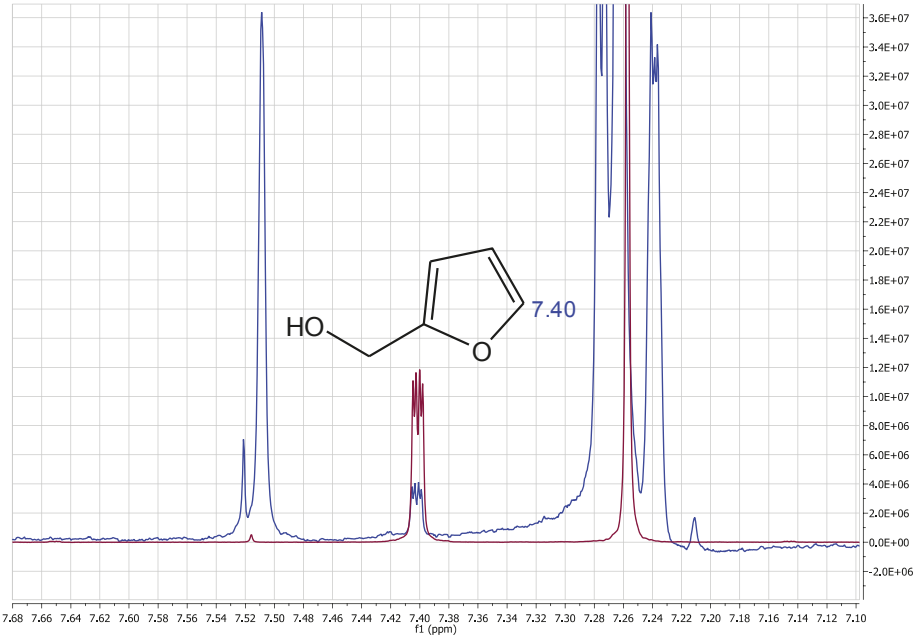

Figure 1. NMR spectra of an authentic coffee sample (**blue line**) containing 408 mg/kg of furfuryl alcohol compared to the reference standard (**red line**).

Table 2. β-Myrcene content in various matrices.

Matrix [Reference]	N	Concentration							Units [a]
		Mean	Median	P90	P95	P97.5	P99	Maximum	
Hops oil [18]	4	479	424	776	852	890	912	927	mg/L
Hops [42]	12	5489	4804	8580	9450	9972	10,285	10,494	mg/kg
Hops [43]	8	15	14	28	29	29	29	29	µg/L
Hops [44]	12	1082	705	2369	2795	3043	3191	3290	mg/kg
Pilsner beer [45]	2	46, 79	-	-	-	-	-	79	µg/L
Beer [46]	2	0.5, 0.6	-	-	-	-	-	0.6	µg/L
Alcoholic beverages [47] [b]	-	1.1	-	-	-	-	-	-	mg/L
Baked goods [47] [b]	-	10	-	-	-	-	-	-	mg/kg
Chewing gum [47] [b]	-	116	-	-	-	-	-	-	mg/kg
Condiment [47] [b]	-	5	-	-	-	-	-	-	mg/kg
Frozen dairy [47] [b]	-	12	-	-	-	-	-	-	mg/kg
Gelatin, pudding [47] [b]	-	20	-	-	-	-	-	-	mg/kg
Meat products [47] [b]	-	5	-	-	-	-	-	-	mg/kg
Non-alcoholic beverages [47] [b]	-	8	-	-	-	-	-	-	mg/L
Soft candy [47] [b]	-	6	-	-	-	-	-	-	mg/kg

[a] The ambiguous unit ppm was interpreted as mg/L for liquids/beverages and as mg/kg for solid food. [b] Number of samples not provided. The data are suggested as being "usual concentrations" found in these food/beverage types.

4. Discussion

4.1. Occurrence of Furfuryl Alcohol

The concentration of furfuryl alcohol was highest in coffee (beans 564 mg/kg and 267 mg/kg in instant coffee powder). Our new data on coffee with an average of 251 mg/kg and a maximum of 408 mg/kg corresponds well to the previous data. Green coffee is free of furfuryl alcohol (confirmed in eight samples with non-detectable levels), so the occurrence of furfuryl alcohol in coffee has been

confirmed as being attributable to the roasting process [5]. This observation parallels the high content of furan found in coffee compared to other foods [24]. Other thermally processed foods, such as bread (187 mg/kg), baked goods (110 mg/kg), ice cream/ices (88 mg/kg), and fried fish (about 10 mg/kg) were also found to contain detectable amounts of furfuryl alcohol. Among beverages, higher concentrations of furfuryl alcohol arising from aging in oak barrels [30] were found in spirits (10 mg/L) than in wine (1.5–3.4 mg/L). However, the content was lower compared to bread, baked goods, fish, and coffee. Relatively lower concentrations (less than 1 mg/kg) were observed in palm sugar, chips, popcorns, sweet potatoes, and vinegar. The variation in the concentration of furfuryl alcohol in the foods/beverages may be related to the type of raw materials and processing conditions used. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) set a group acceptable daily intake (ADI) of 0–0.5 mg/kg body weight for furfuryl alcohol, and suggested the compound as being of no safety concern at current levels of intake when used as a flavouring agent [48]. Despite the concentrations reported here being low for a majority of individual foods and beverages, a cumulative amount of furfuryl alcohol may be ingested from consuming a combination of different foods and beverages. According to the National Toxicology Program (NTP) report [49], exposure of male mice to 32 ppm (equivalent to 60 mg/kg bw/day [50]) of furfuryl alcohol was found to induce tumours of renal tubular epithelium. The postulated mechanism of carcinogenicity of furfuryl alcohol is through activation by sulfotransferases resulting in the formation of a 2-methylfuranyl-DNA adduct [50,51]. According to estimation from the typical intake levels of the food items listed in Table 1, concentrations of toxicological concern are probably not reached. However, food legislation demands to reduce food contaminants as low as reasonably achievable (ALARA principle). More data are clearly necessary to provide exposure estimations and risk assessment for this compound.

4.2. Occurrence of β-Myrcene

A majority of the studies on β-myrcene are qualitative, and the few quantitative data were focusing on hops and beers, despite the widespread occurrence of myrcene in many plants that are used in foods and beverages. Hop oil and chewing gum were found to contain the highest content of β-myrcene compared to other products. The low concentration of β-myrcene in beers is plausible, since there is a very variable extraction of β-myrcene from hops to beer postulated to be in the range of 0.5%–5.6% from cones and 8.4%–25.8% from pellets [52], and hops contain other volatile components such linanool, humulene, and α-terpineol in higher proportions than β-myrcene. Additionally, β-myrcene may be destroyed during the heating processes, and thus a low level is expected in the final beer. The NTP report links β-myrcene with neoplasms of the kidney in male rats and liver cancer in male mice [53]. The daily per capita intake (eaters only) for β-myrcene was estimated as being 164 μg corresponding to 3 μg/kg bw [54].

5. Conclusions

Consistent with the relatively high amounts of furfuryl alcohol (above 10 mg/kg) observed in coffee, baked goods, bread, fish, and some spirit drinks, monitoring these items for furfuryl alcohol is advisable for comprehensive estimation of exposures and the risk of these foods, while more research on the occurrence of β-myrcene in foods and beverages in general is required for meaningful risk assessment.

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Harmful Elements (Al, Cd, Cr, Ni, and Pb) in Wild Berries and Fruits Collected in Croatia

Michaela Zeiner [1,2,*] and Iva Juranović Cindrić [3]

[1] Man-Environment-Technology Research Centre, School of Science and Technology, Örebro University, Gymnastikgatan 1, 70182 Örebro, Sweden
[2] Division of Analytical Chemistry, Department of Chemistry, BOKU—University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria
[3] Department of Chemistry, Faculty of Science, University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia; ijuranovic@chem.pmf.hr
* Correspondence: michaela.zeiner@oru.se; Tel.: +46-019-303779

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Abstract: Fruits and vegetables are considered a beneficial contribution to the human diet. Especially, berries contain a great deal of bioactive compounds, such as anthocyanins, organic acids, tannins, phenols, and antioxidants. Apart from organic substances, inorganic nutrients are also present in fruits. Some metals and metalloids are essential for humans, whilst others may exhibit harmful effects. Wild grown berries, collected in so-called unpolluted areas, are considered to be free of any potentially toxic ingredients. However, due to transmission processes pollutants can also reach remote areas and, furthermore, metal uptake from the soil via roots has to be taken into account. Thus, the presented study focused on the determination of Al, Cd, Cr, Ni, and Pb in lingonberries, blueberries, and rose hips collected in a non-polluted area in Croatia. Neither Cd nor Cr could be found in any sample. Ni levels were mainly up to 25 mg/kg, in a comparable range to the literature data. No health threat is to be expected by eating these fruits and berries regarding Cd, Cr, and Ni. Rose hips, however, contain Pb beyond the stipulated limit in fruits, and also Al is present at a high level (8 mg/g).

Keywords: blueberries; lingonberries; rose hips; aluminium; cadmium; chromium; nickel; lead; provisional tolerable intake

1. Introduction

Berries have been collected for nutritional purposes for a long time. They can be either consumed directly as fresh or dried fruits or in various processed forms, such as jams, syrups, infusions, juices, and jellies, or as ingredient of yoghurts or ice-creams. Diet in the Western world includes vegetables and fruits, as well as other plant parts, such as roots, leaves, stems, and seeds, from more than 40 botanical families [1]. Many demonstrate a presence of phytochemicals in fruit and vegetables, which contribute to good human health by influencing metabolic processes [1], e.g., free radical scavenging, stimulating the immune system, inducing apoptosis, and detoxification. Including fruits and vegetables in the daily diet is recommended in dietary guidelines worldwide. However, the consumers' awareness of nutraceutical components is increasing. Thus, ongoing scientific interest in berries, especially wild grown ones, is needed [2]. Regarding public interest, it was estimated in the 1990s, that one family gathers approx. 20–30 kg of berries per year [3]. Even if gathering berries and fruits by oneself is nowadays still carried out, it has started to change from self-collecting in the woods to the usage of pick-your-own farms, which offer a wide range of berries, such as strawberries, raspberries, or blackberries, and processed products [4,5].

Berry fruits, in general, contain a wide range of micro- and macronutrients, such as fibres, minerals, vitamins, and folate, but their biological properties have been attributed mainly to hydrophilic phenolic-type phytochemicals. Usually not only one bioactive compound determines the positive effects of a fruit, but the presence of multiple phytochemicals exhibiting complementary, synergistic, and/or additive effects results in the wide spectrum of health promoting effects [1,2]. Thus, berry research has been focused mainly on organic composition [6–11].

Regarding inorganic analytes, investigations dealing with nutrients in different berry types, e.g., major and minor essential elements, have been published for decades [12–17]. Using old data, such as from 1944 [12] or 1974 [13], it has to be taken into account that the analytical methodology, as well as the environmental influences, have changed, reducing their significance for current evaluations. In addition to the nutritional value, the content of minerals present also influences food quality parameters, like appearance, taste, texture, stability, and even flavonoid production [18]. Conversely, ripeness affects the mineral composition, and some nutrients have been found to decrease with maturity [15], whereas the level of the harmful element arsenic increased [16]. Furthermore potentially harmful metals and metalloids have to be taken into account. Berries collected in highly contaminated areas have been analysed for this purpose [3,19,20]. Additionally, in remote areas heavy metal pollution may occur due to transmission processes. Thus, food safety is of concern [21]. Ten chemicals have been classified to be of major public health concern, including four metals/metalloids (arsenic, cadmium, lead, and mercury) [22]. In addition to the top-ten, other metals may exhibit harmful effects to humans through food intake, e.g., nickel [23,24]. Therefore, maximum allowed limits have been established for harmful elements, and food on the market should not contain certain metals in levels that might result in exceeding the respective allowable intakes. PTWI (provisional tolerable weekly intake), PTDI (provisional tolerable daily intake), or PTMI (provisional tolerable monthly intake) levels have been published by the FAO/WHO [25].

The aim of the present investigation was to determine the content of five elements: aluminium (Al), cadmium (Cd), chromium (Cr), nickel (Ni), and lead (Pb) in wild berries and fruits, since they are considered to be potentially toxic contaminants. Regarding the methodology, previously optimised digestion and measurement procedures were applied, i.e., acidic microwave-assisted digestion followed by inductively-coupled plasma atomic emission spectrometry (ICP-AES) [26]. Closed-vessel microwave digestion as a sample preparation method has been approved for the determination of 31 elements in foodstuffs [27].

2. Materials and Methods

2.1. Chemicals and Glass/Plastic Ware

Reagents for sample and CRM digestion, i.e., hydrogen peroxide (H_2O_2) and supra pure nitric acid (HNO_3) were purchased from Sigma (Munich, Germany). The standard stock solution used for preparation of calibration standards (ICP Multielement Standard IV) was obtained from Merck (Darmstadt, Germany). Ultra-pure water, resistivity $\geq$ 18 MΩ·cm, was prepared by in-house equipment. Quality control measurements were based on the certified reference material strawberry leaves (CRM: LGC7162) from LGC Standards (Middlesex, UK). All glass- and plasticware used for sample storage and preparation were pre-cleaned with diluted nitric acid.

2.2. Samples and Sample Preparation

Wild berries and fruits, namely blueberries (*Vaccinium myrtillus*), lingonberries (*Vaccinium vitis-idaea*), and rosehip (*Rosa canina*), were collected during summer and fall 2010 when optimally ripe in a non-polluted rural area of Croatia (Slunj: N, 45.07°; E, 15.36°; A: ~280 m). The sampling site covered approx. 200 m^2. For each fruit type five plants, randomly distributed in the sampling area, were sampled by collecting 5 to 15 fruits. Immediately after picking, the fruits were frozen and kept at −20 °C prior to further work-up. After thawing the fruits were rinsed with

ultra-pure water. Rose hips were separated into flesh (mesocarp) and seeds. All fruits were dried at 105 °C for 24 h. Using a metal-free mortar, the samples were then ground and homogenised, resulting in a pooled sample for each plant (i.e., five subsamples for each fruit type) In order to destroy the organic matrix, all samples underwent a previously-optimised acidic microwave assisted digestion [26], whereby approx. 0.25 g to 0.5 g (weighed to the nearest 0.1 mg) were treated with 5 mL nitric acid (c = 7 mol/L), applying the following three-step digestion procedure: (1) 150 °C/10 min, (2) 160 °C/10 min, and (3) 190 °C/20 min. The obtained clear solutions were then brought to 10.0 mL with ultrapure water.

2.3. Apparatus and Measurements

Microwave-assisted digestion of the fruits, CRM, and blanks were done in a MWS-2 Microwave System Speedwave instrument (Berghof Laborprodukte GmbH, Eningen, Germany). The subsequent quantitative determination of metals and metalloids was carried out using a Prodigy High Dispersive ICP-AES spectrometer (Teledyne Leeman, Hudson, NH, USA), using a simultaneous mode, the optimal instrumental conditions are listed in Table 1. The emission lines selected along with the respective characteristics of the analytical method are given in Table 2.

Table 1. Operating conditions of the Prodigy High Dispersive ICP-AES.

Parameter	Settings
Spectrometer	High resolution Echelle polychromator Large format programmable array detector (L-PAD)
RF-Generator	40 MHz "free-running"
Output power	1.1 kW
Argon flows	Coolant: 18 L min^{-1} Auxiliary: 0.8 L min^{-1} Nebuliser: 1.0 L min^{-1}
Peristaltic pump	1.0 mL min^{-1}
Nebuliser	Pneumatic (glass concentric)
Spray chamber	Glass cyclonic
Plasma viewing	Axial
Sample uptake delay	30 s

External standards in the concentration range from 0.0500 mg/L to 5.00 mg/L were used to calibrate all analytes. The standard solutions were prepared by diluting a multi-element standard stock solution (1000 mg/L) with 1% w/w HNO_3. This diluted nitric acid is also the medium of the blank solution, whose intensity was subtracted from all sample values for blank correction. In case of sample concentrations outside the calibration range, appropriate dilutions to the sample were performed with 1% w/w HNO_3.

2.4. Calculations

All solutions, i.e., fruit and berry digests, blanks, and CRM digest were measured thrice. The blank-corrected values (mass concentrations) were converted into contents in mg/kg dried plant material considering dilution factor, final volume, and mass of dry matter. Finally, means and standard deviations were calculated for all samples. In order to see statistically significant differences between the three fruits analysed, ANOVA test was carried out, based on $p < 0.05$. All calculations were performed using Microsoft Excel 2010 and 2013.

2.5. Characterisation of the Analytical Method

In order validate the analysis, parameters of trueness, precision, and day-to-day repeatability were determined from strawberry leaves which were chosen as a certified reference material for plant

matrix. The samples were analysed after calibration on two different days. The recoveries were obtained using the following formula:

$$\text{recovery}_x \text{in } \% = \frac{\text{content}_{x,\text{ found}} \text{ in mg/kg}}{\text{content}_{x,\text{ certified}} \text{ in mg/kg}} \times 100$$

Based on Bouman's procedure [28], the limits of detection (LOD; 3σ) and quantification (LOQ; 10σ) were calculated.

3. Results

3.1. Analytical Method

The limits of detection (LOD) for the dried fruits, based on a digested mass of 0.25 g and a final volume of 10.0 mL, are below 0.3 mg/kg for all analytes except for Al, whose LOD is 2.9 mg/kg. In addition to the LOD values, the recoveries obtained by analysing the CRM (ranging from 85% to 113%) are given in Table 2. These figures of merit are in the range of reported ones for similar studies [26,29]. The coefficients of determination (R^2) of the calibration curves are all beyond 0.9995. The precision presented as relative standard deviation (RSD) for triplicate measurements is below 1.2% for all analytes. The RSD obtained for the day-to-day-repeatability is not higher than 2.5%.

Table 2. Characteristics of the analytical method.

Analyte	Wavelength (nm)	LOD in Digest Solution (mg/L)	LOD in Dried Fruits (mg/kg)	Recovery (%)
Aluminum	308.215	0.074	2.9	110
Cadmium	214.441	0.00071	0.028	113
Chromium	206.149	0.00045	0.018	101
Nickel	231.604	0.0038	0.15	85
Lead	220.353	0.0070	0.28	101

3.2. Elemental Content of Wild Berries and Fruits

The results obtained for the three kinds of berries and fruits for the five metals analysed are summarised in Table 3. In all samples the Cd, as well as Cr, levels were below the respective LODs. For the other analytes (Al, Ni, Pb) the minimum, mean, and maximum values are given. Statistically significant differences in the element content were found for Al and Ni, the p-values are 2.6×10^{-14} and 0.0023, respectively. Conversely, the Pb contents do not differ statistically significantly between the three sample types analysed ($p = 0.24$)

Table 3. Minimum–mean–maximum metal content in fruit material (mg/kg d.w. [1]), n = 5.

Metal	Lingonberries	Rose Hip	Blueberries
Aluminum	34.9–42.5–63.9	7527–8242–8836	1093–1248–1463
Cadmium	<0.028	<0.028	<0.028
Chromium	<0.018	<0.018	<0.018
Nickel	1.81–2.49–12.9	10.6–11.3–23.5	21.0–24.8–56.2
Lead	0.542–0.601–9.28	3.00–3.34–15.3	1.19–1.66–2.42

[1] d.w. = dry weight.

4. Discussion

4.1. Analytical Method

The above described figures of merit for the analytical method applied are in the range for the determination of trace elements in biological samples. LODs in a similar range were also obtained

by other working groups [17,29]. Limit values for Cd and Pb in fruits and vegetables are stipulated (see Table 4). For both metals the calculated LOD is lower than the maximum allowed content. The latter are given for fresh weight. Since the water content of fruits and berries is around 90% [14], the elemental content referred to fresh weight is 10 times lower than referred to dry weight. Based on the characteristic data found, the analytical method chosen has been proven to be appropriate for the given analytical task.

Table 4. Limits for metals in food.

Metal		Limit in Food		Intake Limit [1]	Lit.
Aluminum				1.00 mg/kg bw/week (PTWI)	[25]
Cadmium		Fruits and vegetables	0.050 mg/kg f.w.		[30]
				0.025 mg/kg bw/month (PTMI)	[22]
				0.001 mg/kg bw/day (Rfd)	[31]
Chromium	insoluble Cr(III)-salts			1.500 mg/kg bw/day (Rfd)	[32]
	Cr(VI)			0.003 mg/kg bw/day (Rfd)	[33]
Nickel	soluble salts			0.020 mg/kg bw/day (Rfd)	[34]
				0.020 mg/kg bw/day (Trv)	[23]
Lead		Fruit, excluding cranberries, currants, elderberries and strawberry tree fruit	0.10 mg/kg f.w.		[35]
		cranberries, currants, elderberries and strawberry tree fruit	0.20 mg/kg f.w.		[35]
				PTI value withdrawn 2010	[22]

[1] f.w. = fresh weight; bw = body weight; PTWI = Provisional tolerable weekly intake; PTMI = Provisional tolerable monthly intake; Rfd = Reference oral dose; Trv = Toxicity reference value.

4.2. Elemental Content of Wild Berries and Fruits

Based on the five elements determined, all three fruit types differ statistically significantly from each other regarding Al and Ni content. In the following all elements analysed are discussed in detail.

Aluminium has no known physiological role in the human body. Until the 1970s it was considered to be innocuous, but starting in the 1980s toxic effects to animals, plants, and humans have been reported [36]. Al rarely shows acute toxicity, but chronic intoxications have to be considered, especially due to the fact that Al accumulates with age [37,38]. Diet is regarded as being a significant contributor to the body burden of aluminium [39], and its weekly intake is limited by the FAO/WHO [25]. The Al content found in the fruits and berries analysed was highest in rose hips, followed by blueberries and, finally, lingonberries, the mean values being 8242 mg/kg, 1248 mg/kg, and 42.5 mg/kg, respectively. The data differ from each other in a wide range, even since all samples were collected in the same area having a similar environmental background. Thus, it can be clearly seen that the uptake differs between the plants. Nile and Park do not give any value for Al in berries in their review on berry composition, they just mention that berries are rich in Al [10]. Rose hips used for the preparation of infusions collected in Turkey show a mean Al content of 157 mg/kg [40], whereby it is not reported if the entire fruit or only the flesh (as in the present study) was analysed. However, comparable Al contents were found in strawberries from Pakistan (Lahore region), whereby the values decreased during ripeness from 740 mg/kg to 230 mg/kg d.w. [15]. The PTWI given by the FAO/WHO limits the weekly Al dietary intake with 1 mg/kg bw. Thus, an 80 kg-person should not consume more than 100 g of rose hips per week. For blueberries and lingonberries more than 600 g, and even 2 kg, are to

be eaten resp. in order to reach the maximum, considering no other Al sources. Chinese blueberries contained 41 mg/kg [41], much lower than the results from this study, but in the same order of magnitude than the data obtained for lingonberries. This fact can be explained by the impact of bedrock composition on Al content in plants. A potential health threat might only be caused by rose hip intake. This is a drawback, since rose hips have recently been defined as a functional food based on bioactive ingredients, as well as due to their content of essential elements, such as Ca, Mg, K, S, Si, Se, Mn, and Fe [42].

Cadmium, being a highly toxic metal, is one of ten chemicals classified by the WHO to be a major public health concern [22]. It occurs naturally in soil, but it is also a pollutant in the environment due to anthropogenic impact. Since it is easily taken up and accumulated by plants and crops through the root systems, it may easily enter food [43], especially berries and fruits. In all samples, including blueberries, lingonberries, as well as rose-hips, no Cd was detected, meaning that the contents are below the LOD of 0.028 mg/kg d.w. Commission Regulation (EU) No. 2015/1005 limits Cd in fruits and vegetables to 0.050 mg/kg f.w., corresponding to 0.50 mg/kg d.w. [30]. Thus, all fruits analysed are supposed to be safe for humans regarding this element. The PTMI is 0.025 mg/kg bw, which would be 2 mg per month for an 80 kg person. Considering Cd present in the berries at the LOD level, this corresponds to more than 700 kg of fresh blueberries in one month. An investigation of blackberries, elderberries, autumn olives, and candleberry-myrtles revealed that they also contained Cd only below LOD [17]. Whereas no Cd was found in Azorean blueberry, levels between LOD and limit of quantitation (LOQ) were registered for Madeiran blueberry [17]. A Russian study reports Cd in blue- and cranberries in the range from 0.03 mg/kg to 0.06 mg/kg d.w. [3]. Strawberries collected in Serbia did not contain Cd at detectable concentrations either [29]. Blueberries collected in China were reported to contain 0.034 mg/kg Cd [41], slightly above the LOD of the current study. Rose hips from Turkey have been reported to contain quite a high amount of Cd, namely 0.81 mg/kg d.w. [44]. A working group from Bangladesh found Cd contents ranging from 0.012 mg/kg and 0.216 mg/kg in leafy and non-leafy vegetables [43].

Chromium, as Cr(III), plays a crucial role in human health and is, thus, defined as an essential trace element [45]. Special attention has to be drawn to its interaction in glucose tolerance and Cr supplementation in persons with diabetes, hypoglycaemia, and obesity [46]. In addition to the positive effects of Cr(III), Cr(VI) is a toxic, carcinogenic substance, this fact being reflected in the 500-times lower reference oral dose for the latter species [32,33] compared to the former species. No limit for Cr in foods is given in the Codex Alimentarius Commission, or by Australia, New Zealand, Japan, the United States, and Taiwan [41]. Even if Cr(III) is reported to be mainly found in fruits, vegetables, and grain products [47,48], no Cr could be detected in all samples of this investigation (<0.018 mg/kg). Data for Cr in blueberries are given by Hua et al., who found 0.77 mg/kg [41]. Additionally, in fresh strawberries the Cr content is quite low, ranging from 0.01 mg/kg up to 0.03 mg/kg [29]. Wild berries from Portugal, analysed by Llorent-Martínez and colleagues, found Cr in blackberries, elderberries, autumn olives, and candleberry-myrtles up to 0.6 mg/kg fresh weight [17]. Cranberries from Russia have been reported to contain Cr from 0.02 mg/kg up to 1.5 mg/kg, and blueberries 0.03 to 0.06 mg/kg d.w. [3]. Literature data for rose hips are given by Duran and colleagues, who found 0.80 mg/kg d.w. Cr [44].

Nickel is naturally occurring in soils ranging from 4 to 80 mg/kg in the USA [49] and, for agricultural soil in Velika Gorica, Croatia (N 45.7173°–E 16.0571°; A 107 m), a mean content of 55 mg/kg was found [50]. The main exposure route to nickel is via food intake, approx. 0.100 to 0.300 mg/day are taken up by per adult [23,49]. Apart from contact dermatitis, genotoxicity, haematotoxicity, teratogenicity, immunotoxicity, and carcinogenicity have been identified as harmful effects of Ni [24]. Thus, its intake is limited to 0.020 mg/kg bw per day [23,34]. In the present study the highest Ni content was found in blueberries (25 mg/kg), followed by rose hips (11 mg/kg) and lingonberries (2.5 mg/kg). The daily consumption of 100 g fresh blueberries would lead to an intake of 0.250 mg, being approx. 16% of the allowed limit for an 80 kg person. Thus, no harmful effects are to be expected by the berries analysed. Cranberries from polluted areas (close to a Ni-Cu-smelter)

showed elevated levels (up to 97 mg/kg d.w.), whereas the background contents were in the same range as the results from the current study, i.e., 2 mg/kg to 9 mg/kg [3]. No high Ni accumulation in lingonberries collected in the vicinity of a chromium mine was reported by a Finnish research group [51]. Blueberries from the same contaminated area did not accumulate Ni that much, with contents being in the range from 4 mg/kg to 11 mg/kg for all sampling sites [3]. A Chinese working group found 2.2 mg/kg d.w. Ni in blueberries [41]. Wild berries from Portugal contain Ni in the same order of magnitude, namely from 0.6 mg/kg to 1.6 mg/kg [17], as our results for lingonberries. Additionally, results for strawberries are in the same range [29]. Regarding rose hips, the literature data are available for Turkish fruits, with the mean content being 6.5 mg/kg d.w. [44].

Lead is well known as a toxic element, exhibiting developmental neurotoxicity in young children and cardiovascular effects and nephrotoxicity in adults [35]. Thus, the Pb content in fruits and berries is limited to 0.10 mg/kg f.w. and 0.20 mg/kg f.w., respectively [35], whereas the PTI value was withdrawn in 2010 [22]. The highest maximum and mean level for Pb were found in rose hips (15 mg/kg; 3.3 mg/kg, resp.), exceeding the limit value in foods, which would correspond to approx. 1 mg/kg d.w. This result is comparable to that form Turkish rose hips with 10 mg/kg d.w. [44]. Blueberries' Pb content showed the smallest range, compared to rose hips and lingonberries. Only one sample exceeds the limit in berries (2.4 mg/kg > 2 mg/kg). The mean value of Pb in blueberries (1.66 mg/kg) is similar to the data from Barcan et al., who reported contents ranging from 0.7 mg/kg up to 1.5 mg/kg [3]. Conversely, the Chinese working group found less, their average being 0.135 mg/kg d.w. [41]. Lingonberries contained the least Pb of all fruits analysed, except for one sample (9.3 mg/kg) all results are below 1 mg/kg, thus being within the allowed range for berries. Even less Pb was found in lingonberries from Finland, whose contents ranged from 0.0006 mg/kg to 0.0011 mg/kg f.w. [51], corresponding to approx. 0.006 mg/kg to 0.011 mg/kg dried berries. Wild berries gathered in Portugal all had Pb levels below the LOD (0.012 mg/kg f.w.) [17].

5. Conclusions

Wild berries and fruits collected in a remote and, thus, considered unpolluted area in Croatia have been analysed for potentially toxic elements, revealing that no health threat is to be expected by eating these fruits and berries regarding Cd, Cr, and Ni. Due to high Al and Pb contents in rose hips, their intake should not exceed 100 g per week for an adult, especially in the case of chronic renal failure. Only in cases of high consumption of blueberries are harmful effects by Al and Pb to be expected. Lingonberries were found to have the lowest contents of all metals investigated.

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Perfuorooctane Sulfonate (PFOS), Perfluorooctanoic Acid (PFOA), Brominated Dioxins (PBDDs) and Furans (PBDFs) in Wild and Farmed Organisms at Different Trophic Levels in the Mediterranean Sea

Elena Fattore [1], Renzo Bagnati [1], Andrea Colombo [1], Roberto Fanelli [1], Roberto Miniero [2], Gianfranco Brambilla [2], Alessandro Di Domenico [2], Alessandra Roncarati [3] and Enrico Davoli [1,*]

1 Department of Environmental Health Sciences, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, 20156 Milano, Italy; elena.fattore@marionegri.it (E.F.); renzo.bagnati@marionegri.it (R.B.); andrea.colombo@marionegri.it (A.C.); roberto.fanelli@marionegri.it (R.F.)

2 Toxicological Chemistry Unit, Environment Department, Italian National Institute of Health, 00161 Rome, Italy; roberto.miniero@iss.it (R.M.); gianfranco.brambilla@iss.it (G.B.); alessandro.didomenico@iss.it (A.D.D.)

3 School of Biosciences and Veterinary Medicine, University of Camerino, I-62024 Matelica, Italy; alessandra.roncarati@unicam.it

* Correspondence: enrico.davoli@marionegri.it

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Abstract: The present study shows the results of perfuorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), brominated dioxins (PBDDs) and furans (PBDFs) measured in several marine fish and seafood of commercial interest at different trophic levels of the food chain. The aims were to investigate the level of the contamination in Mediterranean aquatic wildlife, and in farmed fish, to assess human exposure associated to fishery products consumption. Samples of wild fish were collected during three different sampling campaigns in different Food and Agriculture Organization (FAO) 37 areas of the Mediterranean Sea. In addition, farmed fish (gilthead sea bream and European sea bass) from off-shore cages from different marine aquaculture plants. Results showed contamination values of PFOS and PFOA were lower than those detected in sea basins other than the Mediterranean Sea. Concentration values of PFOS were generally higher than those of PFOA; moreover, levels in farmed fish were lower than in wild samples from the Mediterranean Sea. Intake of PFOS and PFOA through fishery products consumption was estimated to be 2.12 and 0.24 ng/kg·BW·day, respectively, for high consumers (95th percentile). Results of 2,3,7,8-substituted congeners of PBDDs and PBDFs were almost all below the limit of detection (LOD), making it difficult to establish the contribution of these compounds to the total contamination of dioxin-like compounds in fish and fishery products.

Keywords: perfluorooctane sulfonate; perfluorooctane acid; PFOS; PFOA; mediteranean fish; toxicological risk

1. Introduction

Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are two chemicals included in the large group of the perfluorinated compounds (PFC) which have been widely produced for industrial purposes since 1950 [1]. They are characterized by a fully fluorinated hydrophobic chain and a hydrophilic head and these properties, in combination with their high chemical stability, make these compounds unique for their ability to repel both water and oils. They have been used in many applications, such as surface treatments for coatings, clothes, carpets, packaging products, cookware, food contact papers, and as additives in the fire-fighting foam. They are considered

emerging pollutants, since they have been detected in human tissues and wildlife with increasing trends [2–4] and seem to meet the criteria of persistence, biomagnification, and long-distance transport, to be included in the persistent organic pollutants (POPs) under the Stockholm convention. Liver is the target organ of toxicity of these chemicals. Toxicity of PFOS and PFOA includes developmental effects, interference with transport and metabolism of fatty acids, immune-suppression, and interference with thyroid hormones. PFOA shows the typical effects of the peroxisome proliferator-activated receptor alpha (PPAR-α) agonists, which include increase of β-oxidation and cytochrome P450 mediated reactions [5]. For both compounds, carcinogenicity has been shown in animal study mediated by a non-genotoxic mechanism.

PBDDs and PBDFs are another group of POPs formed as byproducts of other brominated compounds, such as brominated flame retardants (BFRs) or brominated biphenyls (PBBs), or ex novo in the combustion processes starting from brominated precursors. In addition, for PBDDs, a biogenic origin in the marine environment has been hypothesized [6]. These compounds are of concern because they seem to have the same mechanism of toxicity of the highly toxic 2,3,7,8-substituted congeners of the polychlorinated dibenzo-p-dioxins (PCDDs) and furans (PCDFs) through the binding to the aryl hydrocarbon receptor (AhR) [7,8], which is the protein mediating the dioxin-like toxicity [9]. Indeed, the classical fingerprint of the dioxin-like biological effects, such as wasting syndrome, thymic atrophy, chloracne, teratogenesis, reproductive effects, and immunotoxicity have been observed [10].

One of the main research gaps related to these pollutants is to what extent exposure for humans and other living organisms occurs [11], since few data on environmental occurrence are available, especially for PBDDs and PBDFs. Fish and fishery products are a known source of dietary intake of POPs for general population, since seas and oceans represent the final accumulation tank of such compounds and their tendency to bioaccumulate.

Within a more extensive research project on the welfare of wildlife and farmed fauna in the Mediterranean Sea [12], this paper reports detailed results species and location specific for PFOS, PFOA, PBBD and PBDF analysis in fish and other aquatic organisms collected in different areas of the Mediterranean Sea. The aims were to investigate the level of the contamination in farmed and wild fish at different trophic levels of the food chain and to assess human exposure associated to fish and fishery products consumption.

2. Materials and Methods

2.1. Sampling

Wild aquatic organisms, at different levels of the food chain, were collected during different sampling campaigns in May, November and January, in three areas in the Mediterranean Sea. The different sampling areas were selected based on the anthropic level of the coasts and were located close to: Monopoli, in the Adriatic sea, south Bari; Porto Palo, in the Ionian sea, in front of the city of Pachino; and Bagnara Calabra, in the Tyrrhenian Sea nearby the Eolie Islands. During the first two sampling campaigns, farmed fish (gilthead sea bream and European sea bass) from off-shore cages have been also collected from three different aquaculture plants. In total. 61 samples of aquatic organisms were analyzed for PFOS, PFOA, PBDDs and PBDFs. The species analyzed with the corresponding sampling areas are shown in the Table 1.

For fish of larger sizes (>100 g), the fillet was isolated and analyzed, whereas for species of smaller size, where it was difficult to separate the fillet, the analysis was performed on the whole fish without head, tail and entrails. For shrimps, the analyzed samples consisted of the body without exoskeleton.

Table 1. Aquatic species, with the corresponding sampling areas analyzed in the present study.

No of Samples	Species	Sampling Area
5	Gilthead sea bream (*Sparus aurata* L.)	Farmed fish
5	European sea bass (*Dicentrarchus labrax* L.)	Farmed fish
9	Red Mullet (*Mullus surmuletus* L.)	PP, BC, MO
9	Anchovy (*Engraulis encrasicholus* L.)	PP, BC, MO
3	Pilchard (*Sardina pilchardus* Walb.)	PP
3	Pink shrimp (*Parapenaeus longirostris* Lucas)	PP
4	Bonito (*Sarda sarda* L.)	PP, BC, MO
2	Mackerel (*Scomber scombrus* L.)	PP
9	Hake (*Merluccius merluccius* L.)	PP, BC, MO
3	Horse mackerel (*Trachurus trachurus* L.)	MO, BC
2	Norway lobster (*Nephrops norvegicus* L.)	MO
3	Bullet tuna (*Auxis rochei* Risso)	BC, PP
2	Swordfish (*Xiphias gladius* L.)	PP, BC
2	Bluefin Tuna (*Thunnus thynnus* L.)	PP, BC

Legend: PP, Porto Palo; BC, Bagnara Calabra; MO, Monopoli.

2.2. PFOS and PFOA Analytical Procedure

Fresh samples (0.5 g), after spiking of the internal standard 13C12 PFOS and 13C12 PFOA (Wellington Laboratories, Guelph, ON, Canada) in methanol, were extracted by ultrasounds for 40 min, and centrifugated at 2800 rpm for 10 min. The supernatant (0.5 mL) was transferred to glass vials and added to 0.5 mL Milli-Q water. Instrumental analysis was performed by high pressure liquid chromatography-tandem mass spectrometry (HPLC-MS-MS) Perkin-Elmer Series 200 (Waltham, MA, USA), Applied Biosystem API 3000 (Concord, ON, Canada) with electrospray ionization (ESI). The HPLC conditions were the following: chromatographic column XTerra MS C18 2.1 × 100 mm, 3.5 μm. The mobile phase A was 5 mM ammonium acetate and the mobile phase B was acetonitrile and the flow rate 200 μL/min. Spectrometric conditions have been optimized in multiple reaction monitoring (MRM) mode using a continuous direct infusion of a solution of the analytes. Detailed analytical methodology for PFOS and PFOA quantification will be published elsewhere.

2.3. PBDD and PBDF Analytical Procedure

Homogenized samples (20–60 g) were lyophilized (Thermo MicroModulyo Freeze Dryer, Fisher Scientific, Hampton, NH, USA) and spiked with a mixture of the following labeled internal standard: 2,3,7,8-tetrabromodibenzofuran (2,3,7,8-TBDF)-13C12; 1,2,3,7,8-pentabromodibenzo-p-dioxin (1,2,3,7,8-PBDD)-13C12, 1,2,3,7,8-pentabromodibenzofuran (1,2,3,7,8-PBDF)-13C12, 2,3,4,7,8-pentabromodibenzofuran (2,3,4,7,8-PBDF)-13C12, and 1,2,3,4,7,8-hexbromodibenzofuran (1,2,3,4,7,8-HBDF)-13C12. Labeled and native analytical standards (congeners 2,3,7,8-substituted from tetra to hexa for dioxins and furan and 1,2,3,4,6,7,8-heptabromodibenzofuran) were purchased from Cambridge Isotope Laboratories Inc. (Tewksbury, MA, USA). Lyophilized samples were extracted using an accelerated solvent extractor ASE300 (Dionex, Sunnyvale, CA, USA) by a mixture of n-hexane:acetone (9:1) and three extraction cycles using a 60% vessel flush at 80 °C and 1500 psi. The extracts were completely evaporated until dryness by rotary evaporator and the fat content was determined gravimetrically. Cleanup was carried out overnight adding sulfuric acid on an Extrelut column and subsequently by alumina column, adapting the clean-up procedure for chlorinated dioxins.

Quantification has been performed by high resolution gas chromatography–high resolution mass spectrometry HRGC-HRMS (Thermo Fisher, Waltham, MA, USA) using a thermo Finnigan MAT95 XP mass spectrometry with GC PAL, CTC Analytics auto sampler, in EI^+ and SIM modes, electron energy 38 eV, ion source temperature 280 °C, resolution power 8000–10,000. The selected ions used for quantification and confirmation were M+2 and M+4 for TBDF and TBDD; M+4 and M+6 for PBDF,

PBDD, HeBDF and HeBDD; and M+6 and M+8 for HpBDF. The chromatographic conditions were: capillary column J&W DB-5MS, 30 m × 0.25 mm, film thickness 0.1 µm. Temperature program: 80 °C, 25 °C/min until 180 °C; 3 °C/min until 280 °C; 6 °C/min until 310 °C for 7 min. For the limit of detection (LOD), a signal-to-noise ratio of 3:1 was chosen.

3. Results and Discussion

Sampling details and descriptive statistics for complete sample dataset has been reported elsewhere [12]. Concentration values for PFOA were below the LOD (0.05 ng/g fresh weight) in 37 samples and for PFOS in 11 samples, out of 65, corresponding to a total below LOD of 57% for PFOA and 17% for PFOS of negative results, respectively. Levels ranged from <0.05 to 1.89, and from <0.05 to 5.96 ng/g fresh weight (fw) for PFOA and PFOS, respectively. Levels ranged from < 0.05 to 1.89, and from <0.05 to 5.96 ng/g fresh weight (fw) for PFOA and PFOS, respectively. Mean ± standard deviation concentrations for PFOA and PFOS in the wildlife of the three sampling areas of the Mediterranean Sea were, respectively, 0.09 ± 0.11 and 1.19 ± 0.91 in Porto Palo, 0.06 ± 0.08 and 1.27 ± 1.36 in Bagnara Calabra, and 0.20 ± 0.47 and 1.25 ± 1.06 in Monopoli. As already reported in other investigations on levels of these chemicals in aquatic organisms, concentrations of PFOS resulted higher than those of PFOA [13]. In addition, concentration levels measured in this study are markedly lower than those reported in other geographical areas but comparable to those in muscle fish and other aquatic organisms sampled in the Mediterranean Sea [1,14,15].

Figure 1 shows the PFOA and PFOS concentrations in the samples analyzed. From that figure, it is evident that levels of both chemicals in the farmed sea bass (*Dicentrarchus labrax* L.) and sea bream (*Sparus aurata* L.) are at least one order of magnitude lower than those measured in the wildlife of the Mediterranean Sea. These results seem to indicate that contamination from PFOS and PFOA arises from food through biomagnification rather than directly from water (bioconcentration) since the farmed fish analyzed have been sampled in offshore cages of the aquaculture plants. A larger set of samples should be performed in order to provide better statistics, especially if there is an interest in Species-Specific risk assessment.

Among the wildlife, the highest concentrations of PFOS were found in anchovy (*Engraulis encrasicholus* L.), 2.7 ± 1.5 ng/g fw, followed from horse mackerel (*Trachurus trachurus* L.), 2.4 ± 0.4 ng/g fw, whereas the lowest in hake (*Merluccius merluccius* L.), 0.46 ± 0.29 ng/g fw, and Atlantic mackerel (*Scomber scombrus* L.), 0.29 ± 0.13 ng/g fw (Figure 2). Thus, from these data, it is not evident that organisms at higher position in the food chain have higher contamination levels of PFOS and PFOA. That could be due to the different behavior of these chemicals in the bioaccumulation process, since PFOS and PFOA do not accumulate into the fat tissues, as the typical POPs, but they bind serum and liver proteins. However, further studies with a greater number of samples should be performed to confirm this hypothesis.

Analysis of the edible part allows estimating the human exposure to these pollutants through fish and other sea food consumption. Combining food consumption data for the Italian general population [16] and average PFOS and PFOA concentration, as measured in the present study, the human intake associated to average fishery products consumption were 0.82 and 0.09 ng/kg BW·day, respectively, whereas the intake associated to high consumers (95th percentile) were 2.1 and 0.24 ng/kg·BW·day, respectively. These latest figures represent 0.01% and 1.3% of the corresponding tolerable daily intake (TDI) for PFOS and PFOA, respectively, established by the European Food Safety Authority (EFSA) in 2008 [17]. However, in 2016, US-EPA issued a RfD of 20 ng/kg·BW·day valid both for PFOS and PFOA [18], while EFSA is re-evaluating its former 2008 opinion on the basis of epidemiological evidences in PFOS and PFOA exposed groups [19]. Under such scenarios, the seafood consumption could cover approximately the 10% of the PFOS tolerable intakes [20].

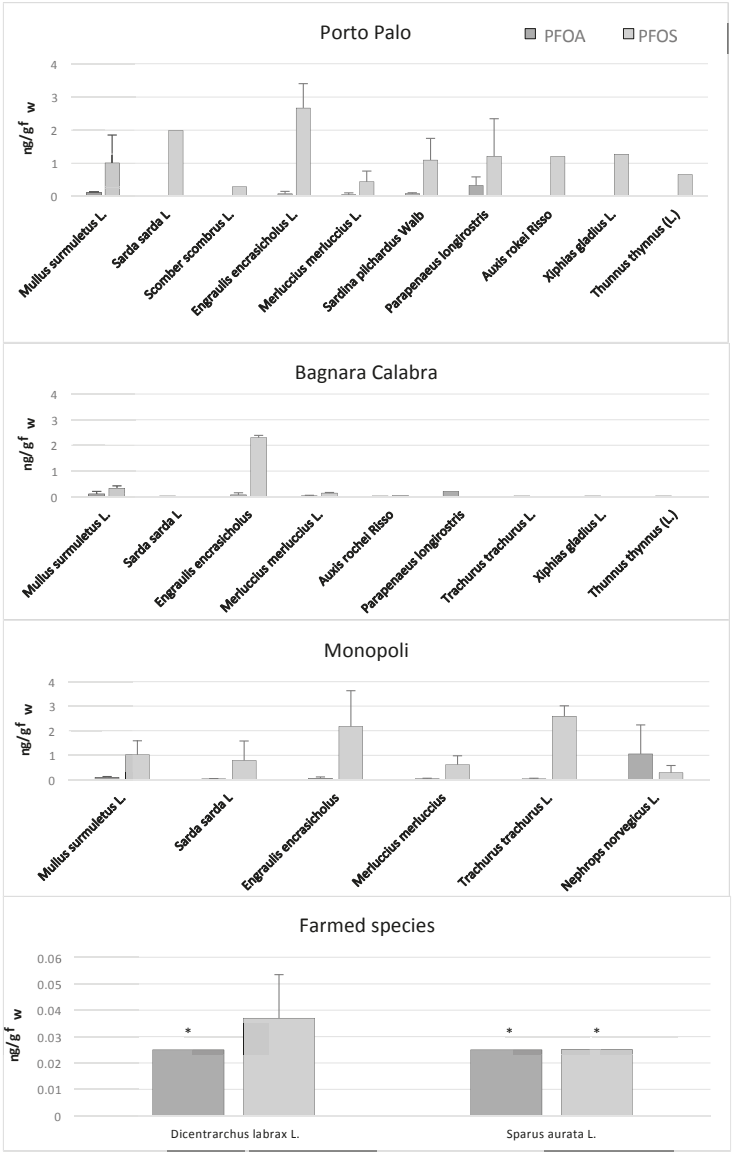

Figure 1. PFOS (perfuorooctane sulfonate) and PFOA (perfluorooctanoic acid) mean concentrations ± standard deviation expressed as ng/g of fresh weight (fw) in wild aquatic organisms from Porto Palo, Bagnara Calabra and Monopoli in the Mediterranean Sea, in farmed fish. * 1/2 Limit of Detection (LOD).

Results of PBDD and PBDF analysis showed concentration values above the LOD for at least a congener in only in 5 samples out of 65. The congeners detected above the LOD values were the 2,3,7,8-TBDF, 2,3,7,8-TBDD, 1,2,3,7,8-PBDF, 2,3,4,7,8-PBDF, 1,2,3,7,8-PBDD, and 1,2,3,4,6,7,8-HpBDF. Concentration values of these congeners ranged from 0.01 to 0.89 pg/g fw and the highest values were found in a wild species, (*Sarda sarda* L.), collected in the sampling area of Monopoli.

Overall, these results indicate that contamination level due to the 2,3,7,8-sostituted congeners of PBDDs and PBDFs, if occurs, is in the range of low ppt; moreover, because of the limited number of samples above the LOD, and the relatively high LOD values (range 0.003–4.5 pg/g fw), it is not possible to make a reliable estimation of the contribution of these compounds to the total contamination of dioxin-like compounds in fish and other aquatic organisms.

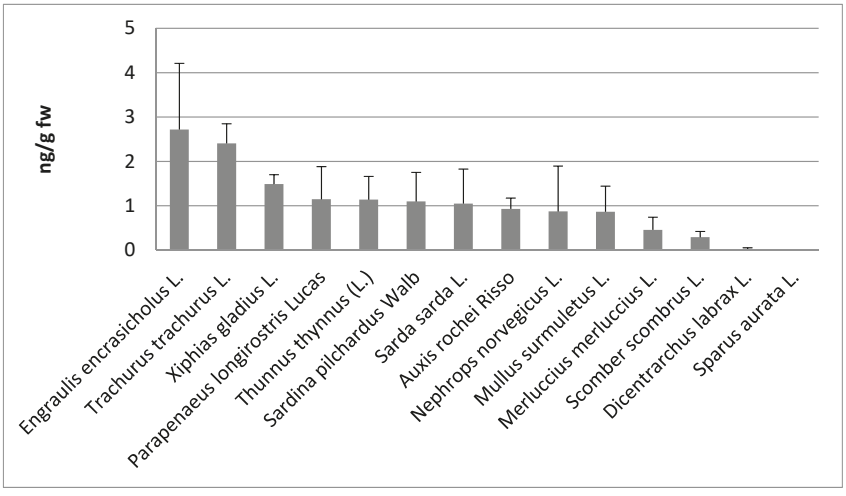

Figure 2. PFOS and PFOA mean concentrations ± standard deviation expressed as ng/g of fresh weight (fw) in different aquatic species from the Mediterranean Sea.

Author Contributions: A.D.D. and R.M. conceived and designed the project. Experiments were conceived, performed and executed by R.F., E.F., R.B., A.C., R.M., G.B., A.R. and E.D., E.F. and R.M. analyzed the data. E.F. and E.D. wrote the manuscript that has been discussed with all authors.

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Conflicts of Interest: The authors declare no conflict of interest.

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toxics

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Article

Characterization of MS/MS Product Ions for the Differentiation of Structurally Isomeric Pesticides by High-Resolution Mass Spectrometry

Alberto Nuñez *, Yelena Sapozhnikova and Steven J. Lehotay

US Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19038, USA; Yelena.Sapozhnikova@ars.usda.gov (Y.S.); steven.lehotay@ars.usda.gov (S.J.L.)

* Correspondence: alberto.nunez@ars.usda.gov; Tel: +1-215-233-6639

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Abstract: Structural isomeric pesticides are used in agriculture and may be challenging to differentiate for accurate identification in pesticide monitoring programs. Due to structural similarity, isomeric pesticides are difficult to separate chromatographically, and thus, their accurate identification may rely solely on mass spectrometric analysis (MS). In this study, we challenged the ability of high-resolution quadrupole-orbitrap (Q-Orbitrap) mass spectrometry to produce and evaluate the tandem mass spectrometry (MS/MS) product ions for the selected five pairs of isomeric pesticides from different classes: Pebulate and vernolate, methiocarb and ethiofencarb, uniconazole and cyproconazole, sebuthylazine and terbuthylazine, and orbencarb and thiobencarb. The use of Q-Orbitrap instrument with a mass error <3 ppm allowed proposed elucidation of the product ion structures with consideration of the ion formulae, data interpretation, and literature searches. Product ions unique to pebulate, vernolate, methiocarb, ethiofencarb, and uniconazole were observed. Elucidation of the observed MS/MS product ion structures was conducted, and the fragmentation pathways were proposed. This information is valuable to increase selectivity in MS/MS analysis and differentiate isomeric pesticides, and thereby reduce the rates of false positives in pesticide monitoring programs.

Keywords: high-resolution mass spectrometry; Orbitrap; structure elucidation; pesticide residue analysis

1. Introduction

Numerous pesticides are used in agriculture worldwide to protect crops and increase harvest yields, with over 1.1 billion pounds used annually in the USA, and nearly six billion pounds (three million tons) usage worldwide [1]. Use of specific pesticides varies by different countries, and certain pesticides may be approved for applications in some countries but banned or no longer used in others. To protect consumer health, maximum residue limits (MRLs), or tolerances in the USA, are established and enforced by regulatory agencies around the world. MRLs depend on pesticide toxicity, physico-chemical properties, and application rates, which can range significantly for the same pesticide in different commodities [2]. Additionally, different countries may have different MRLs for the same pesticide in the same commodity. Hence, reliable identification and accurate measurement of pesticide residues in foods is important in global food trade because false positives and other forms of incorrect results can have detrimental economic and health consequences.

Common approaches for analysis of pesticide residues include liquid chromatography (LC) or gas chromatography (GC) with single or triple quadrupole for tandem mass spectrometry (MS) for separation and identification of targeted contaminants. In recent years, however,

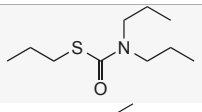
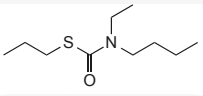
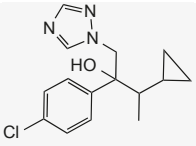
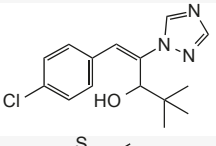
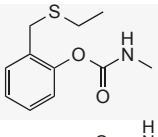
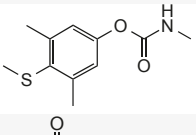
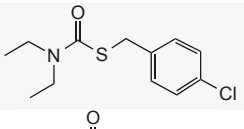
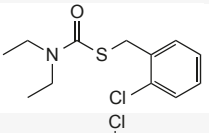
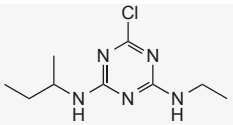
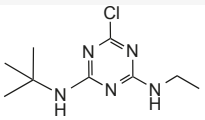
high-resolution accurate mass (HRAM) analyzers are becoming more common in routine analysis of pesticides and other contaminants [3,4]. During the MS/MS method development phase, precursor, and fragment/product ions are selected for analyte identification, and often, the most intense ions are selected. Many pesticides belonging to the same chemical classes (triazines, carbamates, *etc.*) produce the same product ions, and analyte/analyte interferences commonly complicate their accurate quantification. To achieve better selectivity and increase confidence in identification, specific fragments should be selected and studied when possible.

The use of HRAM analyzers in comparison with low resolution quadrupole analyzer instruments facilitates ion mass determination with error <5 ppm required for ion formula determination [5]. Combining this information with literature searches, databases, and data interpretation helps with the assignment of product ions structures. Currently, few published manuscripts on the analysis of contaminants report fragment/product ions with elucidation of the corresponding structures [6–10]. Yet, elucidation of ion structures is important to provide further support for elimination of false positive findings. This becomes even more important when dealing with identification of structurally isomeric pesticides, i.e., pesticides with the same molecular formula and weight, but different atom arrangement.

One example of isomeric pesticides are the carbamate herbicides, orbencarb and thiobencarb. Orbencarb (*S*-(2-chlorobenzyl) *N*,*N*-diethylthiocarbamate) was previously used on cereals, including barley, wheat, rye, maize, soybean, *etc.*, but not currently authorized for use in the European Union (EU) and not registered for use in the USA. Thiobencarb (*S*-(4-chlorobenzyl)-*N*,*N*-diethylthiocarbamate) differs from orbencarb only by Cl positioning on benzene ring (Table 1). It is used for weed control in paddy fields, mostly on rice, and according to Pesticide Action Network of North America, has an active status in the US products [11], but it is not approved for use in the EU. The MRL for thiobencarb is 10–200 ng/g depending on the country and commodity. As isomeric pesticides possess very similar properties, they are usually difficult or impossible to separate chromatographically. For example, both orbencarb and thiobencarb had the same retention time of 19.38 min and the same ions at *m/z* 258, 125, and 100 when analyzed by LC-MS/MS [12]; thus they were not differentiated by either LC separation or MS identification. MS/MS spectra of isomeric compounds are usually very similar and may have few distinct ions to tell them apart. In order to support these differentiations, it is important to show structures for those specific ions in support of the method of analysis. The use of HRAM is essential for the determination of ion formulas to be able to determine structures and fragmentation pathways [13,14].

In this study, we selected five pairs of isomeric pesticides (see Table 1): Pebulate and vernolate, methiocarb and ethiofencarb, uniconazole and cyproconazole, sebuthylazine and terbuthylazine, and orbencarb and thiobencarb, to test the hypothesis of applying HRAM for finding unique product ions to distinguish between the isomers. A Q-Orbitrap HRAM MS instrument with an error <3 ppm was utilized for identification of products ion formulae and the proposal of structures and pathways of fragmentation to differentiate isomeric pesticides. When necessary, the fragmentation patterns were based on product ions obtained from the MS^2 and their selected ions spectra (MS^3) obtained with a triple quadrupole instrument with a linear trap (Q-Trap) mass spectrometer.

Table 1. Isomeric structures of studied pesticides.

Name	CAS Number	Formula	Exact Mass	Structure
Vernolate	1929-77-7	$C_{10}H_{21}NOS$	203.1343	
Pebulate	1114-71-2	$C_{10}H_{21}NOS$	203.1343	
Cyproconazole	94361-06-5	$C_{15}H_{18}ClN_3O$	291.1138	
Uniconazole	83657-22-1	$C_{15}H_{18}ClN_3O$	291.1138	
Methiocarb	2032-65-7	$C_{11}H_{15}NO_2S$	225.0823	
Ethiofencarb	29973-13-5	$C_{11}H_{15}NO_2S$	225.0823	
Thiobencarb	28249-77-6	$C_{12}H_{16}ClNOS$	257.0641	
Orbencarb	34622-58-7	$C_{12}H_{16}ClNOS$	257.0641	
Sebuthylazine	7286-69-3	$C_9H_{16}ClN_5$	229.1094	
Terbuthylazine	5915-41-3	$C_9H_{16}ClN_5$	229.1094	

2. Materials and Methods

2.1. Reagents

Positive ion calibration solution for the Q-Orbitrap instrument was from Thermo Scientific (Rockford, IL, USA). Pesticide standards (purity > 95–99%) and formic acid (purity 98%) were obtained from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (MeCN), methanol (MeOH) and water were Optima LC-MS grade obtained from Fisher Scientific (Phillipsburg, NJ, USA), and deionized water was

obtained from a Millipore (Bedford, MA, USA) Milli-Q system. Stock standard solutions were prepared at 2 mg/mL in MeOH, and working solutions at 0.5 μg/mL were prepared from stock solutions in 1:1 MeCN:H_2O (*v*:*v*) containing 0.1% formic acid.

2.2. Instrumentation and Conditions

Pesticide standard solutions (0.5 μg/mL) were infused at 10 μL/min using a Chemyx syringe pump model Fusion 101 into a Q-Exactive Plus (Thermo Scientific, Madison, WI, USA) mass spectrometer equipped with the electrospray ionization (ESI) probe (HESI-II) in positive mode to obtain MS/MS spectra. Collision energies were optimized and precursor ions were obtained with the mass error of <3 ppm. Capillary temperature was 300 °C with a spray voltage of 3.5 kV and nitrogen as sheath gas set to 60 arbitrary units of the HESI probe. Resolution was set to 140,000 (FWHM) for MS and MS/MS using normalized collision energy (NCE) adjusted to produce the ions of interest. The collision induced dissociation (CID) at the ion source was set at 5 V, except when a specific fragment ion was produced for subsequent MS/MS, in which case the CID was set to 30 V. The instrument was calibrated in the positive mode with a standard solution (Thermo Scientific) and the polysiloxanes *m*/*z* of 371.10124 and 445.12003 were used as lock masses in the MS mode.

Ion formation pathways were also investigated by infusion the selected pesticide standard solutions using a Sciex 6500 Q-Trap mass spectrometer (Sciex, Framingham, MA, USA) to obtain MS^2 and MS^3 spectrum of specific ions of interest. Ion source parameters were: Curtain gas at 10 psi, the ion spray potential was 5,000 V, the source temperature was 350 °C, entrance potential 10 V, collision gas (CAD) was set at medium, and ion source gases were 12 psi (GS1) and 10 psi (GS2).

3. Results and Discussion

Extensive compiled literature on mechanisms of fragmentation is available for electron ionization, also referred as odd electron ionization, but less is found regarding more generalized soft ionization, or even electron ionization, producing protonation, deprotonation, or alkyl adduct formation of the analytes. Recently, a very comprehensive compilation of drugs and pesticides MS/MS data interpretations has been published by Niessen and Correa [10], providing an excellent source for structural identification of MS/MS ions resulting from soft ionization mass spectrometry. In addition, availability of commercial software for spectra interpretation and public compound databases can help in elucidation of structures of product ions, but this approach should be considered with caution and some of these limitations have been described by Wright et al. [15]. Previously, we conducted structural characterization of product ions for 120 veterinary drugs with ESI quadrupole time-of-flight (Q-TOF) MS [7–9]. In the present study, a Q-Orbitrap instrument was used for characterization of product ions to differentiate isomeric pesticides. Occasionally, to better understand fragmentation pathway, product ions were selected for additional fragmentation. This was achieved by using a Q-Trap to obtain the MS^3 spectrum or, alternatively, by using adequate collision dissociation (CID) energy at the ion source of Q-Orbitrap to generate fragmentation for selection and subsequent MS/MS fragmentation. During the discussion, and with the purpose of simplification, the ion masses are used without considering the fractional masses that are reported in figures and schemes.

3.1. Pebulate and Vernolate

Vernolate (*S*-propyl-*N*,*N*-dipropylcarbamothioate) and pebulate (*S*-propyl-*N*-butyl-*N*-ethylcarbamothioate) are thiocarbamate herbicides. Both pesticides' applications ceased in the USA since 2001 and 2009, respectively [16], and in the EU in 2009 [17]. These pesticides are *S*-propyl thiocarbamates bearing a *N*-butyl-*N*-ethyl group (pebulate) and a *N*,*N*-dipropyl group (vernolate) substituent at the nitrogen. MS/MS spectra for these pesticides are presented in Figure 1, showing a very similar profile. The structure of some of these ions have been previously reported [18] and more recently described by Neissen and Castro [10]. The spectrum in Figure 1A for pebulate

has two product ions at *m/z* 57 and 72 that are not observed for vernolate (Figure 1B). Additionally, vernolate has a significant ion at *m/z* 86 that is small for pebulate.

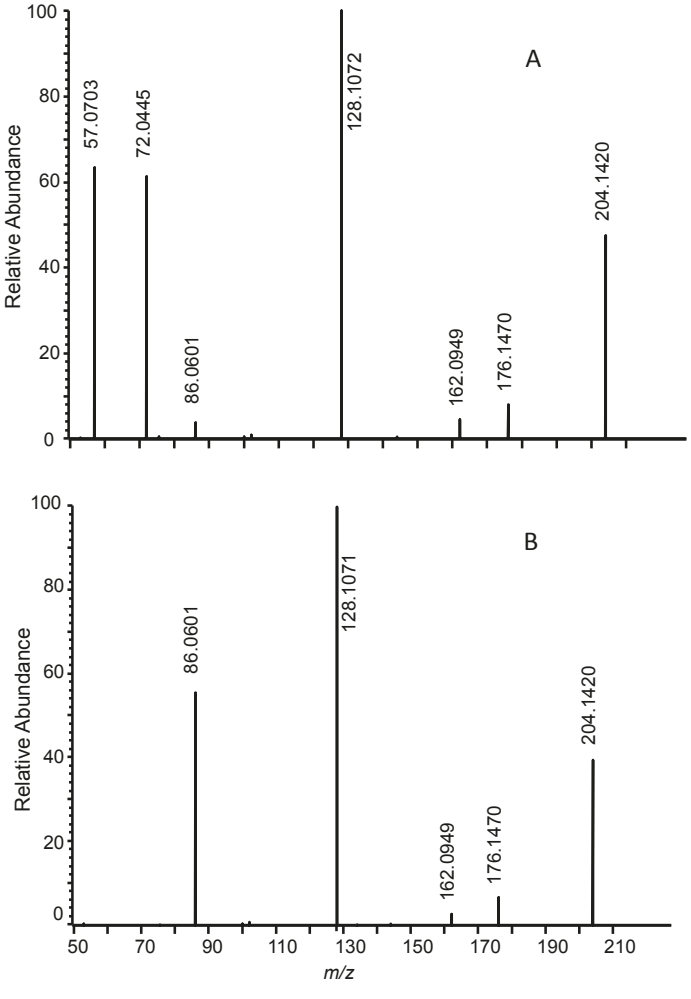
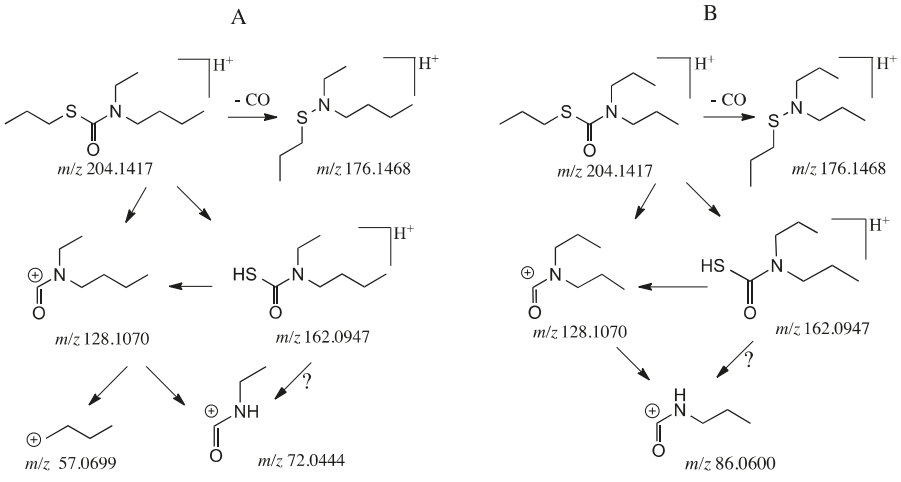


Figure 1. Positive ESI spectra obtained with an NCE 20 eV for: (**A**) pebulate and (**B**) vernolate.

Scheme 1 is the proposed fragmentation pathway for these pesticides indicating formation of two ions at *m/z* 128 and 162 that lead to product ions at *m/z* 57 and 72 for pebulate, and product ion at *m/z* 86 for vernolate. This ion resulted from the loss of butyl group but the equivalent ion resulting from the loss of ethyl group was not observed in the spectra. It is not clear if ion at *m/z* 162 can lead to those product ions, and is thus shown with a question mark in Scheme 1.



Scheme 1. Proposed fragmentation pathway for (**A**) pebulate and (**B**) vernolate.

The small ion at *m/z* 86 in the spectrum of pebulate is difficult to explain based on the fragmentation pattern observed. To further verify its origin, the *m/z* 162 ion was generated by adjusting CID energy at the ion source of Q-Orbitrap, and then selected for MS/MS. The resulting spectrum is shown in Figure 2. This approach generates a set of product ions that are presented in the pathway in Scheme 2. A careful inspection of the spectrum indicated the presence of a small ion at *m/z* 102 (Figure 2 insert). This ion is not expected for pebulate, nor is *m/z* 86 product ion, and consequently, this suggests that pebulate was contaminated with vernolate. This approach is not a true MS^3 because other isobaric ions could be present in the ion source, thus has to be used with caution, especially when low mass ions are selected. Additionally, the inclusion of solvent clusters in MS/MS high-energy collision cells can produce misleading product ions. However, exceptional mass accuracy and resolution of Q-Orbitrap are highly beneficial for assignments of ion formulae to better interpret fragments observed in this manner.

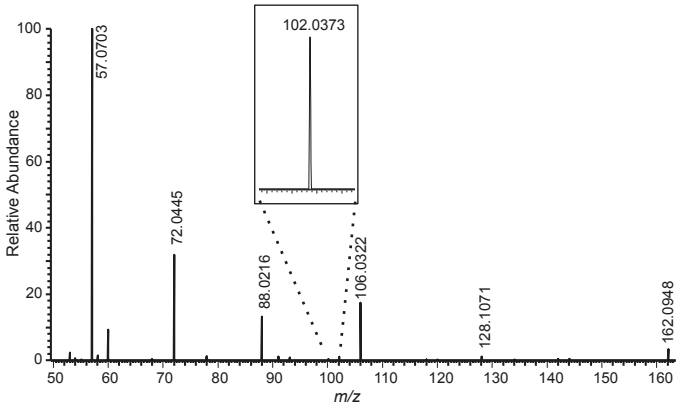
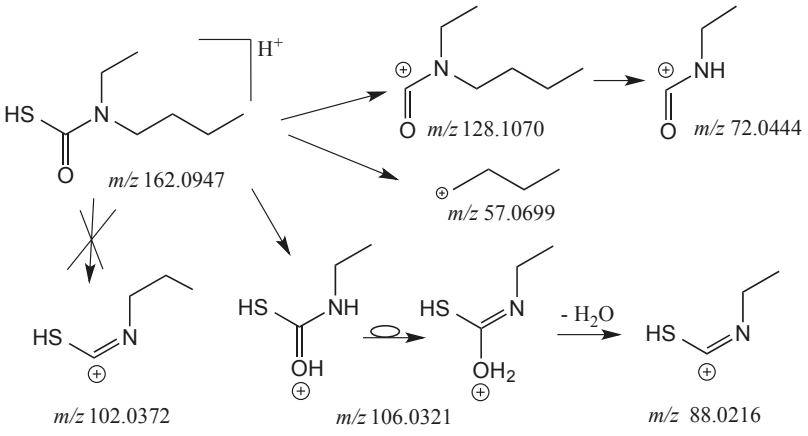


Figure 2. CID/HCD MS/MS spectrum of the selected ion at *m/z* 162 product ion of pebulate. The insert is the zoom region showing ion at *m/z* 102.



Scheme 2. Proposed fragmentation pathway for the CID generated product ion at *m/z* 162 of pebulate.

3.2. Methiocarb and Ethiofencarb

Methiocarb (3,5-dimethyl-4-methylthiophenyl-*N*-methylcarbamate) is a carbamate pesticide, currently approved for use in the EU. It is used for seed treatment, and applied to various agricultural crops, such as maize, lettuce, and fruits. It has an EU MRL of 50 ng/g in food of animal origin, 100 ng/g in grains, and 100–1000 ng/g in fruits and vegetables [17]. Methiocarb does not appear to be used in the USA, and no tolerance is listed in the USA MRL global database [19]. Ethiofencarb (α-ethylthio-*o*-tolyl methylcarbamate) is not approved for use in the EU, and no longer produced or used in the USA.

These two pesticides have MS/MS spectra producing a few fragments at 40–50 eV with product ions that can clearly differentiate between methiocarb and ethiofencarb as shown in Figure 3. However, the ions at *m/z* 107 and 169 are also observed at higher collision energies and only specific ions at *m/z* 121 for methiocarb and ion at *m/z* 164 can be used for differentiation. The pathway for the formation of these product ions is shown in Scheme 3, where ethiofencarb's loss of carbamate group leads to formation of ion at *m/z* 169 that subsequently leads to ion at *m/z* 121 after losing methanethiol [10]. Furthermore, the hydroxybenzyl cation at *m/z* 164 could rearrange to hydroxytropyllium ion [20].

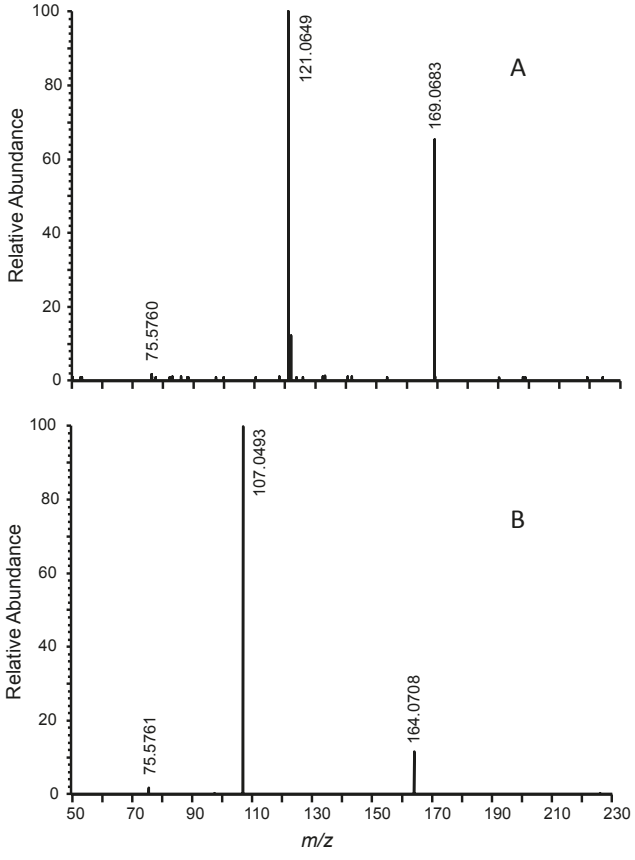
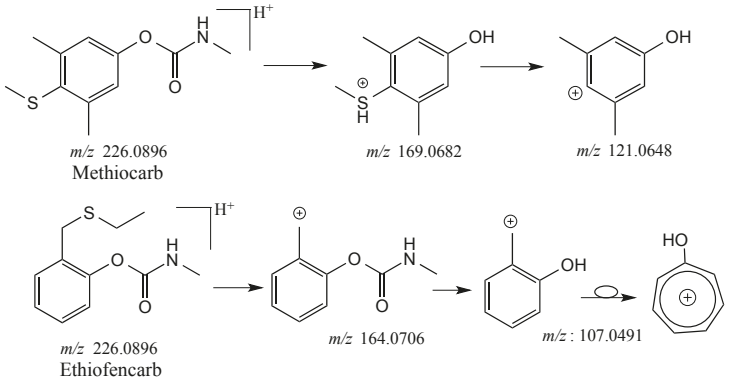


Figure 3. Positive ESI spectra obtained with an NCE 20 eV for (**A**) methiocarb and (**B**) ethiofencarb.



Scheme 3. Proposed pathway for the formation of the differentiation ions for methiocarb and ethiofencarb.

3.3. Uniconazole and Cyproconazole

Uniconazole ((*E*-3*S*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)- 1-penten-3-ol) and cyproconazole (2-(4-chlorophenyl)-3-cyclopropyl-1-(1H-1,2,4-triazol-1-yl)-2-butanol) are triazole fungicides. Uniconazole is not approved for use in the EU, but is used in the USA as plant growth regulator for flowering plants and vegetables (such as tomatoes, peppers, cucumber, and avocado) with the US tolerance of 10 ng/g. Cyproconazole, on the other hand, is approved for use in the USA and EU, and is widely used as a fungicide for cereals, vegetables and fruits, and nuts. Its MRL ranges from 10–1000 ng/g depending on the country and commodity.

In terms of chemical structure, cyproconazole and uniconazole are triazolyl derivatives with a monochlorobenzyl group as shown in Table 1. The most significant fragments for these compounds have been described by Niessen and Correa [10] and fragments include the loss of the triazolyl and the product ion corresponding to its protonated form ($[C_2H_4N_3]^+$). Another significant fragment is the chlorobenzyl ion. Ions like these have been reported as the tropylium cation, but rather high energy is needed to rearrange to this configuration and it formation is questioned [21,22]. Figure 4 shows the spectra for uniconazole and cyproconazole with product ions at *m/z* 70 for the protonated triazolyl cation and *m/z* 125 for the monochlorobenzyl cation. The spectra require 90 eV to produce ions that can be used to differentiate both monochlorobenzyl derivatives. Under this condition, loss of water is not observed, but this loss occurs at lower energy, most likely because the preferred site of protonation is at the triazolyl ring, leading to the elimination of this group (*m/z* 70).

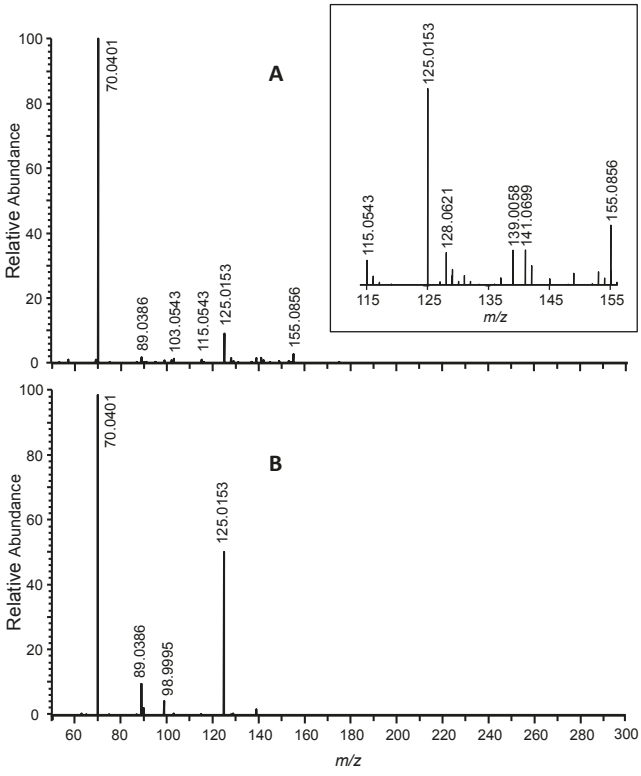
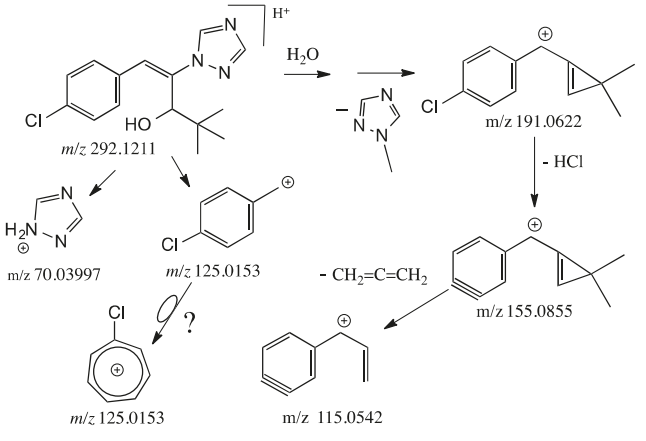


Figure 4. Positive ESI spectra obtained with an NCE 90 eV for: (**A**) uniconazole, with insert expanding the mass range between 115 and 155, and (**B**) cyproconazole.

Both spectra in Figure 4 have the same fragment peaks at different intensities, but uniconazole presented two distinctive product ions at *m/z* 155 and 115 at 70–90 eV that were not found for cyproconazole (Figure 4A insert). On the other hand, cyproconazole did not show any characteristic fragments. Q-Trap isolation and fragmentation of product ion at *m/z* 155 confirmed the subsequent ion at *m/z* 115. The fragmentation pathway required to explain these ions is complex and requires elimination of water, methyl, and triazole groups. The proximity of triazole to the methyl moiety in the terbuthyl group in a 3D model suggests that the methyl can migrate to the triazole and be eliminated as methyl triazole, leading to the ion at *m/z* 191, as shown in Scheme 4. The latter ion is very small and requires less energy to be observed, but seems to lead to the formation of product ion at *m/z* 155 after the loss of HCl. Positive charge in the structure will provide resonance stability to the ion. Loss of 1,2-propadiene from ion at *m/z* 155 produced a product ion at *m/z* 115. These two ions appear to be specific for identification of uniconazole, however, no specific ions were found for cyproconazole to differentiate it from uniconazole.



Scheme 4. Proposed pathway for the formation of the differentiation ions for uniconazole.

3.4. Orbencarb and Thiobencarb

These two pesticides differ in the position of chlorine group at the benzene ring (Table 1); consequently, no specific ions were observed for their differentiation. However, they form an intense product ion at *m/z* 125 (chlorobenzyl), which after loss of HCl produces *m/z* 89 (data not shown). Due to the ortho position of Cl in orbencarb and para position in thiobencarb, the product ion *m/z* 89 has different relative ion abundance ratios for these pesticides. Additionally, a product ion at *m/z* 100 corresponding to $[(CH_3\text{-}CH_2)_2N\text{-}C{=}O]^+$ is affected by the position of Cl, thus producing different ion intensities. Under controlled conditions, fragmentation of ions at *m/z* 125 and *m/z* 100 as precursors can provide additional evidence for distinction of these pesticides. The difference in intensities depends on collision energy, but when using the Q-Trap instrument, thiobencarb showed intensities of approximately 60% and 25% for ions at *m/z* 89 at *m/z* 100, respectively. The ion at *m/z* 89 is in agreement with a chlorobenzyl ion at *m/z* 125 that did not seem to rearrange into a chlorotropyllium ion (see Scheme 4) [21] because both pesticides would have the same possibility of forming this ion at *m/z* 89.

3.5. Sebuthylazine and Terbuthylazine

Sebuthylazine (2-*N*-butan-2-yl-6-chloro-4-*N*-ethyl-1,3,5-triazine-2,4-diamine) and terbuthylazine (2-*N*-tert-butyl-6-chloro-4-*N*-ethyl-1,3,5-triazine-2,4-diamine) are triazine group herbicides, very similar to the well-known herbicide, atrazine. No currently registered pesticide products containing

sebuthylazine were found, and it is listed as obsolete in the EU. Terbuthylazine, on the other hand, is widely used in Europe and 45 other countries to control weeds in corn, potatoes, sorghum, pea, bean, grape, fruit, citrus, etc. with MRLs of 50–100 ng/g [15]. It has become a widely used triazine herbicide in Europe since atrazine was discontinued in 2004. Conversely, terbuthylazine is not registered in the USA [16].

Sebuthylazine and terbuthylazine are monochlorotriazines, and other well-known herbicides from this class include atrazine, propazine, and cyanazine. Fragmentation pathway of protonated monochlorotriazines has been previously extensively investigated and described [12,23]. MS/MS ions observed for both pesticides included *m/z* 174, 104, 132, and 96, similar to the ones previously reported, but no distinct product ions for differentiation of these isomers were observed.

4. Conclusions

Analysis of isomeric pesticides requires the identification of product ions that are unique to each isomeric form. The use of HRAM mass spectrometry provided the molecular ion formula that led to the proposal of pathways and structure for these distinctive product ions for pebulate-vernolate, uniconazole-cyproconazole, and methiocarb-ethiofencarb. Thiobencarb and orbencarb can only be differentiated according to the relative intensities of the ions at *m/z* 89 and 100, but for sebuthylazine and terbuthylazine no differentiation could be established. The importance of identifying specific ions and determining their structures provides further support for elimination of false positives, and consequently, avoiding wrongful prosecution by providing further evidence for the analytical results in legal proceedings.

Author Contributions: Conceptualization, A.N. and Y.S.; Methodology, A.N. and Y.S.; Software, A.N.; Validation, A.N., Y.S. and S.J.L.; Investigation, Y.S. and S.J.L.; Writing-Original Draft Preparation, A.N.; Writing-Review & Editing, A.N., Y.S., and S.J.L.

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Conflicts of Interest: The authors declare no conflict of interest.

Disclaimer: Mention of brand or firm name does not constitute an endorsement by the US Department of Agriculture above others of a similar nature not mentioned.

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Pressurized Solvent Extraction with Ethyl Acetate and Liquid Chromatography—Tandem Mass Spectrometry for the Analysis of Selected Conazole Fungicides in Matcha

Renata Raina-Fulton * and Aisha A. Mohamad

Department of Chemistry & Biochemistry, Trace Analysis Facility, University of Regina; 3737 Wascana Parkway, Regina, SK S4S 0A2, Canada; aam837@uregina.ca

* Correspondence: renata.raina@uregina.ca; Tel.: +1-306-585-4012; Fax: +1-306-337-2409

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Abstract: The extraction of powdered nutraceuticals is challenging due to the low water content and high concentration of matrix components that can lead to significant matrix effects in liquid chromatography-positive ion electrospray ionization-tandem mass spectrometry (LC-ESI$^+$-MS/MS). In this study we assess the feasibility of using pressurized solvent extraction with ethyl acetate to reduce the co-extraction of polar matrix components. Pigment attributed to chlorophyll was removed with in-cell clean-up utilizing Anasorb 747, Florisil®, and C18. Visible inspection of the extracts showed that pigment was removed from matcha, a powdered green tea sample. Pressurized solvent extraction with in-cell clean-up can be utilized to remove pigments from powdered samples such as nutraceuticals. Average matrix effect of the 32 target analytes that observed mass spectrometric signal suppression or soft MS signal enhancement was $-41 \pm 19\%$ with the majority of analytes having a protonated molecular ion with m/z of 250 to 412. As generally moderate signal suppression was observed for conazole fungicides and structurally related compounds analyzed by LC-ESI$^+$-MS/MS, it is recommended that matrix matched or standard addition calibration is used for quantitation. Catachins, other polyphenols, and caffeine are expected to contribute to the matrix effects observed in LC-ESI$^+$-MS/MS. Diniconazole, fenbuconazole, and tebufenozide were the only target analytes with severe MS signal enhancement. Low levels (0.002–0.004 mg/kg) of prothioconazole-desthio and flusilazole were detected, along with trace levels of tebuthiuron in matcha.

Keywords: matcha; conazole fungicides; pressurized solvent extraction; pesticide residue analysis

1. Introduction

Conazole fungicides are a critical group of fungicides used on a wide variety of crops including tea products. They are predominated de-methylation inhibitors and include triazoles and imidazole fungicides. Triazoles are most widely used for blister blight in teas produced in Asia. There has been a rise in commercially available powder nutraceutical products which may be consumed in multiple ways including in drinks (tea or mixed in with other drinks) or food products (e.g., baked goods and chocolate). This necessitates the need for analytical methods capable of dealing with finely powdered samples with high pigment levels.

We have previously reviewed methods for analysis of fungicides in nutraceutical products and presented some of the analytical challenges to a wide variety of fungicides currently in use [1,2]. The analysis of conazole fungicides has been accomplished by LC-MS/MS, GC-MS/MS or GC-MS methods, and is more prone than other pesticides to a variety of issues including stability issues of target analytes, pH sensitivity, carry-over problems largely attributed to strong adsorption of conazole

fungicides on surfaces including tubing and other components of LC-MS/MS or GC-MS systems, and isobaric interferences in MS detection even when using tandem MS methods [1,3–9]. Tandem mass spectrometry provides good sensitivity and selectivity for the analysis of conazoles fungicides. Chromatographic resolution is still required due to the structural similarity and presence of isotopes for many conazoles such that isobaric interferences are common. The number of individual conazole fungicides registered for use worldwide is also increasing with small modifications to the structure such that there is a higher potential for isobaric interferences in MS detection. In addition until recently few deuterated or C-13 isotopes of conazole fungicides were commercially available such that conazoles used for medical or veterinary applications were previously utilized as internal standards [1,4]. Due to the ability of LC-MS/MS to provide short analysis times and good MS sensitivity and selectivity it is often preferred when a wide range of conazole fungicdes are analyzed. Selected conazoles may have better or similar sensitivity when GC-MS methods are used for their analysis [1,7].

Methods for the extraction and analyses of pesticides in dry nutraceutical products are more limited than other food products [2]. In addition to the very complex matrix of nutraceutical products, these products are predominantly sold in dry powder form or as tablets or capsules which is less compatible to extraction methods. There are high concentrations of pigments. Tea products such as matcha have high levels of chlorophyll, caffeine, and polyphenols [10–12]. Most extraction and clean-up methods for fruits and vegetables which have higher water content are based on quick, easy, cheap, effective, rugged, and safe (QuEChERS) or modified QuEChERS approaches, and when dry powders are extracted a wetting step is required for the acetonitrile salt-out extraction typically followed by filtration [7–9]. Sample size must be controlled for dispersive solid phase extraction (dSPE) clean-up to avoid saturation of sorbent materials. A variety of sorbents have been used for clean-up of tea matrices in dSPE or SPE including graphitized carbon black (GCB), graphite carbon/aminopropylsilanized silica gel (carbon-NH_2), primary secondary amine (PSA) or silica, and are efficient for the removal of chlorophyll, catechins and caffeine in infused teas and other sample matrices [9,12–15]. Some methods have been able to successfully use graphitized carbon black in sample cleanup for analysis of selected conazoles, while others have found lower recoveries [8]. Planar pesticides including selected conazole fungicides are known to strongly bind to graphitized carbon black which is the most commonly used sorbent to remove pigments in extracts.

Pesticides in tea products have been predominately analyzed after infusion or a wetting step. The Japan Official Method and modified versions of this method for pesticide residues utilize a wetting step with 20 mL of water per 5 g sample for a 30-min period followed by homogenization with acetonitrile and subsequent filtration [13,14]. The salt-out acetonitrile extraction step is followed by a portion of the extract removed for subsequent clean-up. These approaches rely on good transfer rates of the fungicides from the solid matrix into water such that the fungicides must have high water solubility. Poor recoveries of <20% have been reported for conazole fungicides even with a wetting step in the extraction procedure in a variety of sample matrices including teas [14]. Desired recoveries for pesticide residue analyses methods are 85–110%. Only selected methods have completed the extraction of some pesticides or catechins in tea directly into methanol or 50/50 v/v% ethyl acetate/hexane [3,16]. Analysis of strobilurin fungicides that were extracted with ethyl acetate using pressurized solvent extraction (PSE) has been accomplished for particles collected on filters and matcha [4,17]. PSE is an alternative extraction approach to QuEChERS that allows for direct extraction of target analytes into an organic solvent without the need for the powdered sample of low water content to undergo a time-consuming wetting step or for the extract obtained to be filtered prior to clean-up. Using PSE with ethyl acetate as the extraction solvent recoveries of conazole fungicides and deuterated internal standards (diazinond$_{10}$) are 85–110% with within batch recoveries of $\pm$10% [4]. The objective of this study was to evaluate an adapted pressurized solvent extraction method with in-cell clean-up of matcha green tea powders targeting initial removal of pigment. We focused on the evaluation of recoveries and the matrix effects of the remaining matrix in the extract in the quantitation of a large range of conazole fungicides by LC-ESI$^+$-MS/MS.

2. Materials and Methods

2.1. Materials

Ethyl acetate, acetonitrile, and methanol were of pesticide grade and supplied by Fisher Scientific. Deionized water (18 MΩ resistivity) was from a Nanopure Diamond system (Barnstead International, Dubuque, IA, USA). Aqueous solvents were passed through a 0.45 μm membrane filter from Nuclepore (Watman, Florham Park, NJ, USA). Formic acid (>88.0%) was obtained from VWR Scientific (West Chester, PA, USA). Solids or stock solutions at 100 μg/mL of pesticide standards of all test analytes were purchased from Chem Service, Inc. (West Chester, PA, USA). Solid of propiconazole-phenyld$_3$ was purchased from Sigma-Aldrich (Oakville, ON, Canada). Solids of individual pesticides (~1 mg) were dissolved in 1mL of methanol with further dilution to prepare individual stock solutions at 100 μg/mL in methanol and stored at −4 °C. A further standard stock solution at 1 μg/mL of all target analytes was prepared in methanol for use in preparation of calibration standards.

Filters used for weighing the matcha solid were LABX Berkshire Engineering Clean, 10 cm diameter, and filters used in the 66 mL ASE extraction were glass fiber 934-AH^{TM}, 3.0 cm diameter (VWR Scientific). Matcha is a powdered green tea product and samples were obtained from three different manufacturers that distributed product within Canada and were labeled as organic products. Anasorb 747 (40/80 mesh) was obtained from SKC Inc. (Eighty Four, PA, USA). C18 and Florisil® were obtained from Sigma-Aldrich (Oakville, ON, Canada).

2.2. Sample Preparation

Pressurized solvent extraction with in-cell clean-up was used to extract the target analytes from the matcha samples (see Figure 1). An ASE 100 pressurized solvent extraction system (Dionex, Sunnyvale, USA) was used for extraction with the following extraction parameters: temperature 100 °C; static mode time of 30 min at 1500 psi; four static cycles; 60% flush volume; purge time with nitrogen (UHP) at the end of 600 s. The 66 mL extraction cell was loaded from bottom to top as follows: 934-AH^{TM} filter; 2 g of C18; filter; 4 g Florisil®; filter; 30 g Anasorb 747; two filters; 1 g of Matcha weighed in folded cleanroom grade LABX filter paper (10 cm). The LABX filter paper holding the pre-weighed matcha sample was folded to a diameter of 3 cm to fit into the extraction cell.

The extraction solvent was ethyl acetate with total volume of extraction after the four static cycles of ~130 mL. A 1 mL volume of 2-propanol was added to the extract as a keeper for the drying step. The sample extract was then dried to 4–5 mL in a SPE apparatus under slight vacuum, transferred to a 15 mL vial with methanol used to rinse the extraction bottle three times, and dried to approximately 1 mL at 0.5 mL/hr. The extract was diluted by a factor of 0.43 (65/150) with addition of internal standard (propiconazole-phenyld$_3$ at 50 ng/mL) for final analysis. Standard addition calibration with internal standard was used for final analysis of the three brands of organic matcha samples. For standard addition calibrations the standard amounts added were from MDL to 80 ng/mL.

For recovery evaluation 1 g pre-weighed matcha powdered samples were spiked with 100 μL of 1 μg/mL conazole standard mix (equivalent to 0.1 mg/kg) and allowed to dry prior to loading into the extraction cell. Calibration standards (solvent-only) and matrix matched standards were prepared for evaluation of matrix effects from 0.6 to 100 ng/mL with matrix added at 1/10 dilution. The matrix was obtained from the extraction of a matcha sample with no detectable fungicides.

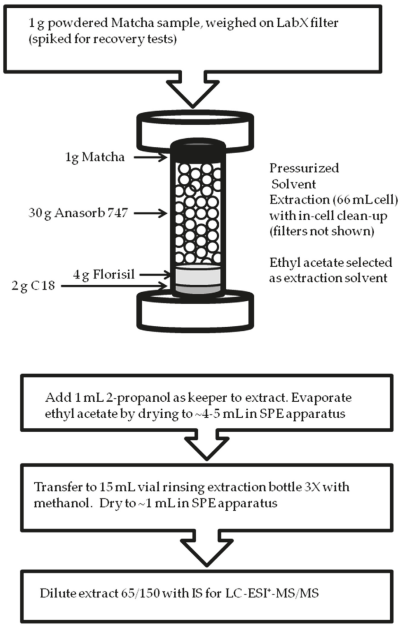

Figure 1. Sample preparation method for conazole fungicide analysis.

2.3. LC-ESI$^+$-MS/MS Analysis

LC analysis was performed with a Waters (Milford, MA, USA) LC system consisting of a 1525 µm binary pump and a column heater at 21 °C. A LEAP Technologies autosampler (Carrboro, NC, USA) was used for 5 µL injections at 100 µL/s and 1 pre- and post-cleans with ethyl acetate followed by methanol to minimize carry-over. A guard column (4 × 2.0 mm id, Gemini) was connected to the analytical column, Synergi Polar-RP, 550 × 2.00 mm id, 2.5 µm particle size (Phenomenex, Torrance, CA, USA). A pre-injection of 5 µL of 2-propanol was completed prior to each sample run at the initial conditions (3 v% methanol in 0.05 v% formic acid aqueous mobile phase) with a flow rate of 0.15 mL/min held for 10 min. The pre-injection of 2-propanol was completed at the initial mobile phase conditions to reduce carry-over issues. A mobile phase gradient was used for the separation of target analytes with initial conditions at 3 v% methanol in 0.05 v% formic acid aqueous mobile phase. The gradient of 0.1 v% formic acid in acetonitrile was changed linearly as follows: 0 to 1.5 min 0%; 2.5 min at 20%; 3 min at 35%; 10 min at 45%; 16 min at 50%; 18 min at 60%; 20 min at 75%; 25 min at 80% and held to 30 min. The mobile phase is re-equilibrated to 0% acetonitrile in 15 min and held for an additional 5 min prior to the pre-injection of 2-propanol. All analytes eluted in the first 25 min.

The Waters LC system was connected to a Waters Quattro Premier triple quadrupole mass spectrometer, operated in electrospray positive ion mode. The temperature of the source was set to 120 °C, desolvation temperature to 350 °C, desolvation gas flow was 750 L/h, and cone gas flow was 150 L/h. The optimized settings for ESI$^+$ were capillary voltage of 3.1 kV; and Rf lens, 0.1 V. The collision gas used for SRM was argon (UHP) at 0.15 mL/min or 4×10^{-4} mbar. Cone voltage and collision energy were set up in the MS method as previously report [4], and shown in Table 1 for target analytes. Infusion experiments were conducted for each individual target analyte to determine the SRM conditions with a syringe pump flow rate of 50 µL/min. Table S1 (see Supplementary Material) shows the regression coefficient of the matrix matched calibration curve for each analyte at the quantitative SRM. The ratio of response for SRM1/SRM2 are within relative standard deviation criteria of <20% for all target analytes.

Table 1. Selected Reaction Monitoring Transitions (SRMs) for Target Analytes Analyzed by LC-ESI$^+$-MS/MS.

Target Analyte	Retention Time (min)	Quantitative SRM, Confirmation SRM (Cone Voltage, Collision Energy)	Method Detection Limits with Matrix Matched Standards (mg/kg)
Benzotriazole	8.24	120→65 (40,17), 120→92 (40,17)	0.0006
Sulfathiazole	8.46	256→155 (20,15), 256→92 (20,25)	0.002
Imazamox	9.09	306→261 (40,20), 306→217 (40,20)	0.010
Sulfamethizole	9.34	271→156 (20,15), 271→92 (20,25)	0.002
Tebuthiuron (thiadiazolylurea herbicide)	10.48	229→172 (25,15), 229→116 (25,25)	0.001
Tricyclazole (benzothiazole fungicide)	12.06	190→163 (35,20), 190→136 (35,25)	0.002
Sulfentrazone	15.36	387→307 (35,20), 389→309 (35,20)	0.010
Imazalil	15.8	297→159 (20,25), 297→201 (20,20)	0.010
Thioconazole	15.93	391→130 (20,20), 391→360 (20,10)	0.010
Azaconazole	16.71	300→159 (30,25), 300→231 (30,15)	0.001
Triadimenol	18.19	296→70 (15,15), 298→70 (15,15), 296→99 (15,10)	0.002
Paclobutrazol (plant growth regulator with triazole moiety)	18.58	294→70 (30,20), 295→70 (25,20), 296→70 (15,15)	0.010
Triticonazole	19.39	318→70 (20,15), 320→70 (20,20)	0.010
Cyproconazole	19.76	292→70 (30,15), 294→70(30,20)	0.002
Hexaconazole	20.58	314→70 (25,20), 316→70(25,20)	0.010
Uniconazole (uniconazole-P)	20.94	292→70 (30,15), 294→70 (30,20)	0.010
Etaconazole	21.58	328→159 (30,25), 330→161 (30,25), 328→187 (30,30)	0.001
Prochloraz	21.61	376→70 (15,25),378→70 (15,25), 376→308 (15,15)	0.010
Myclobutanil	21.73	289→70 (25,15), 291→70 (25,15)	0.010
Triadimefon	21.73	295→70 (25,20), 297→70 (25,20)	0.002
Prothioconazole (analyzed as prothioconazole-desthio)	21.75	314→70 (25,20), 312→70 (25,20), 312→125 (25,20)	0.005
Tebuconazole	21.94	308.5→70 (35,20), 310.5→70 (35,20), 308.5→125 (35,20)	0.001
Bromuconazole	22.01	376→159 (30,25), 378→159 (30,25)	0.010
Penconazole	22.12	284→70 (25,15), 284→159 (25,15)	0.010
Metconazole	22.15	321→70 (30,20), 323→70 (30,20)	0.010
Diniconazole	22.46	326→70(35,25), 328→70(35,25), 326→159 (35,20)	0.0006
Epoxiconazole	22.46	330→121 (25,20), 332→121 (25,20), 330→123 (25,20)	0.010
Tetraconazole	22.46	372→159 (30,25), 372→70 (30,25)	0.010
Biteranol	22.73	338→99 (15,15), 338→269 (15,15)	0.002
Propiconazole	22.73	342→159 (30,25), 342→69 (30,25)	0.010
Flusilazole	22.94	316→165 (30,25), 316→248 (30,15)	0.0006
Fenbuconazole	23.12	337→70 (30,20), 337→125 (30,20)	0.001
Tebufenozide (insecticide)	23.12	353→133 (12,17), 353→297 (12,17)	0.002
Difenoconazole	23.64	406→251 (30,25), 408→253 (30,25)	0.010
Etoxazole	25.02	360→57 (35,25), 360→141 (35,30), 360→177.5 (35,20)	0.010
Propiconazole-phenyld$_3$ (internal standard)	22.80	347→164 (50,25), 349→166 (50,25), 347→69 (50,25), 349→69 (50,25)	NA

3. Results

3.1. Cell Design for Pressurized Solvent Extraction of Matcha

Matcha was used as a model case for analysis of conazole fungicides and structurally related pesticides in powdered samples with high levels of pigments in the sample matrix. Potential interferences in MS detection from the matrix of matcha samples includes chlorophyll, caffeine, catechins and other polyphenols which if present in samples injected would co-elute in a reversed-phase LC separation [10,11,13,14,16]. These matrix components can lead to interferences in MS detection and signal suppression or enhancement in MS detection. Powdered samples may require additional contact time with solvents to ensure complete extraction based on our prior work on extraction of fungicides from particles collected on glass fiber filters [17]. The pressurized solvent extraction procedure with in-cell clean-up was designed to firstly remove the pigment attributed to chlorophyll from the sample. The sample was loaded at the top of the extraction cell held in LABX filter paper for easier removal of the solid after extraction. Two glass fiber filters (3 cm diameter) were placed between the sample and Anasorb 747 sorbent to remove residue solids that pass through the LABX filter paper holding the matcha powdered sample. Ethyl acetate was selected as it provides good recoveries for conazole fungicides from other solid materials and the use of a less polar solvent than acetonitrile, acetonitrile/water or methanol reduces co-extraction of more polar matrix components that can lead to suppression of MS signal and interferences in MS detection. Pressured solvent extraction with ethyl acetate has been used in the extraction of catechins from teas including matcha with lower levels of caffeine found in the extracts [16]. PSE with ethyl acetate or liquid–liquid extractions have also been used to extract selected conazole and strobilurin fungicides from other teas, and solid sorbents used in air sampling [2–4]. Ethyl acetate has also been used with QuEChERS rather than acetonitrile for extraction of fungicides from fruit and vegetable matrices [9]. To reduce co-extraction of less polar matrix components such as fat soluble co-extracts more polar solvents such as acetonitrile or acetone are selected for extraction and have been used to extract fungicides from soil, plant and animal based foods [18,19].

The extraction cell size (66 mL) was selected to enable 30 g of Anasorb 747 to be loaded in the cell (directly below the sample) and this amount of sorbent was required to remove the green pigment coloration of the extracts. Without the use of Anasorb 747 extracts were too high in chlorophyll content such that after preconcentrated (drying) step there was precipitation. To remove the residual color in the ethyl acetate extracts 4 g of Florisil® was required which was placed in the extraction cell below the Anasorb 747. We choose to also add 2 g C18 to aid in removal of some potential matrix components including pigment. Conazole fungicides recoveries have been shown to be good with both C18 and Florisil® SPE clean-up [3,4,13–15]. Filters were placed in the extraction cell before and after each sorbent layer to allow the sorbents to be removed separately. Visible inspection of the sorbent materials after extraction showed that Florisil® and to a lesser extent C18 were removing the residue pigment after the solvent had passed through the Anasorb 747. Graphitized carbon black and primary secondary amine (PSA) were also tested but were not used as the extracts obtained still had green coloration after the pressurized solvent extraction step. PSA has been found to work less effectively for clean-up in the presence of high pigment levels [15]. The use of Anasorb 747 over graphitized carbon black for pressurized solvent extraction was preferred for improved flow characteristics (larger particle size (20/40 mesh) and was the most efficient sorbent tested at removing most of the color of the extracts. Other carbon based sorbents that have been used in the literature include Carbon-X in a SPE format for green tea supplements [15]. Based on our prior procedure where we extracted fungicides from particles on filters we selected four static stages followed by a 60% flush after each static stage to ensure adequate contact time of the solvent with the finely powdered matcha sample. Fungicides are known to strong bind to many solid sorbent materials. An addition extraction with 4 static stages showed no visible color in the extract and also no presence of pesticides.

3.2. Modifications to the LC-ESI$^+$-MS/MS Analysis

We modified our existing LC-ESI$^+$-MS/MS method to include some new fungicides or additional structurally related pesticides that have become commercially available since our initial development (see Table 1) [4]. Sulfentrazone, which is a triazolone herbicide, has few existing methods [5,6]. We also re-optimized the separation for shorter total analysis time from ~45 min to 25 min with the addition of a small percentage of methanol rather than 5% 2-propanol in the aqueous mobile phase. In the prior method 2-propanol was added in the aqueous mobile phase to reduce carry-over issues, however, additional of methanol to the aqueous mobile phase rather than 2-propanol results in better chromatographic resolution for isobaric compounds. To avoid issues with carry-over from sorption of matrix or conazole fungicides in the LC-MS/MS system we completed a pre-injection of 2-propanol prior to each run for 10 min at initial mobile phase conditions.

Good chromatographic resolution was obtained for all compounds with the same or similar SRMs. The conazole fungicides shown in Figure 2A,B have similar molecular weight range are chlorinated and produce the m/z = 70 fragment that is attributed to the triazole moiety. Good separation of these compounds and retention time stability must be obtained to avoid false positive identification as particularly cyproconazole and uniconazole-P give response at SRM of 292>70 and 294>70 (quantification and confirmation SRMs). Co-elution of these fungicides with isobaric interferences occurs and worsens with matrix issues [1,4,17,20]. Similarly hexaconazole and prothionconazole-desthio elute in a similar retention time range and give response at 314>70 (Figure 2C) as well as 312>70 and 316>70. Prothioconazole-desthio has a significantly higher abundance than hexaconazole at 314>70. A small amount of 2-propanol could be added to the aqueous mobile phase to further reduce potential sorption of matrix or conazoles in the LC-MS/MS system with a recommendation to kept the percentage below 2% if methanol is also used in the aqueous mobile phase to ensure adequate chromatographic resolution of isobaric fungicides without the need for long analysis times. When matrix or conazole sorption occurs in the system this is usually evident by a shift to longer retention times and broadening of peak shapes.

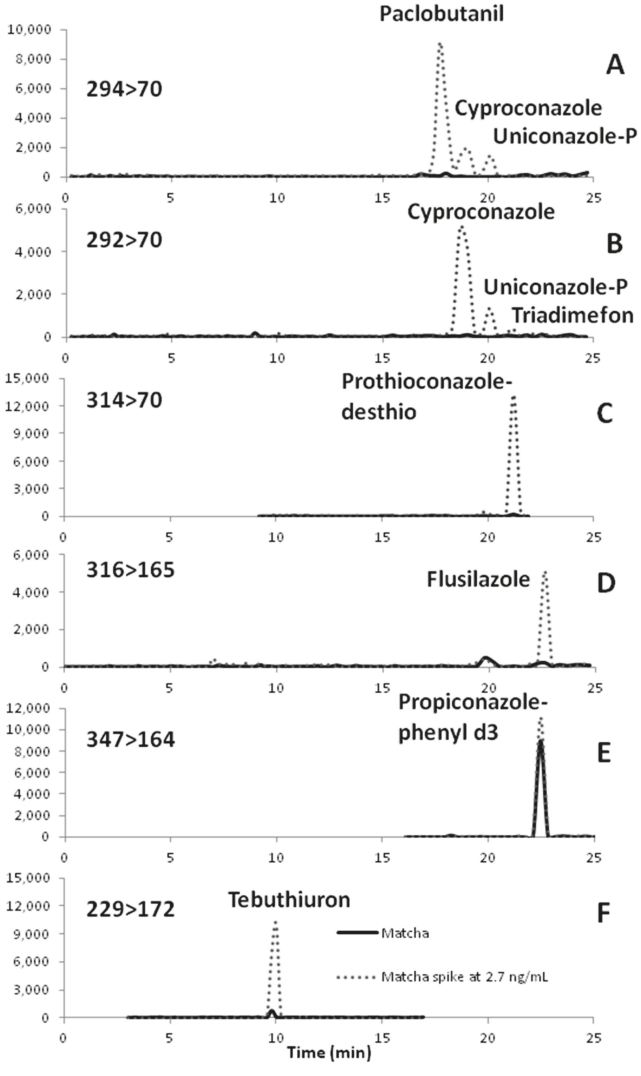

Figure 2. Selected Reaction Monitoring (SRM) Chromatograms of Matcha and Matcha Spiked with Standard Solution. Sample size of 1 g of Matcha with pre-concentrated of pressurized solvent extraction extract to a volume of 1 mL with extract diluted by factor of 0.43 with methanol. Sample extract is spiked at level of 2.67 ng/mL of standard mixture of conazole fungicides and structurally similar pesticides. Internal standard (propiconazole-phenyld_3) added to sample at concentration of 50 ng/mL. SRM Chromatograms: A, SRM 294>70; B, SRM 292>70; C, SRM 314>70; D, SRM 316>165; E, SRM 347>164; F, SRM 229>72. See Table 1 for retention times of analytes.

3.3. Method Detection Limits and Calibration

Table 1 shows the quantitative and confirmation SRM for all target analytes. All new conazole or structurally similar analytes (imazamox, tricyclazole, sulfentrazone, etaconazole, etoxazole) were separated from each other. Etaconazole at higher concentrations can give a response at 328→70, and 326→70, however the response of diniconazole is much stronger at these SRMs and the two

conazoles are separated. Table 1 shows the method detection limits (MDLs) as determined by the lowest concentrations of standard (matrix matched) that deviate from the regression line by <25%. The method detection limits for 29 of the 35 analytes were slightly higher than we previously reported with solvent-based standards [4], but still in a desirable range of 0.0006–0.010 mg/kg. The new target analytes (imazamox, sulfentrazone, and etacoanzole) had detection limits of 0.010 mg/kg. Method detection limit for etaconazole and tricyclazone were lower at 0.001 and 0.002 mg/kg, respectively. The ratio of SRM1/SRM2 were determined on the same day of analysis from matrix matched standards, with relative standard deviation <20% (see Supplementary Material Table S1). Our working calibration range was MDL to 0.080 mg/kg. Correlation coefficient of calibration curves obtained from matrix matched standards was >0.94 (see Table S1). Tetraconazole observed a lower r^2 (0.91) which was attributed to severe matrix effects.

3.4. Matrix Effects and Recoveries of Pressurized Solvent Extraction

To evaluate the extent of matrix suppression or enhancement on MS signal in LC-ESI$^+$-MS/MS the slope of best fit line obtained with calibration standards of the same concentration in calibration standards with matrix added (m_{matrix}) and those obtained with solvent-only ($m_{solvent}$) were compared. The matrix effect (ME) was calculated using the following formula:

$$\mathrm{ME} = ((\mathrm{mmatrix} - \mathrm{msolvent}) - 1) * 100\% \quad (1)$$

The matrix was obtained from an extract of a matcha powder sample which was shown to have no target analytes detectable (signal/noise ratio < 3). Most fungicides observed soft (+20 to −20%) or moderate matrix effects (±20–50%). Signal suppression rather than enhancement in MS detection was more commonly observed after removal of the chlorophyll from the sample extract. Average matrix effect of 32 target analytes with suppression or soft signal enhancement was $-41 \pm 19\%$. The majority of these target analytes have a mass to charge of 250 to 412 for the protonated molecular ion. Diniconazole, fenbuconazole, and tebufenozide were the only analytes with severe MS signal enhancement (102%, 81%, 225%, respectively). A larger number of analytes (6 of 8) with severe signal suppression (range −55% to −75%) eluted between 20–25 min in the separation. Febuconazole, diniconazole, and difenconazole also observed shifts in the baseline (see Figure S1) and had low recoveries (see Table 2). For conazole fungicides matrix matched standards or standard addition calibration and use of an internal standard are necessary to provide reliable quantitation. Addition of higher amounts of matrix than 50% of solvent composition of injected sample lead to instability of retention times such that the samples for subsequent analysis were diluted generally by a factor of 0.43 (65/150) with methanol.

Table 2 shows that acceptable recoveries (70–120%) were obtained for selected conazole fungicides including tebuthiuron, triadimenol, myclobutanil, and triadimenfon. The recovery of prothioconazole-desthio which was detected in one of the matcha samples was 69% with a relative standard deviation >20%. Cyproconazole and paclobutanil also had recoveries in the 50–70% range (see Table 2). While these recoveries are not ideal they are still an improvement over methods reported for this difficult sample matrix [2]. Other less commonly analyzed conazoles observed low recoveries (not listed in Table 2 due to recoveries <20%), although MS signal suppression was moderate indicating that either these fungicides were strongly adsorbed on Anasorb 747 or not adequately removed from the powdered matcha sample. Additional extraction with ethyl acetate did not show any detectable levels of fungicides.

Table 2. Matrix Effect and Detected Concentration of Selected Fungicides in Matcha.

Target Analyte (SRM)	Recovery, Spiked at 0.01 mg/kg [1] (Average ±SD, n = 4)	% Matrix Effect	Detected Concentration in Matcha (mg/kg) [2]
Selected Analytes with MS Signal Suppression or Soft Enhancement			
Tebuthiuron (229→172)	80.7 ± 4.70	−19%	ND
Sulfentrazone (387→307)	64.0 ± 18.3	−35%	ND
Triadimenol (296→70)	109.5 ± 11.7	−32%	ND
Paclobutanil (295→70)	51.8 ± 14.0	−38%	ND
Cyproconazole (292→70)	69.3 ± 12.9	−37%	ND
Uniconazole (292→70)	23.9 ± 12.9	−29%	ND
Myclobutanil (291→70)	84.9 ± 38.3	−53%	ND
Triadimenfon (295→70)	96.1 ± 34.6	−44%	ND
Hexaconazole (314→70)	12.1 ± 19.8	−75%	ND
Prothioconazole-desthio (314→70)	69.2 ± 29.2	−54%	0.0035
Flusilazole (316→165)	40.4 ± 28.9	6%	0.0024
Propiconazole (342→159)	41.9 ± 25.0	−54%	ND
Etaconazole (330→161)	49.1 ± 11.3	−5%	ND
Azaconazole (300→159)	32.3 ± 11.0	−33%	ND
Difenconazole (406→251)	20.4 ± 87.9	−47%	ND
Analytes with Severe Signal Enhancement			
Diniconazole (326→70)	21.5 ± 5.40	102%	ND
Fenbuconazole (337→70)	29.9 ± 8.10	81%	ND

[1] Matrix Matched Standards; [2] Standard addition calibration; ND (not detected) < MDL.

For subsequent analysis of matcha product samples (labeled as organic) from three different manufacturers standard addition calibration was utilized with propiconazole-phenyld$_3$ for the internal standard. Target analytes that were at or near our method detection limits included tebuthiuron, prothioconazole-dethio, and flusilazole. Figure 2C–E shows the SRM chromatograms for sample injection with and without standard addition for prothioconazole-desthio, flusilazole, and tebuthiuron. Two of the three commercial products did not contain any target analytes above the method detection limit. Tebuthiuron levels were just below our MDL for the injected sample at a dilution factor of 0.43 (see Figure 2). With improved extraction and clean-up the analysis of these target analytes would be more reliable and based on the current method detectable amounts are in the range of 2–4 μg/kg which is comparable to other reports of conazole fungicides in green tea leaves [2,21]. There have been no prior reports of detectable levels of conazole fungicides or tebuthiuron in matcha.

4. Discussion

An additional clean-up step following the pressurized solvent extraction with in-cell clean-up was not used. SPE clean-up following extraction and pre-concentration of the extract is a commonly used approach to further remove matrix. Given the sorbents used in the in-cell clean-up the most common alternative sorbent for SPE clean of extracts would be silica to remove residual caffeine in the ethyl acetate extracts, however it has already been reported that a large number of conazole fungicides have poor recoveries with this sorbent material [13,15]. Filtration with polyvinyldifluoride membrane filters has been shown to reduce epicatechin and epigallocatechin gallate concentrations in acetonitrile extracts [10], and this could be further evaluated for recoveries of conazole fungicides in different solvents used for pressurized solvent extraction.

Ethyl acetate is a less polar solvent than typically used in modified QuEChERS methods, Official Japan method, or modification of this method that involve a wetting step of the solid followed by an acetonitrile salt-out extraction. It has been shown that ethyl acetate extracts obtained with pressurized solvent extraction have lower caffeine content but high concentrations of catechins and polyphenols [16]. Other options for further removal of polyphenols without removal of conazole fungicides includes the use of other non-carbon based sorbents such as polymeric resins or green extraction resins such as chotisan which still need to be evaluated for application to extraction of nutraceuticals [21]. Including a more nonpolar solvent such as hexane or cyclohexane with ethyl acetate as an extraction solvent mixture for pressurized solvent extraction may also further reduce the

presence of catechins and other polyphenols in the extract solvent [3,19]. Difenoconazole has been shown to be efficiently extracted with both ethyl acetate and 50/50 v/v mixtures of ethyl acetate and hexane from chrysanthemum flower tea [3]. Poor recoveries of difenconazole and matrix effects in our study indicate that both MS interferences and strong sorption onto sorbents used for clean-up are the most likely sources of low recoveries of conazole fungicides. Poor extraction recoveries <20% were observed for all target analytes not reported in Table 2 with the potential source including strong adsorption onto the Anasorb 747 (a carbon based material) or poor contact of the solvent with the powdered sample which may also result in larger (>20%) relative standard deviation of recoveries for some conazole fungicides. MS signal suppression was moderate for most analytes, and matrix matched calibration curves in the desired calibration range from MDL-0.080 mg/kg had correlation coefficients >0.94 (see Table S1).

The standard deviation of the recoveries of conazole fungicides from matcha were also larger for some fungicides (>20%) with recoveries in the range of 60–70% or lower. There were also noticeable differences in matrix interferences in repeated extractions of the same manufacturer's sample of matcha as seen in baseline shifts in some SRM chromatograms. Lower extraction recoveries for biteranol, cyproconazole, simeconazole, and triadimenol for the matcha samples relative to other tea samples have also been reported [14]. To obtain relative standard deviation for recoveries in the desired range (<20%) for all target analytes it may be necessary to increase the contact of the solvent with the fine powdered sample by mixing the matcha with a sorbent such as a polymeric resin or Florisil®. This may also allow us to reduce the amount or need for Anasorb 747 for removal of chlorophyll in the extracted solvent.

5. Conclusions

The clean-up of extracts from powdered nutraceutical samples is a challenge. Extracts of matcha infused in water have inheritably better relative standard deviation of recoveries of selected conazoles as only the water soluble components are extracted, however other conazoles still show poor recoveries [14]. Evaluation of a wider range of conazole fungicides and other structurally similar pesticides showed that there are similar issues when using PSE with poor extraction recoveries for the less commonly analyzed conazole fungicides. In general matrix suppression of the MS signal was moderate indicating a need for matrix matched or standard addition calibration. PSE with in-cell clean-up can be used to remove pigments from powdered nutraceutical products such as matcha. There are more commercially available products with high pigment levels such that new extraction and clean-up methods need to be developed. This analysis shows that conazole fungicides strongly adsorb to many of the sorbent materials used for pigment removal such that alternate sorbents from carbon based materials need to be evaluated in the future. Matcha is a very difficult sample matrix and selected conazole fungicides can be extracted directly into ethyl acetate with subsequent in-cell clean-up to remove chlorophyll. The first detection of flusilazole, prothioconazole-desthio and trace levels of tebuthiuron are presented. The MS signal suppression that is still present is attributed to the presence of polyphenols including catechins that future extraction and clean-up methods need to address. This work shows that issues with MS signal suppression are also caused by other components of matcha than pigment. Further reducing the polarity of the extraction solvent while maintaining good recoveries for conazole fungicides, and clean-up with Florisil®, polymeric or alternative green sorbent materials should be evaluated. Of the carbon based sorbents and other sorbents evaluated Anasorb 747 was best able to remove high amounts of the green pigment from ethyl acetate extracts such that filtration was not required after the pre-concentration of extracts. Anasorb 747 also has potential for re-use after clean-up to reduce sample analysis costs. To improve the method development strategy utilizing green analytical approaches, improvements in PSE with in-cell clean-up are needed to deal with difficult sample matrices without the need for subsequent off-line clean-up steps [22,23]. To further reduce matrix effects caused by more polar matrix components, a membrane filter could be tested for use with PSE in-cell clean-up. Some membrane filters strongly absorb polyphenols, and this

may aid in reducing matrix issues. Pressurized solvent extraction is well suited to address filtration in the extraction cell as extracts are filtered as the solvent is pushed out of the extraction cell with nitrogen gas.

Supplementary Materials: The following are available online at http://www.mdpi.com/2305-6304/6/4/64/s1, Figure S1: Selected Reaction Monitoring Chromatograms of Diniconazole With and Without Matrix Added, Table S1: Method Detection Limit, Regression Coefficient of Matrix Matched Calibration Curve, and Percentage Matrix Effects for Target Analytes Analyzed by LC-ESI$^+$-MS/MS.

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Optimization of Sample Preparation for Detection of 10 Phthalates in Non-Alcoholic Beverages in Northern Vietnam

Thanh-Thien Tran-Lam [1], Yen Hai Dao [1], Duong Thanh Nguyen [1], Hoi Kim Ma [2], Trung Quoc Pham [1] and Giang Truong Le [1,*]

[1] Institute of Chemistry, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Cau Giay, Hanoi 100000, Vietnam; thanhthien307@gmail.com (T.-T.T.-L.); dhy182@gmail.com (Y.H.D.); ntduong182@gmail.com (D.T.N.); phamquoctrung0811@gmail.com (T.Q.P.)

[2] University of Science, Vietnam National University HCMC, Linh Trung Ward, Thu Duc District, Ho Chi Minh City 720400, Vietnam; makimhoi@gmail.com

* Correspondence: hoasinhmoitruong.vast@gmail.com; Tel.: +84-024-3212-1834

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Abstract: A novel method was developed for the sensitive, cheap and fast quantitation of 10 phthalates in non-alcoholic beverages by liquid–liquid extraction (LLE) combined with gas chromatography tandem mass spectrometry (GC-MS/MS). The best results were obtained when *n*-hexane was used as extraction solvent. A central composite design (CCD) was applied to select the most appreciated operating condition. The method performance was evaluated according to the SANTE/11945/2015 guidelines and was linear in the 0.1 to 200 µg/L range for 10 phthalate compounds, with $r^2 > 0.996$ and individual residuals <15%. Repeatability (RSD_r), within-laboratory reproducibility (RSD_{wr}), and the trueness range were from 2.7 to 9.1%, from 3.4 to 14.3% and from 91.5 to 118.1%, respectively. The limit of detection (LOD) was between 0.5 to 1.0 ng/L and the limit of quantitation (LOQ) was between 1.5 to 3.0 ng/L for all 10 compounds. The developed method was successfully applied to the analysis of non-alcoholic beverages.

Keywords: phthalate; non-alcoholic beverages; liquid–liquid extraction; response surface methodology; GC-MS/MS

1. Introduction

Vietnam is a developing country with a high demand for soft drinks. According to the Vietnam beverage association (VBA) the average consumption of non-alcoholic beverages is over 43 L/person/year and the market is expected to develop from 4 billion liters in 2017 to an estimated 5 billion liters in 2020. With the positive growth of beverages market, consumers increasingly pay more attention to the quality of bottles along with the impact on human health due to long storage time and high temperature conditions.

Polyvinyl chloride (PVC) and polyethylene (PE) are main raw materials of plastic bottles that are widely used in a broad variety of beverages in many countries around the world [1]. In the manufacture process, phthalates are used to produce flexible and durable plastics. It is estimated that in 2017 the global consumption of phthalate compounds was nearly 8 million tons [2]. Because phthalates do not form chemical bonds in the plastic's network, they are easily released and migrate into food and beverages at different stages such as packaging, bottling and production [3–5]. As a result, the consumers can inevitably be exposed to phthalates via eating and drinking. The presence of phthalates was detected in more than 95% of human urine samples in numerous countries around the world [6,7]. Bioaccumulative potential, toxicity and adverse effects of phthalates on experimented

animals have been reported in previous studies [8,9]. Phthalates are known as endocrine disrupters, severely affecting respiratory, hepatic and reproductive organs [10–13].

In 1999, the United States Environmental Protection Agency (U.S. EPA) added 8 phthalates to the list of hazardous chemicals, of which di (2-ethylhexyl) phthalate (DEHP) was in the category of carcinogenic substances of level II [14]. The Chemicals Act (REACH) of the European Council have introduced three phthalate compounds including DEHP, di-*n*-butyl phthalate (DBP) and benzyl butyl phthalate (BzBP) into Section 52, Appendix XVII (Annex XVII of the REACH Regulation) since 2007 in order to restrain the production, trade and use of these compounds [15]. Since then, the list of prohibited phthalate substances has increased steadily. RoHS 2 EU/65/2011 amended in 2016 remains the restrictions of DEHP, BzBP, and DBP concentration <0.1% (enacted since 2011) and will be forbidden in all electrical and electronic devices as of 22/7/2019.

Currently, there has been no research and statistics in Vietnam about phthalates concentrations and the risk of exposure to consumers due to these compounds being contained in plastic bottles. To support consumers becoming more aware of the hazards of these toxins and selecting healthy food, the assessment of phthalates in types of non-alcoholic beverage drinks is vitally important.

Therefore, in this study, the presence of 10 phthalates in 148 samples of non-alcoholic beverages divided into 6 groups in Vietnam was investigated by liquid–liquid extraction and gas chromatography-tandem mass spectrometry (GC-MS/MS) [16]. We also applied response surface methodology (RSM) to plan the sample preparations.

2. Materials and Methods

2.1. Chemicals and Materials

Individual neat crystal phthalates standards, including Dimethyl phthalate (DMP), diethyl phthalate (DEP), dipropyl phthalate (DPP), diisobutyl phthalate (DiBP), benzyl butyl phthalate (BzBP), di-*n*-hexyl phthalate (DnHP), di (2-ethylhexyl) phthalate (DEHP), di-*n*-octyl phthalate (DnOP), dicyclohexyl phthalate (DCHP) and di-*n*-butyl phthalate (DBP) and three isotope titrants (dimethyl phthalate-3,4,5,6-d4 (DMP-d4), diisobutyl phthalate-3,4,5,6-d4 (DiBP-d4) and di (2-ethylhexyl) phthalate-3,4,5,6-d4 (DEHP-d4)) were obtained from Sigma (St. Louis, MO, USA). The purities of phthalate standards and isotope internal standards were guaranteed above 98%. Only glassware was used in all analytical procedure. All the containers such as volume flasks, centrifuge tubes, pipettes and extraction funnels are rinsed carefully by methanol, ethyl acetate and *n*-hexane. Standard stock solution of 10 phthalate compounds and the isotope titrants were prepared by dissolving each compound in *n*-hexane to obtain solutions with concentration of 1000 mg/L and then diluted to 10 mg/L. The internal isotope solutions were prepared at the same procedure to the concentration of 100 mg/L. Standard solutions containing 0.1, 1.0, 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0 μg/L of the 10 phthalates in *n*-hexane were prepared daily and used for the preparation of calibration curves.

Methanol and acetonitrile were purchased from Thermo Fisher (Waltham, MA, USA). Dichloromethane, *n*-hexane, sodium sulfate, sodium chloride and sodium hydroxide were obtained from Merck (Waltham, MA, USA) with purity of above 95%. Ultrapure water was prepared by Milli-Q® Gradient A10 (Merck Millipore, Burlington, MA, USA).

Fourteen commercial mineral water, 17 carbonated drinks, 29 functional drinks, 32 juice drinks, 33 tea drinks and 23 fermented milks were purchased from the retail market in Hanoi, Vietnam. All non-alcoholic beverages were assigned with unique marks and protected from the light until needed.

2.2. Instrumentation and Chromatographic Conditions

All the phthalates determination was performed using a GC-MS/MS system (Thermo Fisher Scientific, Waltham, MA, USA), a Trace GC 1310 gas chromatograph, a TriPlus RSH Autosampler and TSQ 8000 mass spectrometer (Thermo, Waltham, MA, USA) and controlled by a computer running

TraceFinder software. A DB5-MS (30 m × 0.25 mm × 0.25 μm) gas chromatography column from Agilent (Santa Clara, CA, USA) was used to separate phthalates. Oven temperature was set initially at 100 °C (hold for 1 min), then increased to 280 °C at 10 °C/min and to 310 °C at 5 °C/min. At 310 °C, temperature was maintained for 5 min. Helium was used as a carrier gas in a constant flow of 1 mL/min and the injection volume was 1 μL with an autosampler in splitless mode. The total of analysis time was 20 min. Solvent delay was 1 min. The GC was interfaced by a heated transfer liner (310 °C) to the mass spectrometer in electron ionization mode with an electron energy of 70 eV. Inlet temperature was 290 °C and inject volume was 1 μL. The criteria for the identification of phthalates were based on both the same retention times as the standard within ±2% and correctly relative abundance of two characteristic ions within ±15%. Data processing was done by TraceFinder software from Thermo Fisher Scientific. Identifying and quantifying ions, retention time, and collision energy are listed in Table S1.

2.3. Sample Preparation Procedure

We injected 5.00 mL of samples into a 15 mL centrifuge tube, and then added 10 μL of internal isotope and 1.5 mL of methanol. The mixtures were mixed well by vortex and transferred to the extraction funnel. Next, 15 mL *n*-hexane was added to the funnel, and the mixtures were shaken vigorously for 7 min. After standing for 5 min to separate phases, 0.5 mL of 10% NaCl solution was added to remove the emulsion. The *n*-hexane solvent phases were transferred to 50 mL centrifuge tube. The procedure was repeated one more time, then the solutions after 2 extractions were transferred into erlenmeyer flasks and mixed vigorously. Next, 15 g Na_2SO_4 was added and shaken seriously to remove water completely. The remaining solutions were evaporated to about 5 mL by a rotary evaporator, and then dried by nitrogen until dry. Finally, the dried samples were dissolved in 1 mL *n*-hexane, filtered through a 0.22 μm Polytetrafluoroethylene (PTFE) filter and analyzed by means of the GC-MS/MS.

2.4. Experimental Design

Several trials were conducted to optimize a liquid–liquid extraction process for the quantitative analysis of phthalates in soft drinks. The D-optimal was selected to confirm the significant variables ($V_{solvent}/V_{sample}$ ratio, number of extractions, NaCl concentration and extraction time). The response was the sum of all phthalate peak areas. Two different full factorial designs were created at three levels: low (−1), medium (0) and high (+1). MODDE 12.1 software was used to design experimental matrices, calculate regression values and analytical variance. The D-optimal planning method was used with three continuous variables and one intermittent variable. A total of 29 experiments were done and the experimental results are shown in Table 1.

Table 1. Independent variable and experiment level.

Coded	Independent Variable	Level Experiment		
		−1	**0**	**1**
X1	$V_{solvent}/V_{sample}$ ratio	5.0	6.0	7.0
X2	Number of extractions	1	2	3
X3	NaCl (M)	0.1	0.2	0.3
X4	Time of extraction	5	10	15

The relationship between the response function Y and the coded variables (X1, X2, X3, and X4) is indicated in the following equation:

$$Y = \beta_0 + \beta_i \sum x_i + \beta_{ii} \sum x^2{}_i + \beta_{ij} \sum x_i x_j \quad (1)$$

where Y is a response function; x_i and x_j are independent variables; β_0 is a constant; and β_i, β_{ii}, and β_{ij} are linear, quadratic, and interactive coefficients, respectively.

The appropriate fitting model for the response was selected based on the comparison of various statistical parameters such as R^2, Q^2, lack of fit and adequate precision.

2.5. Figure of Merit

Validating the analysis method in this research followed the instruction of European SANTE 11945/2015. The parameters evaluated in the validating process for carbonated beverages and fat drink samples are linearity, linear range, recovery, precision, limit of detection (LOD) and limit of quantitation (LOQ). Quantification was carried out by the internal calibration method. To assess the specificity, blank samples were tested based on the extraction process in order to evaluate false positive phenomenon and contamination of the chemicals. To appraise the linear range, 7 values of mix standard solution of 10 phthalate compounds with the concentration of 1.0, 5.0, 10.0, 20.0, 50.0, 100.0 and 200.0 μg/L were prepared. LOD is defined as the three times the standard deviation of eleven consecutive blank injections divided by the slope of the calibration curve (LOD = (3 × (SD_{blank}) (slope of the calibration cure))) and LOQ is calculated based on the lowest spike level for which the criteria for trueness (i.e., 70–120%) and precision (<20%) met. Precision is calculated using 15 determinations (i.e., three concentration levels in quintuplicate). The repeatability (RSD_r) is calculated from the results of four replicate experiments in a single day of standard 1, 10 and 100 μg/L and the within-laboratory reproducibility (RSD_{wr}) is calculated from results obtained over four consecutive days. The trueness is calculated depending on the method of standard addition with the help of three different concentrations (1, 10 and 100 μg/L).

3. Results and Discussion

3.1. Selecting Extraction Solvent and Optimizing the Method of Solvent Evaporation

The analytical method of phthalates in beverage samples is based on the liquid–liquid extraction technique, and thus the recovery of the compounds relies on two fundamental factors: (i) removing solvent to extract compounds out of the matrix, and then eliminating the matrix and (ii) evaporating the solvent to concentrate the samples after extraction.

3.1.1. Solvent Evaporation Method

One of the most common disadvantage of the liquid–liquid extraction technique is a high volume of extraction solvent. It is, therefore, required to have a method that evaporates only the solvent but not the analytical substance. Herein, we implemented the evaluation of three solvent evaporation methods: (1) using nitrogen to remove the solvent, (2) using the rotary evaporator system, and (3) combining these two methods. The standard solution was mixed in 30 mL *n*-hexane, which was carried out in experimental conditions as mentioned above. The result was reflected through the recovery of 10 phthalate compounds (Figure 1).

The recovered efficiency of 10 phthalates when using the vacuum rotary evaporator to evaporate solvent ranged from 12% to 62%; while using the nitrogen gas flow, the figure was from 32% to 72%. However, when combining these methods, the recoveries of all 10 phthalates were higher than single methods, ranging from 91% to 105%. This combination saved analysis time, and reduced the evaporation of substances as well as contact time between the substances and surrounding atmosphere. Therefore, we incorporated vacuum rotary evaporator into nitrogen gas to evaporate the solvent in the sample preparation process.

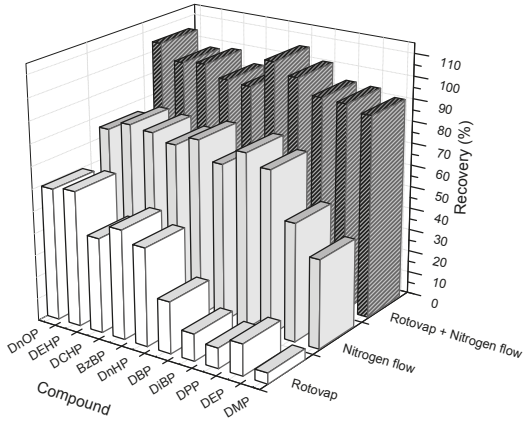

Figure 1. Comparison of recoveries between variable solvent evaporation methods. Note: Dimethyl phthalate, DMP; Diethyl phthalate, DEP; Dipropyl phthalate, DPP; Diisobutyl phthalate, DiDP; Benzyl butyl phthalate, BzBP; di-*n*-hexyl phthalate, DnHP; di(2-ethylhexyl) phthalate, DEHP; di-*n*-octyl phthalate, DnOP; Dicyclohexyl phthalate, DCHP and di-*n*-butyl phthalate, DPBP.

3.1.2. Selecting the Extraction Solvent

The requirements of extraction solvents using in liquid-liquid extraction technique are dissolving well the analytical compounds, having strong affinity to the compounds and preventing matrix effect. In this study, we assessed the extraction ability of numerous solvents such as *n*-hexane, chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2) and ethyl acetate ($CH_3COOC_2H_5$). The isotope standard solution was prepared and added to the drinks which contain fats. These mixtures and each of the mentioned solvents were evaporated by the combination of the rotary evaporator and nitrogen gas. The results of solvent selection were based on the sum of the chromatographic peak area of the three isotopes, and are shown in Figure 2.

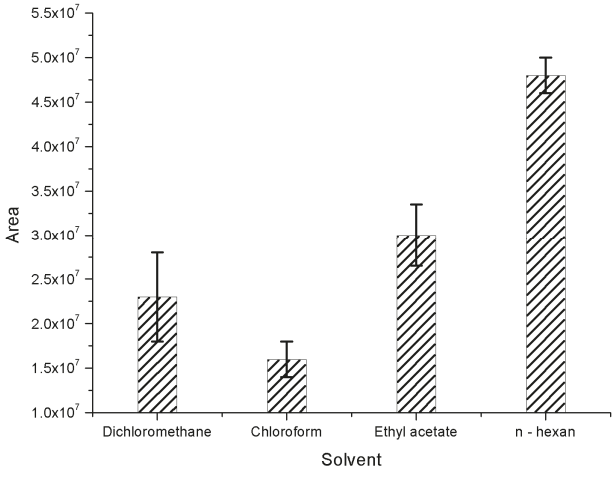

Figure 2. Total peak area of 3 isotope internal standards when using different solvents.

As shown in Figure 2, the total peak area of three internal standard substances of using *n*-hexane as an extraction solvent was much higher than those of dichloromethane, chloroform and ethyl acetate.

Although chloroform is a perfect candidate for extraction of many substances, in this case, when using this solvent in the extraction of beverage samples, other chemicals are also extracted into the organic phase leading to a decrease of the internal standard peak. Therefore, it cannot detect the signal of the internal standard peak. This problem is similar to that of dichloromethane and ethyl acetate solvents [17–20]. Nonetheless, the obtained area when using *n*-hexane was the highest one because this solvent has better capability to extract the analytical compounds to the organic phase and more relatively eliminates the matrix effect than utilizing dichloromethane. To sum up, we decided to use *n*-hexane as the extraction solvent in this study.

3.2. D-Optimal

Analysis of variance is widely used to predict the suitability of a model with experiment results. The obtained results (Table 2) indicated that the predicted values of the model were not conflict with the experiments. The coefficient of determination of R^2 was 0.932 and the coefficient of determination adjustment R^2_{adj} was 0.910. The suitability of the model was also shown in *P* values and Fisher test. $P_{regression}$ value was 0.000 (<0.05), and $P_{Lack\ of\ fit}$ was 0.221 (>0.005), which showed that the obtained model was consistent with the experiment.

Table 2. Analysis of variance (ANOVA).

Recovery	DF	SS	MS (Variance)	F	*P*	SD
Total corrected	28	1199.89	42.8532			6.54624
Regression	7	1118.59	159.798	41.2734	**0.000**	12.6411
Residual	21	81.3055	3.87169			1.96766
Lack of Fit (Model error)	19	79.1989	4.16836	3.9573	**0.221**	2.04166
Pure error (Replicate error)	2	2.10667	1.05333			1.02632
	N = 29	Q^2 =	0.857	Cond. no. =	1.752	
	DF = 21	R^2 =	0.932	RSD =	1.968	
	Comp. = 2	$R^2_{adj.}$ =	0.910			

Note: degrees of freedom, DF; sum of squares, SS; mean square, MS; Fisher, F; probability value, P; and standard deviation, SD.

The three-dimensional response surface shows the effect and interaction of the two factors on the target function. Figure 3a shows the combined effect of the $V_{solvent}/V_{sample}$ ratio and NaCl concentration. Figure 3b shows the image effect of NaCl concentration and time of extraction. Interaction between the $V_{solvent}/V_{sample}$ ratio and time of extraction is shown in Figure 3c. In general, when the value of the variables increases, the efficiency of the phthalate extraction rises and eventually reaches equilibrium.

The contribution of these factors on the extraction efficiency is shown in Figure 3d. The solvent/sample ratio was the biggest influence (54.8%), followed by extraction time (35.8%), and final NaCl concentration (9.4%).

The optimal tool of MODDE 12.1 software was used for the optimization. The results are shown in Table 3. Experimental result was obtained at optimum conditions, yield was 92.51 (95% confidence). This proves that the model was highly meaningful, allowing good experimental results.

Table 3. Optimization of phthalate extraction process.

$V_{solvent}/Vs_{ample}$ Ratio	Number of Extractions	NaCl Concentration (M)	Solvent	Drying Method	Time of Extraction (Min)	% Recovery Efficiency	
						Predicted	Experiment
6.5	2	0.42	*n*-hexan	Rotovap + nitrogen flow	14	90.7	91.1

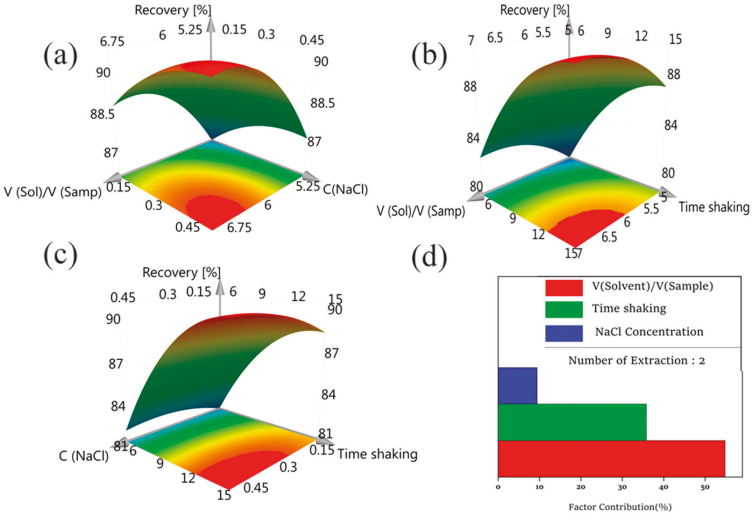

Figure 3. Response surface plots for the central composite design (CCD). Note: (**a**) $V_{solvent}/V_{sample}$ ratio vs. NaCl concentration; (**b**) NaCl concentration vs. time of extraction; (**c**) $V_{solvent}/V_{sample}$ ratio vs. time of extraction and (**d**) The phthalate extraction rises vs. eventually reaches equilibrium.

3.3. Method Performance

To evaluate the efficiency of the proposed method, a number of parameters of the method were investigated and manifested in Tables 4–6. The triple quadrupole detector provided a high degree of selectivity. The linear ranges of these phthalate compounds were built up from 1 to 200 µg/L. Additionally, the weights $1/x^2$ shown through the correlation coefficient (r^2) were greater than 0.996, a non-significant lack of fit and individual residuals deviation of <13% proved the quality of the method. The lowest LOD of these phthalate substances was 0.5 ng/L and the highest was 3.0 ng/L. The maximum of retention time was ±0.06 min, which was below the maximum tolerance deviation stated in SANTE guidelines (±0.1 min). The repeatability (RSD_r) and within-laboratory reproducibility (RSD_{wr}), which expressed percent relative standard deviation (%RSD), ranged from 1.0 to 9.1% and from 2.7 to 12.3%, respectively. All detected RSD values were smaller than 15% that meet the SANTE guideline of RSD ≤ 20%. The trueness of this method was appraised through the recovery values by adding standard solution to carbonated beverage and fat beverage samples at three different concentrations. The average recoveries of 10 phthalate compounds are demonstrated in Table 3 and are within the range required by the SANTE guidelines (between 70% and 120%).

Table 4. Linear dynamic range (µg/L), determination coefficients (r^2), residuals, retention times, limit of detection (LOD) and limit quantitation (LOQ).

Compound	Linear Dynamic Range (µg/L)	r^2	Maximum Individual Residual (%)	Retention Times (min)	LOD (ng/L)	LOQ (ng/L)
DMP	0.1–200	0.997	10.3	6.58	1.0	3.0
DEP	0.1–200	0.996	11.2	8.16	1.0	3.0
DPP	0.1–200	0.998	9.8	10.12	1.0	3.0
DiBP	0.1–200	0.999	11.5	12.00	0.5	1.5
BzDP	0.1–200	0.997	7.3	15.52	1.0	3.0
DnHP	0.1–200	0.997	12.9	15.42	1.5	4.5
DEHP	0.1–200	0.999	9.1	17.02	0.5	1.5
DnOP	0.1–200	0.999	8.2	18.42	1.0	3.0
DCHP	0.1–200	0.996	10.2	16.90	1.0	3.0
DBP	0.1–200	0.999	11.1	12.33	1.0	3.0

Table 5. Repeatability (RSD_r) and within-laboratory reproducibility (RSD_{wr}) for peak areas evaluated at three concentration levels.

Compound	RSDr (n = 5)			RSDwr (n = 5 × 4 Days)		
	1 μg/L	10 μg/L	100 μg/L	1 μg/L	10 μg/L	100 μg/L
DMP	3.6	1.0	3.2	9.1	4.1	5.1
DEP	6.3	4.3	3.8	6.7	4.7	3.4
DPP	7.5	5.4	4.3	8.5	3.3	3.8
DiBP	2.6	1.7	5.7	14.3	3.3	3.3
BzDP	1.6	2.8	3.6	8.6	2.7	3.3
DnHP	5.6	6.0	3.0	12.2	2.8	2.7
DEHP	2.4	3.7	4.4	10.2	7.0	2.8
DnOP	3.8	5.7	8.2	11.2	6.7	4.1
DCHP	5.2	7.4	9.1	12.3	8.6	4.7
DBP	1.9	2.3	8.8	9.9	6.7	3.3

data is presented as % RSD.

Table 6. Trueness results for 10 phthalate compounds in non-alcoholic beverages matrices.

Spiking Level Compound	1		10		100	
	Gas (M/R)	Fat (M/R)	Gas (M/R)	Fat (M/R)	Gas (M/R)	Fat (M/R)
DMP	109.2/7.3	103.2/3.4	94.9/4.3	100.6/9.1	99.2/3.8	103.3/3.8
DEP	97.2/3.8	92.6/6.3	98.3/1.0	98.9/8.8	108.3/1.0	109.3/5.7
DPP	102.2/4.2	101.2/7.5	91.5/1.9	101.4/2.3	94.0/1.7	103.1/5.6
DiBP	107.1/9.1	102.8/2.6	92.3/5.2	105.2/7.4	102.2/4.3	114.7/10.1
BzDP	99.6/6.9	99.3/1.0	113.3/3.8	101.3/5.7	108.5/1.9	97.5/1.6
DnHP	105.6/3.6	100.5/1.9	111.1/2.4	96.5/3.7	100.2/2.8	108.9/8.8
DEHP	114.7/10.1	105.1/2.4	101.1/5.6	99.7/6.0	101.1/5.7	103.2/7.5
DnOP	110.5/8.6	102.5/3.7	99.5/1.6	1002/2.8	94.3/5.2	118.1/12.3
DCHP	117.6/6.9	103.0/1.7	102.8/2.6	103.0/1.7	107.7/6.0	96.6/6.3
DBP	118.1/12.3	109.2/2.8	103.2/7.5	96.1/5.4	100.6/3.6	105.4/8.2

Note: M: Mean (% recovery); R: Relative standard deviation (%).

3.4. Levels of Phthalates in Samples

Non-alcoholic drink samples were analyzed based on the above sample preparation method. The results are shown in Table 7 and Figure 4. As described in Table 4, DBP and DEHP were also detected in all of the 148 collected beverage samples which were analyzed, while DnOP were found in 33% of samples. The appearance of phthalate compounds ranged from 1% to 100% so that almost all of the samples were contaminated by phthalates. As can be seen in Figure 4, DEHP was the phthalate which primarily presents in the samples (>35%), followed by DBP and DEP in mineral water, fruit juice, tea, fermented milk and functional drink. Conversely, in carbonated drink samples, DnOP was the most abundant phthalate substance (>50%). In relation to fermented milk, DMP and DEHP were comparatively in the same proportion (45.5 and 47.6%). It was recognizable that there was the extensive appearance of DMP, DnOP and DBP. DnHP virtually did not appear in these kinds of beverage drinks. DnOP was chiefly found in carbonated drink samples (54.3%). Additionally, DMP was mainly detected in fermented milk samples (45.5%).

The concentrations of phthalate compounds presenting in non-alcoholic beverages are also illustrated in Table 8. In 6 groups of experimental samples, DEHP was the phthalate substance containing the highest mean as well as medium value among all of the samples. The mean and medium were 91.6 and 64.5 μg/L. The variation of concentrations of 148 samples ranged from 0.092 to 466.6 μg/L, which were much higher than those of DBP (22.1 and 18.8 μg/L, the variation varies from 0.093 to 73.5 μg/L). The DMP, BzBP, DPP. DiBP, DnOP and DCHP contents were nd–131.9 μg/L, 0.30–21.5 μg/L, nd –0.52 μg/L, nd–1.9 μg/L, nd–200.4 μg/L and nd–0.60 μg/L, respectively.

Table 7. Detection of frequency [n (%)] of phthalates in non-alcoholic beverages in Hanoi.

Non-Alcoholic Beverages	N	DMP	DEP	DBP	BzBP	DEHP	DnHP	DPP	DiBP	DnOP	DCHP
Mineral water	14	14 (100)	14 (100)	14 (100)	14 (100)	14 (100)	0	14 (100)	14 (100)	0	14 (100)
Carbonated drinks	17	14 (82)	17 (100)	17 (100)	16 (94)	17 (100)	0	17 (100)	17 (100)	17 (100)	17 (100)
Functional drinks	29	19 (66)	26 (90)	29 (100)	28 (97)	29 (100)	0	25 (86)	29 (100)	0	29 (100)
Juice drinks	32	31 (97)	32 (100)	32 (100)	32 (100)	32 (100)	0	32 (100)	0	0	0
Tea drinks	33	0	33 (100)	33 (100)	33 (100)	33 (100)	0	0	0	0	0
Fermented milks	23	23 (100)	23 (100)	23 (100)	23 (100)	23 (100)	0	0	0	0	0
Total	148	101 (68)	145 (98)	148 (100)	146 (99)	148 (100)	0	88 (59)	60 (41)	49 (33)	60 (41)

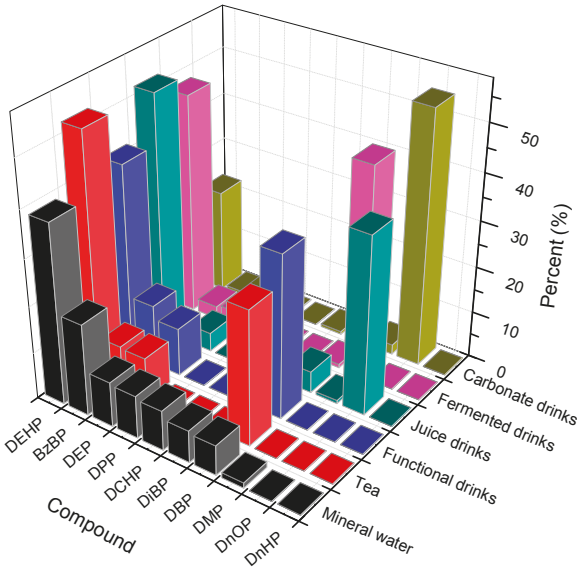

Figure 4. The distribution of 10 phthalate compounds in different kinds of samples. Note: Dimethyl phthalate, DMP; Diethyl phthalate, DEP; Dipropyl phthalate, DPP; Diisobutyl phthalate, DiDP; Benzyl butyl phthalate, BzBP; di-*n*-hexyl phthalate, DnHP; di (2-ethylhexyl) phthalate, DEHP; di-*n*-octyl phthalate, DnOP; Dicyclohexyl phthalate, DCHP and di-*n*-butyl phthalate, DPBP.

Table 8 also shows that the concentrations of phthalate compounds studied on different targets were considerably different. The DEHP in fruit juice samples had the highest mean (230.8 μg/L) and median (222.7 μg/L) among other types of beverage drinks in this experiment. Moreover, the mean and median of DEP (17.9 and 17.3 μg/L) in fruit juice drink were also far higher than other beverages. In terms of fermented milk, DMP content was detected in a range of 12.3 to 131.9 μg/L, and the average and median were 68.0 and 65.7 μg/L, respectively.

Table 8. Phthalate concentrations in different types of non-alcoholic beverages (μg/L).

Non-Alcoholic Beverages		Mineral Water	Carbonated Drinks	Functional Drinks	Juice Drinks	Tea Drinks	Fermented Milk
DMP	Range	0.02–0.05	1.1–5.5	0.04–0.08	0.1–5.6	Nd	12.3–131.9
	Mean	0.03	3.3	0.06	3.3		68
	Median	0.04	3.4	0.05	3.2		65.7
DEP	Range	0.05–0.57	0.38–13.0	1.6–17.9	3.0–33.4	0.9–21.1	0.6–10.2
	Mean	0.3	4.8	10	17.9	11.2	5
	Median	0.29	3.4	11	17.3	10.3	3.8
DBP	Range	0.09–0.95	0.18–43.5	0.16–64.7	1.6–73.4	6.0–4.3	0.81–1.35
	Mean	0.61	19.8	30.3	20.6	34.9	1.1
	Median	0.59	19	26.3	17.8	34.7	1.1
BzBP	Range	0.30–0.95	0.31–3.4	0.41–21.5	0.4–6.4	0.8–21.9	1.1–8.4
	Mean	0.61	1.4	11.8	3.1	11.5	4.2
	Median	0.59	1.2	11.7	3	11.3	3.7
DEHP	Range	0.46–1.8	0.09–71.0	0.72–96.1	27.7–466.6	15.4–87.8	8.3–151.2
	Mean	1.2	28.6	35.9	230.8	63.4	71.2
	Median	1.2	20.5	21	222.7	72.9	67.6
DnHP	Range	Nd	Nd	Nd	Nd	Nd	Nd
	Mean						
	Median						
DPP	Range	0.13–0.44	0.05–0.52	Nd–0.06	0.03–0.21	Nd	Nd
	Mean	0.28	0.3	0.01	0.11		
	Median	0.28	0.33	0.01	0.1		
DiBP	Range	0.14–0.41	0.04–1.9	Nd–0.14	Nd	Nd	Nd
	Mean	0.22	0.76	0.07			
	Median	0.15	0.83	0.06			
DnOP	Range	Nd	0.98–200.4	Nd	136–197	Nd	Nd
	Mean		69.2		169.1		
	Median		56.9		169		
DCHP	Range	0.13–0.60	0.02–0.09	Nd–0.15	Nd	Nd	Nd
	Mean	0.26	0.07	0.02			
	Median	0.16	0.07	0.01			

The distribution of the total phthalate concentration in non-alcoholic drinks was different among the sample matrices. Juice drinks had the highest phthalate concentration, followed by fermented milk and tea. As shown in Figure 5, DEHP was a major contributor leading to the phthalate contamination in non-alcoholic beverage, similar to the previous study [21]. The contamination of phthalates depended on the characteristics of the samples. The sample containing preservatives (potassium benzoate) had higher phthalate concentration than that which did not use preservatives [22]. Furthermore, the sample carrying high fat content was easier to contaminate by phthalate [23]. When comparing the data above, the identification of the sources of phthalate contamination was ambiguous because of other factors such as temperature, pH, light, turbidity and storage time [5,24,25].

Among all phthalates, DEHP is the most popular substance appearing in non-alcoholic beverages in similar studies. Figure 6 illustrates the degree of DEHP contamination in recent studies and the container of these products is not necessarily made from plastic. According to the research of Ustun et al., DEHP concentrations of soda, lemonade, mineral water and high-taste water in Turkey ranged from 73 to 2312 ng/g and the highest DEHP concentration was found in Cola soft drink [21]. In contrast, based on the study of Sireli et al., DEHP concentration in fruit juice drink varies from 1.1 to 44.3 ng/g, which is much lower than Ustun' research [26]. Wu et al. reported that the DEHP content in energy drink and tea ranged from 15 to 83 ng/g [27]. DEHP concentration was remarkably high in the study of Truong et al. of chocolate and high-fat drinks (111–1753 ng/g) [23]. In our research,

DEHP concentration varied from 0.1 to 466.6 ng/g, remained within the range of the above studies and predominantly concentrated in milk-containing fruit juice sample.

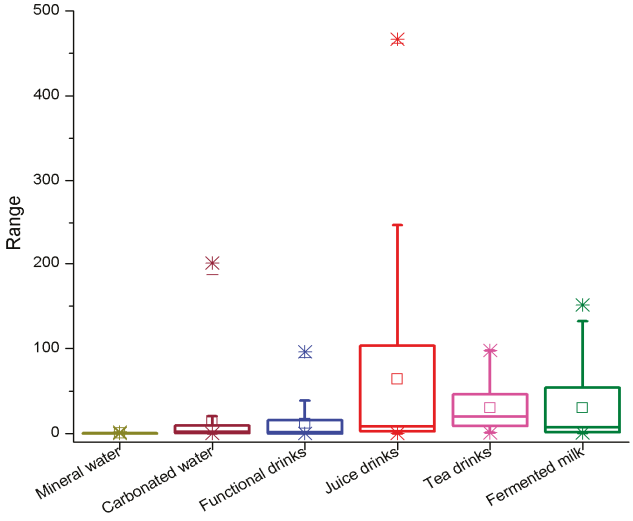

Figure 5. The distribution of the total phthalate concentration in non-alcoholic drinks.

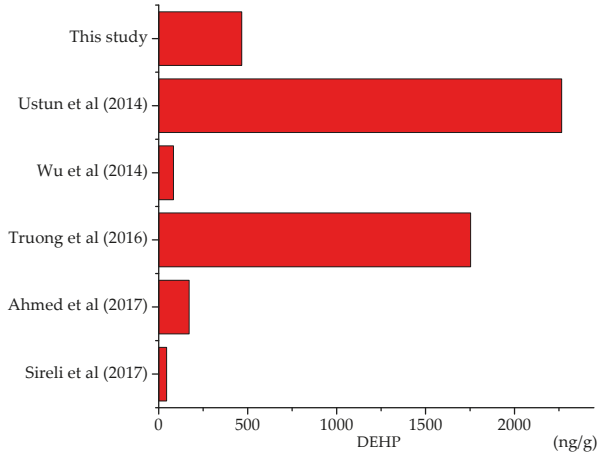

Figure 6. Concentrations of DEHP in similar studies.

3.5. Exposure to Phthalates

Assessing phthalate concentration in non-alcoholic beverages has been investigated by many researchers around the world. However, in Vietnam, there are no specific statistics on phthalate content in daily beverage drinks. Identification of the existence as well as frequency of the occurrence of phthalate compounds in the matrices totally depends on instrument detection limit (IDL) and method detection limit (MDL) of the study, but comparison of phthalate contamination in non-alcoholic beverages still has scientific meaning.

Relying on the studies of Guo [28] and Sireli [26], we calculated the daily intake of DEP, DBP, BzBP and DEHP in Vietnam following the formula below:

$$\mathrm{EDI} = \frac{\mathrm{CQ}}{\mathrm{bw}} \mathrm{r}_{\mathrm{uptake}} \quad (2)$$

where EDI (μg/kg × day) is the estimated daily intake from drinking beverages, C (ng/g) is the phthalate concentration in beverages, r is the gastrointestinal uptake factor and bw (kg) is the body weight. In this study, average beverages intake was 150 g/day, r_{uptake} was 1 and an average bw of 50 kg was used for Vietnam population. The result is shown in the Table 9.

Table 9. Characteristics of the investigated phthalates.

Compound	EDI (μg/kg × Day)	TDI (μg/kg × Day)
DEP	1.50×10^{-4}–0.1002	0.800 (U.S. EPA)
DBP	2.70×10^{-4}–0.2202	0.100 (U.S. EPA)
BzBP	9.00×10^{-4}–0.0645	0.800 (U.S. EPA)
DEHP	2.70×10^{-4}–1.3998	0.020 (U.S. EPA)

The daily intake of DEHP when investigating phthalates in beverages in Vietnam was higher than TDI (U.S. EPA), but the contamination of DEP, DBP and BzBP was significantly lower than the threshold of regulation. The phthalate concentration in non-alcoholic beverages did not give rise to serious consequences for adult health. However, the beverages such as fruit juice and fermented milk, which were analyzed, are consumed daily by pregnant women. Because of this, there is likely to be a mother-to-child exposure through the placenta [29] leading to the phenomenon of hormonal disturbance in children [30].

4. Conclusions

In this research, we focused on the assessment of phthalate compounds in beverages, products whose consumption has grown dramatically in Vietnam, and thus the phthalate contamination factor for non-alcoholic drink was not exactly reflected the exposure level. Phthalate exposure in daily life possibly originates from different sources such as air [31–33], food [28,34], beverages [35,36] as well as cosmetics [37]. As a consequence, in this study, we solely concentrated on evaluating phthalate in beverage drinks, products which are consumed in huge quantities in Vietnam.

Liquid–liquid extraction and the GC-MS/MS analysis technique were optimized and conducted successfully in determining 10 phthalate compounds in different kinds of non-alcoholic drinks. The good recoveries (70–120%), RSDs of all the analysis samples and matrices were lower than 15% and low LOQ (0.5 ng/L) was confirmed. This method was utilized to analyze 10 phthalate substances in 148 non-alcoholic drink samples. The result showed that 100% of the samples were contaminated by DEHP and DEP, and almost all samples were polluted by phthalates. The result of phthalate contamination in this study did not reflect accurately the exposure of phthalates in beverage drinks because of other influencing factors. Therefore, it is necessary to implement more in-depth research to assess properly phthalate contamination during the production process, storage conditions, and when the human body is exposed to these products.

Supplementary Materials: The following are available online at http://www.mdpi.com/2305-6304/6/4/69/s1, Table S1: Identifying, quantifying ions, retention time, and collision energy of 13 phthalates.

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Conflicts of Interest: The authors declare no conflict of interest.

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Potential Antagonistic Effects of Acrylamide Mitigation during Coffee Roasting on Furfuryl Alcohol, Furan and 5-Hydroxymethylfurfural

Dirk W. Lachenmeier [1,*], Steffen Schwarz [2], Jan Teipel [1], Maren Hegmanns [1], Thomas Kuballa [1], Stephan G. Walch [1] and Carmen M. Breitling-Utzmann [3]

[1] Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weissenburger Strasse 3, 76187 Karlsruhe, Germany; jan.teipel@cvuaka.bwl.de (J.T.); maren.hegmanns@cvuaka.bwl.de (M.H.); thomas.kuballa@cvuaka.bwl.de (T.K.); stephan.walch@cvuaka.bwl.de (S.G.W.)

[2] Coffee Consulate, Hans-Thoma-Strasse 20, 68163 Mannheim, Germany; schwarz@coffee-consulate.com

[3] Chemisches und Veterinäruntersuchungsamt Stuttgart, Schaflandstr. 3/2, 70736 Fellbach, Germany; Carmen.Breitling-Utzmann@cvuas.bwl.de

* Correspondence: Lachenmeier@web.de; Tel.: +49-721-926-5434

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Abstract: The four heat-induced coffee contaminants—acrylamide, furfuryl alcohol (FA), furan and 5-hydroxymethylfurfural (HMF)—were analyzed in a collective of commercial samples as well as in *Coffea arabica* seeds roasted under controlled conditions from very light Scandinavian style to very dark Neapolitan style profiles. Regarding acrylamide, average contents in commercial samples were lower than in a previous study in 2002 (195 compared to 303 µg/kg). The roasting experiment confirmed the inverse relationship between roasting degree and acrylamide content, i.e., the lighter the coffee, the higher the acrylamide content. However, FA, furan and HMF were inversely related to acrylamide and found in higher contents in darker roasts. Therefore, mitigation measures must consider all contaminants and not be focused isolatedly on acrylamide, specifically since FA and HMF are contained in much higher contents with lower margins of exposure compared to acrylamide.

Keywords: coffee; acrylamide; furfuryl alcohol; furan; 5-hydroxymethylfurfural; risk assessment

1. Introduction

Acrylamide is a heat-induced contaminant with frequent occurrence in foods and beverages [1–4]. It has been classified by the International Agency for Research on Cancer (IARC) as probably carcinogenic to humans (group 2A) [5]. The EFSA suggested that its margin of exposure indicates a concern for neoplastic effects based on animal evidence [6]. Coffee is an important topic in reduction of acrylamide, because its consumption may lead to 20–30% of total daily intake [7].

Following the first findings of acrylamide in foods and research into its formation mechanism [8,9], it was quickly discovered that coffee behaves differently from all other foods. While typically, the acrylamide content rises with color or browning degree due to its origin as a Maillard reaction product, for coffee, its content decreases from light to very dark roasts [10]. The maximum of acrylamide is formed very early in the roast and then decreases until the desired roasting degree is reached. Experimental studies have shown that the final acrylamide content purely depends on the roasting degree but not on the profile by which this degree is achieved (i.e., neither very slow nor very quick roasting methods have any influence) [10]. Currently, literature offers only speculation into the breakdown product of acrylamide during roasting or the reaction leading to its degradation [11].

Acrylamide is a product formed during coffee roasting by the Maillard reaction, a major pathway comprising the reaction between asparagine and reducing sugars [12,13]. The formation capacity is

limited by the amount of asparagine [14], which is the reason for higher acrylamide contents found in *Coffea canephora* ("robusta") coffee due to its higher asparagine content.

Mitigation options may start with agronomy (e.g., species and variety selection, fertilization etc.) and roasting, but have also included strategies during processing such as asparaginase addition or lactic acid bacteria, none of which left the feasibility stage [15]. Careful removal of defective coffee beans is recommended, because these contain significantly higher amounts of asparagine (>2 fold), which is a major precursor of acrylamide formation [7,16]. Storage of coffee may lead to considerable reduction, but the final brew preparation is believed to have little influence due to the excellent water-solubility of acrylamide [15]. Some authors suggested that the variation detected in commercial samples may predominantly reflect differences in storage time [17]. Supercritical fluid extraction can be applied to reduce acrylamide by up to 79% [18]. Vacuum processing was suggested as a measure to reduce acrylamide in medium roasted coffee by 50% [19].

From all these factors, roasting was the predominant focus of previous research, and consistent findings hint that an increased roasting degree leads to a decrease in acrylamide formation [10,14,20–24].

Following several years of voluntary industry action with minimization concept [25], mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food were recently implemented in an EU regulation [26]. The producers need to identify the critical roast conditions to ensure minimal acrylamide formation. They also need to ensure that the level of acrylamide in coffee is lower than the benchmark level of 400 µg/kg.

Besides acrylamide, coffee may contain further heat-induced contaminants that were also classified by IARC. Namely, furan [27], and furfuryl alcohol (FA) [28,29] are possibly carcinogenic to humans (group 2B). For 5-hydroxymethylfurfural (HMF), some evidence of carcinogenic activity was found in animal experiments [30,31], but the compound has not yet been classified by IARC. Out of these, furan is the compound in coffee studied most intensely, including large surveys [32–34], while less research is available on furfuryl alcohol [35–38] and HMF [39].

2. Materials and Methods

2.1. Analytical Methodology

The analysis of acrylamide was conducted according to the standard method EN 16618:2015 using liquid chromatography in combination with tandem mass spectrometry (LC/MS/MS) [40]. In deviation to this standard, samples were defatted with a mixture of isohexane and butyl methyl ether. Furthermore solid-phase extraction (SPE) was only used for clean-up, not for concentrating the acrylamide [11]. With this method, a limit of detection (LOD) of 10 µg/kg, and a limit of quantification (LOQ) of 30 µg/kg can be achieved. A repeatability relative standard deviation (RSDr) of 6% was determined within our laboratory. The method was applied successfully in several proficiency tests.

Analysis of furan was conducted using headspace-GC-MS and quantification with internal standard (furan-d_4) as previously described [41]. A multipoint calibration (0.65–12.94 mg/kg) was used for quantification in SIM-Mode on a GC 7890B with MSD 5977B (Agilent Technologies, Waldbronn, BW, Germany) instead of the previously used standard addition. With this method, a LOD of 0.36 mg/kg and a LOQ of 1.2 mg/kg was achieved (0.5 g coffee sample weight). A RSDr of 3.5% was determined within our laboratory.

Analysis of furfuryl alcohol (FA) and 5-hydroxymethylfurfural (HMF) was accomplished using nuclear magnetic resonance (NMR) spectroscopy as previously described [42]. The within-laboratory RSDr was 6% for FA and 8% for HMF. LOD and LOQ were 12 and 39 mg/kg for FA and 6 and 23 mg/kg for HMF, respectively.

2.2. Samples and Roasting Experiments

Samples were obtained from official sampling for food control purposes in the German federal state Baden-Württemberg from all stages of trade, mainly supermarkets and artisanal roasters. For roasting experiments, two directly imported single estate terrace coffees (*Coffea arabica* and *canephora*) were supplied by Amarella Trading (Mannheim, BW, Germany).

Twelve separate 2.4 kg batches of coffee beans were roasted using an FZ-94 Laboratory Roaster (CoffeeTech, Tel Aviv, Israel). Roasting was conducted using either pure *Coffea arabica* or pure *Coffea canephora* samples. The roasting profiles (e.g., regarding temperature endpoints) were based on expert roasters' experience as best suitable for the intended coffee roast type. The systematically different roast profiles were recorded and controlled using Artisan v1.5.0 (Artisan-Scope.org, Poing, BY, Germany, 2018, https://artisan-scope.org).

2.3. Risk Assessment Methodology and Statistics

Risk assessment was conducted using the margin of exposure (MOE) methodology according to the method for comparative risk assessment previously published for alcoholic beverages [3]. Statistical correlations were assessed using linear regression analysis calculated with OriginPro V7.5 (OriginLab Corporation, Northampton, MA, USA) with R being the correlation coefficient and p being the significance of Pearson's test for linear relation. p values below 0.05 are assumed as being significant.

3. Results

3.1. Results of Roasting Experiments

Two green coffee samples (*Coffea arabica and canephora*) were subjected to roasting using six different profiles, namely coffee roasting (quick and slow drying), espresso roasting (quick and slow drying) as well as Scandinavian roasting (very light roasting) and Neapolitan roasting (very black roasting). The roasting profiles for the *C. canephora* roasting are shown in Figure 1. Profiles for *C. arabica* roasting were similar (data not shown).

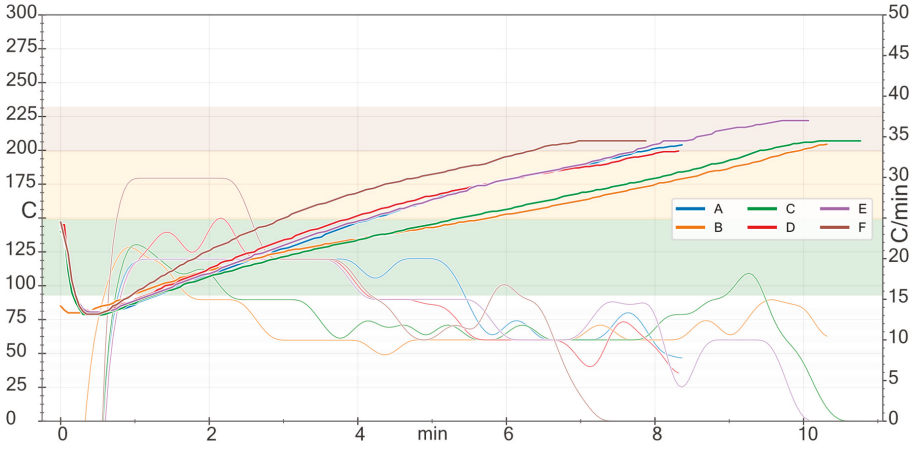

Figure 1. Profiles of experimental coffee roasting (A: Coffee quick drying; B: Coffee slow drying; C: Espresso slow drying; D: Scandinavian coffee; E: Espresso Neapolitan; F: Espresso quick drying).

Some numerical descriptors of the roasting profiles are provided in Table 1 as well as the analytical results for the samples. The individual roasting profile had a significant influence on the contents of the process contaminants. The area under the curve (AUC) is inversely related to acrylamide content ($R = -0.59$; $p = 0.045$; $n = 12$), while the contents of furfuryl alcohol ($R = 0.78$; $p = 0.003$; $n = 12$) and

furan ($R = 0.63$; $p = 0.027$; $n = 12$) are positively correlated to this roasting parameter, independent of the coffee species. Furan ($R = 0.65$; $p = 0.021$; $n = 12$) and furfuryl alcohol ($R = 0.82$; $p = 0.001$; $n = 12$) are significantly positively correlated to drop temperature. The other parameters were not significantly correlated with any analyte.

Table 1. Indicators of roasting (data for *C. canephora* roast; *C. arabica* data similar) and analytical results of roasted coffee (*C. arabica*/*C. canephora*).

Profile	Charge [1] [°C]	Drop [2] [min]	Drop [2] [°C]	AUC [3] [°C·min]	Acrylamide [µg/kg]	Furfuryl Alcohol [mg/kg]	Furan [mg/kg]	HMF [mg/kg]
Scandinavian coffee	145	08:22	200	555	470/480	70/93	<1.2/2.5	40/47
Coffee quick drying	140	08:24	204	566	200/390	124/94	1.7/2.7	74/49
Coffee slow drying	85	10:21	205	673	210/420	128/92	1.5/2.6	62/43
Espresso quick drying	147	07:55	203	625	170/300	170/117	2.5/4.9	66/47
Espresso slow drying	140	10:48	207	762	150/290	173/133	2.6/5.0	78/42
Neapolitan espresso	145	10:06	222	796	130/250	223/189	3.6/7.6	84/32

[1] Temperature at charge of roaster. [2] Drop = end of roast. [3] Area under the curve (indicator how much total energy the beans have received during roasting).

There is an inverse linear statistically significant relationship between acrylamide and furfuryl alcohol ($R = -0.85$; $p < 0.001$; $n = 12$), and between acrylamide and HMF ($R = -0.73$; $p = 0.007$; $n = 12$). None of the other pairs for contaminants were significantly correlated; however, in tendency, acrylamide and furan were also inversely correlated, while furfuryl alcohol is positively correlated with HMF and furan.

3.2. Results of Commercial Sample Analyzes

The full results of analysis are provided in Appendix A, Table 1. The results are summarized in Table 2. From the sub-group of samples analyzed for both acrylamide and furfuryl alcohol, an inverse linear relationship was detected ($R = -0.59$; $p = 0.008$; $n = 19$). However, no correlation between HMF and acrylamide was detected, while HMF and furfuryl alcohol were positively correlated ($R = 0.50$; $p = 0.007$; $n = 28$). The data set of furan analysis was too small for meaningful statistical analysis.

Despite the low number of samples, the comparison of results in Table 2 suggests that the acrylamide content in roasted coffee and in instant coffee may have decreased over the years. None of the samples has exceeded the new EU benchmark levels.

Table 2. Comparison of acrylamide analysis results from 2002 with current results (summary from Annex A, Table S1).

Category according to EU Regulation 2017/2158	Year of Analysis	Number of Samples	Average [µg/kg]	Median [µg/kg]	90th Percentile [µg/kg]
Roast coffee	2002 (data from [11])	5	303	313	461
Roast coffee	2015	4	118	130	138
Roast coffee	2018	22	195	165	306
Instant (soluble coffee)	2013	6	642	686	831
Instant (soluble coffee)	2015	7	483	356	805
Instant (soluble coffee)	2016	5	379	269	664
Instant (soluble coffee)	2018	13	555	600	842
Coffee substitutes exclusively from cereals	2013–2018	6	401	418	563
Coffee substitutes from a mixture of cereals and chicory	2012–2018	16	587	525	805

3.3. Comparative Risk Assesment of Heat-Induced Contaminants in Coffee

Finally, the results of comparative risk assessment using the margin of exposure methodology are shown in Table 3. The risk assessment uses survey data from the literature due to the restricted, non-representative sampling in the current study.

While the contents of acrylamide and furan are much lower than the contents of furfuryl alcohol and HMF, the toxicity thresholds of both compounds are also much lower, with acrylamide being the compound showing effects at the lowest concentration of all four compounds. Nevertheless, due to the higher exposure, HMF and furfuryl alcohol have the lowest margins of exposure. Three of the compounds, acrylamide, furfuryl alcohol and HMF, have MOEs below 10,000. Furan falls below this threshold only in worst-case scenarios (P95 exposure) and can be seen as a compound with lower risk. However, HMF is believed to operate by a non-genotoxic mechanism and hence an uncertainty factor of 100 (instead of 10,000 for genotoxic compounds) may be sufficient to exclude public health concerns.

Table 3. Risk assessment of several roasting contaminants in coffee.

Contaminant	Average/P95 Content in Roasted Coffee	Average/P95 Exposure for Drinking 1 Cup of Coffee [1]	Toxicological Threshold [2]	Average/P95 Margin of Exposure (MOE) [3]
Acrylamide	249/543 µg/kg [6]	0.05/0.10 µg/kg bw/day	0.18 mg/kg bw/day (BDML10) [43]	3800/1700
Furfuryl alcohol	251/392 mg/kg [35]	0.05/0.07 mg/kg bw/day	53 mg/kg bw/day (NOEL) [44]	1110/710
HMF	689/1688 mg/kg [39]	0.13/0.32 mg/kg bw/day	79 mg/kg bw/day (BMDL10) [30]	600/250
Furan	38/107 µg/L [33]	0.12/0.14 µg/kg bw/day [33]	1.28 mg/kg bw/day (BMDL10) [45]	42,134/3113 [33]

[1] Calculated assuming 14 g of coffee powder per 0.2 L cup (according to ISO 6668 [46]) and assuming 100% extraction yield, except for furan for which data from brewed beverage analyses were available. Average bodyweight 73.9 kg [47]. The data for furan were probabilistically calculated and taken from [33]. All other values were own calculations using point estimates. [2] NOEL: no-observed effect level; BMDL10: benchmark dose lower confidence limit for 10% response. [3] MOE = Toxicological threshold/exposure. Values pessimistically rounded to significance. The higher the MOE, the lower the risk. A MOE > 10,000 is typically interpreted as low risk for genotoxic carcinogens, while >100 is used for non-genotoxic compounds with thresholded effects.

4. Discussion

Roasting properties of coffee are basically dependent on the amount of heat transferred into the coffee beans during roasting and on the roasting time [17]. A good indicator for the achieved heat transfer rate is the area under the curve of the roasting profile. These values show a negative correlation with acrylamide during our roasting experiment, confirming the inverse relationship of roasting energy and acrylamide [10,14,20–24]. In contrast, the other contaminants under study (furfuryl alcohol, furan and HMF) appear to be positively related to the roasting energy, meaning the highest contents are typically found in the strongest roasts (espresso).

Interestingly, despite early findings that acrylamide in coffee decreases with the roasting degree, there is still considerable misinformation about this topic. Some small artisanal coffee roasters even advertise on their webpages that their "mild" roasting process with temperatures rising only up to 200 °C would result in lower acrylamide contents. The contrary being clearly the case, however.

Compared to results from our institutes published in 2002 (average acrylamide content in coffee: 303 µg/kg, median 313 µg/kg; 90% percentile 461 µg/kg) [11], the contents found during this study were lower. In Germany, the minimization of acrylamide has been most advanced of all EU member states [25]. Manufacturers should therefore not be challenged, even if the current benchmark level should become the new legal maximum limit [25]. Our results confirm this assumption, since none of our official samples exceeded the current benchmark level.

Some authors have questioned the influence of species, e.g., Mojksja and Gielecinska [22], who found no significant difference in acrylamide contents between Arabica and Robusta coffee. Our restricted results of two pure *C. canephora* coffees (260–270 µg/kg) lie actually above the average acrylamide contents of all coffee samples (196 µg/kg), which is consistent with the majority of

literature [14,24,48]. However, in our case a comparison is confined due to the fact that the species is unknown in most of the analyzed commercial samples. It may be speculated that the difference is caused by the lower quality of commercial *C. canephora* coffee with a higher degree of defective beans. However, the comparison of our high-quality single estate terrace coffees (Table 1) also points to higher levels of acrylamide in *C. canephora*.

There are only few studies available on the correlation of other contaminants with acrylamide. Kocadagli et al. [49] studied the kinetics of both acrylamide formation and HMF formation and found similar tendencies, meaning both acrylamide and HMF are reduced by more intense roasts. This is in contrast to our results, which detected this behavior only for acrylamide but not for HMF. An explanation may the different methodology in Kocadagli et al. [49], which did not apply a commercial coffee roaster but only an oven at 220 °C for 5–60 min. We therefore believe that our results may have a higher relevance for commercial coffee roasting. Nevertheless, there remains some uncertainty in HMF exposure from coffee. For example, the survey reported by Arribas-Lorenzo [39] from Spain found higher HMF levels than our study with less samples. According to the German Federal Institute for Risk Assessment (BfR) evaluation, the levels of HMF in foods were suggested to exhibit no identifiable health risk for the consumer [50]. However, the BfR did not include coffee in its evaluation of HMF due to a lack of food monitoring data necessary for exposure assessment.

For other heat-induced contaminants besides acrylamide, no action has been typically taken to reduce levels and there are also no EU benchmark or maximum levels for heat-induced contaminants besides acrylamide. Therefore, focus and research activity have been mainly aimed at acrylamide in the past. The Codex Code of Practice to reduce acrylamide in foods currently does not provide guidance for coffee because to date "no commercial measures for reducing acrylamide in coffee are currently available" [15,51]. While this opinion is probably outdated, as various measures have shown to be effective (see introduction), our findings suggest that indeed no measures should be implemented that solely focus on acrylamide. Using a holistic risk assessment approach, all major heat-induced contaminants in coffee need to be modelled prior to pointing out any measure. Otherwise it could well mean that the benefit gained by reduction of acrylamide might be outweighed by the elevated risk of other contaminants such as furfuryl alcohol that are concomitantly increased by the applied measure. As other authors have shown [7,20], holistic risk-benefit analysis would be most preferable as the mitigation of acrylamide might not only lead to increased formation of other contaminants such as furfuryl alcohol [36], but may also lead to reduced contents in beneficial compounds in coffee such as antioxidants.

Compared to other lifestyle factors such as tobacco smoking or alcohol drinking, the cancer risk from coffee (if any exists) appears to be rather low. According to IARC, epidemiological studies even suggest a lack of carcinogenicity of drinking coffee for cancer of the liver [52,53], which is the major target organ of heat-induced contaminants. Bladder cancer was the only cancer site for which an increased risk had been observed in some earlier epidemiological studies, leading to an IARC grouping as 2B in 1991 [54]. However, more recent well-conducted epidemiologic studies were unable to replicate the association with bladder cancer, and coffee consumption has been removed from the classification as a possible/probable human carcinogen [52,53].

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Full analytical results of samples measured between 2012–2018 for acrylamide, furfuryl alcohol and HMF.

Sample ID	Sample Description	Category according to EU Regulation 2017/2158	Year	AA (µg/kg)	FA (mg/kg)	Furan (mg/kg)	HMF (mg/kg)
12119400	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2012	803	-	-	-
12119400-1	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2012	792	-	-	-
12119400-2	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2012	806	-	-	-
12119400-3	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2012	759	-	-	-
130122855	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2013	664	-	-	-
130123291	100% soluble coffee	Instant (soluble) coffee	2013	866	-	-	-
130123334	Coffee, soluble	Instant (soluble) coffee	2013	495	-	-	-
130124497	Coffee substitute, soluble	Coffee substitutes exclusively from cereals	2013	436	-	-	-
130124499	Coffee, soluble	Instant (soluble) coffee	2013	744	-	-	-
130127835	100% soluble coffee	Instant (soluble) coffee	2013	796	-	-	-
130128813	Coffee, soluble	Instant (soluble) coffee	2013	325	-	-	-
130128818	100% soluble coffee	Instant (soluble) coffee	2013	628	-	-	-
130130022	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2013	591	-	-	-
130132127	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2013	214	-	-	-
130132150	Coffee substitute, soluble	Coffee substitutes exclusively from cereals	2013	619	-	-	-
130237309	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2013	387	-	-	-
150231135	Coffee, soluble	Instant (soluble) coffee	2015	1135	-	-	-
150231200	Coffee, soluble	Instant (soluble) coffee	2015	199	-	-	-
150231825	Coffee substitute, soluble	Coffee substitutes exclusively from cereals	2015	508	-	-	-
150231835	Turkish coffee	Roast coffee	2015	127	-	-	-

Table 1. *Cont.*

Sample ID	Sample Description	Category according to EU Regulation 2017/2158	Year	AA (µg/kg)	FA (mg/kg)	Furan (mg/kg)	HMF (mg/kg)
150309974	Coffee	Roast coffee	2015	70	-	-	-
150309977	Coffee, decaffeinated	Instant (soluble) coffee	2015	335	-	-	-
150334870	Espresso Italiano	Roast coffee	2015	132	-	-	-
150334875	Coffee, soluble	Instant (soluble) coffee	2015	356	-	-	-
150334880	Coffee, soluble	Instant (soluble) coffee	2015	320	-	-	-
150337674	Coffee	Roast coffee	2015	141	-	-	-
150337675	Coffee, decaffeinated	Instant (soluble) coffee	2015	585	-	-	-
150337676	Coffee	Instant (soluble) coffee	2015	452	-	-	-
160450717	Malt coffee	Coffee substitutes exclusively from cereals	2016	370	-	-	-
160451307	Coffee, soluble	Instant (soluble) coffee	2016	223	-	-	-
160451426	Coffee, soluble	Instant (soluble) coffee	2016	273	-	-	-
160451967	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2016	361	-	-	-
160452173	100% soluble coffee, 100% Arabica	Instant (soluble) coffee	2016	269	-	-	-
160452684	Coffee substitute, soluble	Coffee substitutes exclusively from cereals	2016	74	-	-	-
160454844	Coffee, soluble	Instant (soluble) coffee	2016	206	-	-	-
160472527	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2016	407	-	-	-
160472529	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2016	431	-	-	-
160509176	Coffee, soluble	Instant (soluble) coffee	2016	925	-	-	-
160509194	Espresso	Roast coffee	2016	447	-	-	-
160574673	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2016	347	-	-	-
180352602	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2018	650	-	-	-
180352683	Coffee	Roast coffee	2018	160	108	1.7	46
180365240	Costa Rica Arabica coffee	Roast coffee	2018	170	108	2.0	46
180379248	India Monsooned	Roast coffee	2018	150	74	5.4	34

Table 1. *Cont.*

Sample ID	Sample Description	Category according to EU Regulation 2017/2158	Year	AA (µg/kg)	FA (mg/kg)	Furan (mg/kg)	HMF (mg/kg)
180398113	Coffee	Roast coffee	2018	210	156	2.3	52
180409591	Arabica-Robusta-mixture, coffee	Roast coffee	2018	130	133	2.6	48
180420281	coffee, organic	Roast coffee	2018	110	122	-	57
180420519	Coffee	Roast coffee	2018	95	104	3.5	46
180433746	Coffee	Roast coffee	2018	310	76	-	53
180439193	coffee, Ethiopia	Roast coffee	2018	150	-	-	-
180444177	Coffee	Roast coffee	2018	170	121	-	42
180447473	Coffee	Roast coffee	2018	110	116	-	57
180453665	Coffee Sumatra	Roast coffee	2018	120	119	-	52
180468077	Coffee	Roast coffee	2018	130	122	-	51
180478363	Coffee substitute, soluble	Instant (soluble) coffee	2018	730	-	-	-
180481476	Coffee substitute, soluble	Instant (soluble) coffee	2018	110	64	-	40
180486743	Coffee substitute, soluble	Instant (soluble) coffee	2018	670	-	-	-
180486745	Coffee substitute, soluble	Instant (soluble) coffee	2018	460	-	-	-
180489109	Coffee substitute, soluble	Instant (soluble) coffee	2018	420	-	-	-
180489247	Coffee substitute, soluble	Instant (soluble) coffee	2018	440	-	-	-
180492032	100% soluble coffee, 100% Arabica	Instant (soluble) coffee	2018	870	-	-	-
180492048	Coffee, soluble	Instant (soluble) coffee	2018	660	-	-	-
180494672	Coffee, soluble	Instant (soluble) coffee	2018	910	-	-	-
180504580	Coffee, soluble	Instant (soluble) coffee	2018	420	-	-	-
180520043	100% soluble coffee, 100% Arabica	Instant (soluble) coffee	2018	690	-	-	-
180533580	Pure Canephora coffee	Roast coffee	2018	260	42	-	40
180533581	Pure Canephora coffee	Roast coffee	2018	270	37	-	49
180533582	Arabica coffee	Roast coffee	2018	270	78	-	46
180533583	Arabica coffee	Roast coffee	2018	460	55	-	43

Table 1. *Cont.*

Sample ID	Sample Description	Category according to EU Regulation 2017/2158	Year	AA (µg/kg)	FA (mg/kg)	Furan (mg/kg)	HMF (mg/kg)
180533584	Arabica coffee	Roast coffee	2018	330	60	-	35
180539910	Coffee, soluble	Instant (soluble) coffee	2018	600	-	-	-
180552699	100% Organic Arabica coffee	Roast coffee	2018	120	-	-	-
180619399	Coffee substitute, soluble	Coffee substitutes exclusively from cereals	2018	400	-	-	-
180619831	Coffee, soluble	Instant (soluble) coffee	2018	240	-	-	-
180628887	Espresso	Roast coffee	2018	210	-	-	-
180631120	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2018	460	-	-	-
180631416	Coffee	Roast coffee	2018	250	-	-	-
180638375	Espresso	Roast coffee	2018	100	99	-	38
180643299	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2018	220	-	-	-
180643300	Coffee substitute, soluble	Coffee substitutes from a mixture of cereals and chicory	2018	1500	-	-	-

AA: Acrylamide; FA: Furfuryl alcohol; HMF: 5-Hydroxymethylfurfural; "-": parameter not analyzed in that sample.

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Magnetic Stirring Assisted Demulsification Dispersive Liquid–Liquid Microextraction for Preconcentration of Polycyclic Aromatic Hydrocarbons in Grilled Pork Samples

Jitlada Vichapong [1,*], Yanawath Santaladchaiyakit [2], Rodjana Burakham [3] and Supalax Srijaranai [3]

1 Creative Chemistry and Innovation Research Unit, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Mahasarakham University, Maha Sarakham 44150, Thailand
2 Department of Chemistry, Faculty of Engineering, Rajamangala University of Technology Isan, Khon Kaen Campus, Khon Kaen 40000, Thailand; sanyanawa@gmail.com
3 Materials Chemistry Research Center, Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand; rodjbu@kku.ac.th (R.B.); supalax@kku.ac.th (S.S.)
* Correspondence: jitlada.v@msu.ac.th; Tel.: +66-43-75-4246; Fax: +66-43-75-4246

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Abstract: A simple microextraction method, magnetic stirring assisted demulsification dispersive liquid–liquid microextraction, for preconcentration of five polycyclic aromatic hydrocarbons (fluorene, phenanthrene, anthracene, fluoranthrene, and pyrene) was investigated prior to analysis by high performance liquid chromatography. In this method, a mixture of extraction solvent and disperser solvent was rapidly injected into sample solution. The magnetic stirrer agitator aided the dispersion of the extraction solvent into the sample solution. After the formation of an emulsion, the demulsifier was added, resulting in the rapid separation of the mixture into two phases. No centrifugation step was required. Several parameters affecting the extraction efficiency of the proposed method were studied, including addition of salt, kind and volume of extraction solvent, volume of demulsifier solvent, and extraction times. Under the optimum conditions, high enrichment factor, low limit of detections (LODs) and good precision were gained. The proposed method was successfully applied to analysis of polycyclic aromatic hydrocarbon residues in grilled pork samples.

Keywords: magnetic stirring assisted demulsification dispersive liquid–liquid microextraction; polycyclic aromatic hydrocarbons; grilled pork; high performance liquid chromatography

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute of a large class of organic materials which are formed of two or more fused aromatic rings [1–3]. These compounds are generally drawn from incomplete combustion or high-temperature pyrolysis of organic materials, such as coal, petrol, wood, garbage, tobacco, meats or other organic foods [4]. Both the European Union (EU) and the United States Environmental Protection Agency (USEPA) have their own list of 16 PAHs as "priority organic pollutants", due to their high toxicity to the human health [5]. PAHs are non-polar, very hydrophobic compounds, with low water solubility. Therefore, in the aquatic environment they commonly exist in relatively low concentrations [6]. Consequently, powerful analytical methods are required to extract, separate, and identify these target analytes in the environment [7].

Several chromatographic methods including gas chromatography [8,9] and high-performance liquid chromatography [10,11] have been regularly used for separation and quantification of PAHs in various samples. Although these are sensitive and selective methods, PAHs usually occur in ppb

level or lower in complex mediums containing various interfering compounds [12]. Therefore, sample preparation, matrix removal and preconcentration of the target analytes are needed before analysis in order to obtain sensitive and accurate results.

Conventional sample preparation methods such as solid-phase extraction (SPE) [13,14] and liquid–liquid extraction (LLE) [15,16] have been used for preconcentration and clean-up before analysis of PAHs. Unfortunately, these methods are tedious, time-consuming, and require large amounts of samples and toxic organic solvents [2]. Therefore, much effort has been made to develop a simple, sensitive and environmentally friendly sample preparation method termed dispersive liquid–liquid microextraction (DLLME) [17]. This method is based on the use of high density of extraction solvents such as chlorinated solvent. However, these are toxic and harmful to human health and environment. The other DLLME mode is based on the low density of extraction solvent. However, the main drawback of DLLME modes was the requirement of a centrifugation step. Recently, a new DLLME technique without a centrifugation step was introduced, the low-density based demulsification DLLME [18,19]. After the mixture of extraction solvent and disperser solvent was added into the aqueous solution, the cloudy solution was immediately separated by adding demulsifiers. No centrifugation step was required.

There are various agitators, such as vortex [20], ultrasound [21], and in-syringe [22], that have been used to enhance the dispersion and to accelerate the formation of fine droplets of extraction solvent. Zhang et al. [23] proposed a simple magnetic stirring assisted dispersive liquid–liquid microextraction (MSA-DLLME) method. After the injection of extraction solvent and disperser solvent into an aqueous solution, the sample was magnetically stirred. Consequently, the centrifugal step was not required. In this present study, the magnetic stirring assisted demulsified dispersive liquid–liquid microextraction combined with HPLC was investigated for extraction, preconcentration and simultaneous determination of PAHs. The magnetic stirrer was used to increase the dispersion and mass transfer between two phases. Special attention was paid to parameters providing the highest extraction efficacy of the extraction procedure. We carefully evaluated salt addition, kind and volume of extraction solvent, kind and volume of disperser solvent, volume of demulsifier solvent and extraction times. The proposed method was successfully applied to the determination of PAHs in grilled pork samples. To our knowledge, this was the first time that the simple magnetic stirring assisted demulsified dispersive liquid–liquid microextraction technique was used in the determination of trace levels of PAHs.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and reagents used were of analytical reagent grade or better. The analytical standards of polycyclic aromatic hydrocarbon including fluorene, phenanthrene, anthracene, fluoranthrene and pyrene were purchased from Sigma-Aldrich (Darmstadt, Germany). The stock standard solution (1000 mg·L^{-1}) was prepared by dissolving in methanol (MeOH). The working solution of standard was prepared daily by dilution with deionized water. Deionized water was prepared using RiOs^{TM} Type I Simplicity 185 (Millipore Waters, Newford, MA, USA) with the resistivity of 18.2 MΩ.cm. Acetonitrile (ACN) and MeOH (HPLC grade) were obtained from Merck (Darmstadt, Germany). All solvents for HPLC were filtered through 0.45 μm filters (Millipore Corp., Newford, MA, USA) and degassed in an ultrasonic bath. NaCl, anhydrous Na_2CO_3 and anhydrous Na_2SO_4 were purchased from Ajax Finechem (Auckland, New Zealand), and CH_3COONa (Carlo Erba, France) was used. 1-Dodecanol and 1-octanol were provided by Merck (Darmstadt, Germany).

2.2. Chromatographic Conditions

The HPLC system was comprised of a Waters 1525 binary HPLC pump (Newford, MA, USA), a Rheodyne injector and photodiode array detector (PDA). The Empower 3 software was chosen

for data acquisition. A LiChrospher® 100 RP-8 endcapped (4.6 × 150 mm, 5.0 μm) column (Merck, Darmstadt, Germany) was employed for the separation of all analytes, and was maintained at room temperature. The mobile phase was comprised of 67% acetonitrile in water with isocratic elution at a flow rate of 1 mL·min^{-1}. The injection volume was 20 μL. The detection of all target analytes was set at 254 nm. Five polycyclic aromatic hydrocarbons were separated within 10 min with the elution order of fluorene (t_R = 6.87 min), phenanthrene (t_R = 7.03 min), anthracene (t_R = 7.98 min), fluoranthrene (t_R = 8.98 min) and pyrene (t_R = 9.46 min).

2.3. Sample Preparation of Grilled Pork Samples

Grilled pork samples were collected from market in Maha Sarakham province, Northeastern Thailand. One gram of spiked and non-spiked were weighed and transferred to a centrifuge tube. Then, 10 mL of extraction solvent (2 mol·L^{-1} KOH in ethanol) was added to hydrolyze the sample. This sample was ultrasounded with fixed power for 5 min at 45 °C, followed by centrifugation (15 min, 4,000 rpm). The supernatant was collected into another centrifuge tube. The solid residue was extracted again with 3 mL of extraction solvent. All volumes of supernatant were gathered, the funnel was frozen for 1 h at −18 °C to precipitate the fat of the solution. Approximately 5 mL of solution were kept and followed by centrifugation. After that, an aliquot was applied for magnetic stirring assisted demulsified dispersive liquid–liquid microextraction under the selected conditions and analysis by a HPLC system.

2.4. Magnetic Stirring Assisted Demulsified Dispersive Liquid–Liquid Microextraction

A 10-mL aliquot of standard solution of each polycyclic aromatic hydrocarbon or grilled pork sample was mixed with 20% (*w*/*v*) of NaCl and then mixture solution containing 75 μL of extraction solvent (1-dodecanol) and 500 μL dispersive solvent (ACN) was quickly injected into the sample solution. The solution was then stirred at 1500 rpm to increase the mass transfer between two phases. An emulsion (water/extraction solvent/dispersive solvent) was formed. After that, 500 μL of ACN (as de-emulsifier solvent) was injected into the solution to break down the emulsion. The extraction was found to float to the top of the solution immediately. The extraction phase was kept and injected to HPLC for analysis.

2.5. Method Validation

The method validations such as linearity of calibration graph, limit of detection (LOD), limit of quantitation (LOQ), precision and enrichment factors (EFs) were studied. The linear range of standard calibration was conducted between 0.0005 and 1 mg·L^{-1} of PAH. LODs and LOQs were defined as the lowest detectable concentration with a signal to noise ratio of 3:1 and 10:1, respectively. EFs were defined as the concentration ratio of the analytes in the settled phase after performing microextraction methods and its initial concentration in the aqueous phase. The relative standard deviation (RSD, %) for intra-days and inter-day of extraction of PAHs were determined at three different concentration levels (0.10, 0.25 and 0.35 mg·L^{-1} of each PAHs) using 5 injections. The enrichment factor (EF) was defined as the concentration ratio of the analytes in the settled phase (Cset) and in the aqueous sample (Co).

2.6. Statistical Analysis

Data results are given as the mean ± standard deviation (SD) of three measurements (n = 3). In all graphs, a linear regression analysis was conducted using Microsoft Excel 2013 software (London, EC1V 2NX, United Kingdom).

3. Results and Discussion

3.1. Optimization of Magnetic Stirring Assisted Demulsified Dispersive Liquid–Liquid Microextraction Condition

In order to improve monitoring of PAH using magnetic stirring assisted demulsified dispersive liquid–liquid microextraction coupled with HPLC analysis, various experimental parameters including addition of salt, kind and volume of extraction solvent, volume of demulsifier solvent and extraction times were evaluated. The aqueous solution (10.00 mL) containing 100 ng·mL^{-1} of each PAH was used for optimization. All the experiments were performed in triplicate and the mean of the results were used for optimization.

Generally, the addition of salt decreases the solubility of analytes in aqueous solution [24,25] and therefore increases their partitioning into the organic phase for liquid phase microextraction. In this study the addition of various salts (1.0 g) such as sodium chloride (NaCl), sodium sulphate (Na_2SO_4), sodium carbonate (Na_2CO_3), sodium acetate (CH_3COONa) were considered and compared to a control, without salt addition. A comparison of extraction efficiency in terms of peak area with various kinds of salt is shown in Figure 1. Without salt addition, phase separation did not complete. The results demonstrated that the addition of NaCl and Na_2SO_4 provided no significant difference in the peak area of all PAHs except fluorene and phenanthrene. Therefore, NaCl was added in varying concentrations for further study. The effects of the amount of NaCl range from 0.5 to 2.5 g on the extraction efficiency of target analytes were also studied. The result (shown in Figure 2), was that the extraction efficacy of the analytes slightly increased as NaCl increased from 0.5 to 2.0 g, and trended to be decrease when the amount of NaCl was greater than 2.0 g. Finally, 2.0 g was chosen as the amount of NaCl in following experiments.

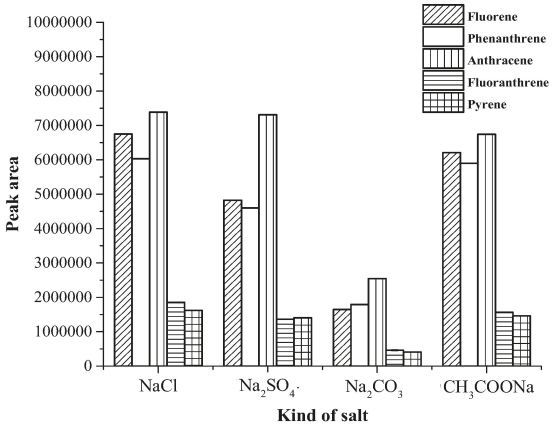

Figure 1. Effect of addition of salt on the extraction efficacy.

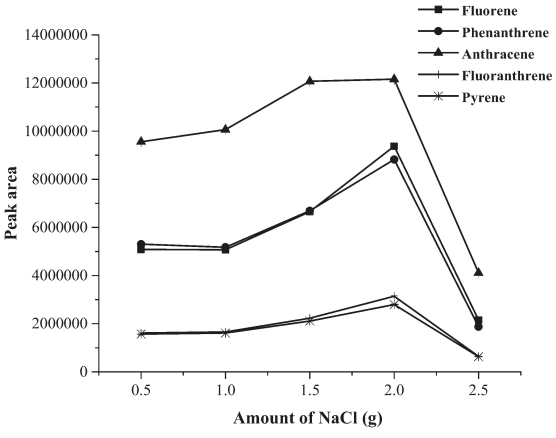

Figure 2. Effect of NaCl amount on the extraction efficacy.

Choosing a suitable extraction solvent is important for obtaining an efficient extraction procedure [26]. In this study, low density immiscible solvents (density = *d*) were investigated, 1-octanol ($d = 0.8270$ g·mL^{-1}), 1-undecanol ($d = 0.8298$ g·mL^{-1}), and 1-dodecanol ($d = 0.8309$ g·mL^{-1}). The cloudy solution was observed after a quick injection of a mixture of extraction solvent and dispersive solvent into the sample solution, the solution was then mixed using the magnetic stirrer. While using 1-octanol as an extraction solvent, it was found that phase separation did not occur. Thus, it was not suitable to be used for extraction solvent. In comparison of extraction efficacy between 1-undecanol and 1-dodecanol, it was found that 1-dodecanol provided high extraction efficacy in terms of peak area (data not shown). Thus, 1-dodecanol was chosen as the extraction solvent for further study. The effect of the 1-dodecanol volume was investigated in the range of 50–150 µL. It was found that, 50 µL of 1-dodecanol cannot complete phase separation. The peak area of all the target compounds decreased as the extraction solvent increased beyond 75 µL. Consequently, 1-dodecanol 75 µL was used as an optimum extraction solvent.

The selection of disperser solvent is important parameter in DLLME technique. This solvent should be miscible in both phases (extraction solvent and aqueous phase), moreover it should disperse the extraction solvent into the aqueous solution to form a cloudy state. To simplify the choosing process, the dispersive solvent should also be used as the demulsifier to break up the oil-in-water emulsion [27]. Various kinds of solvent such as ethanol, acetone, acetonitrile and methanol, were studied. The results are shown in Figure 3. It was found that acetonitrile as the disperser and demulsified solvent provided highest peak area. Thus, acetonitrile was chosen as disperser and demulsified solvent.

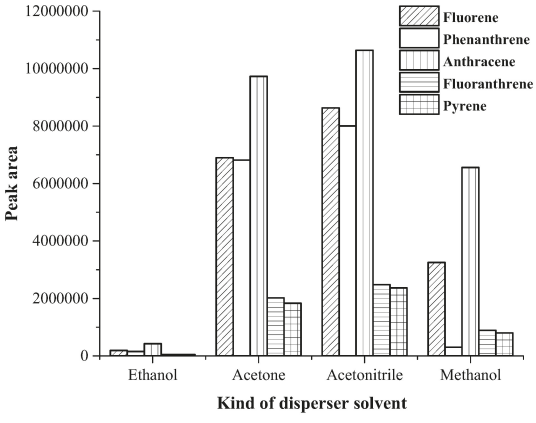

Figure 3. Effect of kind of disperser solvent on the extraction efficacy.

The volume of disperser solvent was studied within the range of 250–1,000 μL. It was found that the extraction efficiency slightly increased with increasing the volume of disperser solvent up to 500 μL and then decreased (as shown in Figure 4). Therefore, 500 μL of acetonitrile was used as disperser solvent. Moreover, volume of demulsified solvent was varied in the range 250–1,250 μL. It can be observed that a volume of 500 μL of demulsified solvent provided highest extraction efficiency in terms of peak area (as shown in Figure 5). Thus, acetonitrile 500 μL was selected as demulsified solvent.

In the two immiscible phase system, the mass transfer between the aqueous solution and extraction solvent depended on the extraction time and agitation [28]. The extraction time was defined as time that the sample was magnetic stirring agitated [29]. Extraction times on the extraction efficiency were evaluated for 1–10 minutes (data not shown) at 1500 rpm. The maximum peak area with the least standard deviation was obtained at four minutes, therefore it was selected as the optimum extraction time.

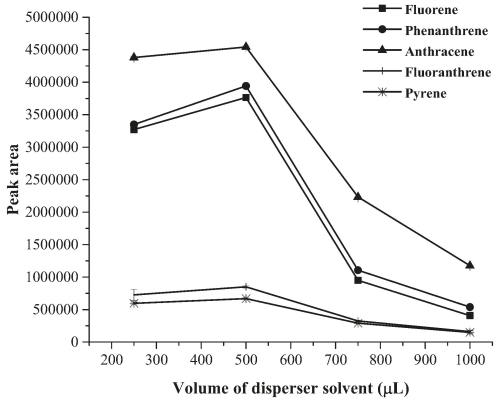

Figure 4. Effect of volume of disperser solvent on the extraction efficacy.

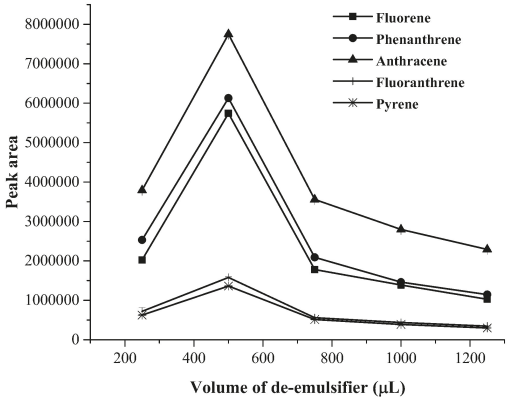

Figure 5. Effect of volume of de-emulsifier solvent on the extraction efficacy.

In summary, the optimum extraction conditions were sample solution 10.00 mL, 2.0 g NaCl, 1-dodecanol 75 μL used as extraction solvent, acetonitrile used as disperser (500 μL) and de-emulsified solvent (500 μL), and an extraction time of four minutes.

Table 1. Analytical performance of magnetic stirring assisted demulsified dispersive liquid–liquid microextraction method for determination of polycyclic aromatic hydrocarbons.

Analyte	Linear range ($\mu g \cdot mL^{-1}$)	Linear equation	R^2	EF	LOD ($\mu g \cdot mL^{-1}$)	LOQ ($\mu g \cdot mL^{-1}$)	Intra-day [a] (n = 5)		Inter-day (n = 3 × 5)	
							t_R	Area	t_R	Area
Fluorene	0.0005–1 (0.03–1) [b]	$y = (7 \times 10^6 x) + 87{,}587$	0.9992	65	0.0001	0.0003	0.15 (0.17)	2.18 (2.51)	0.39 (0.50)	6.72 (7.44)
Phenanthrene	0.0005–1 (0.03–1)	$y = (7 \times 10^6 x) + 87{,}587$	0.9973	64	0.0001	0.0003	0.17 (0.19)	2.79 (3.08)	0.22 (0.29)	2.73 (3.19)
Anthracene	0.0005–1 (0.03–1)	$y = 401{,}767x + 32{,}354$	0.9993	60	0.0003	0.0005	0.24 (0.39)	2.58 (3.70)	0.37 (0.60)	5.68 (6.25)
Fluoranthrene	0.0005–1 (0.03–1)	$y = (2 \times 10^6 x) + 20{,}850$	0.9989	66	0.0003	0.0005	0.20 (0.49)	3.55 (4.34)	0.33 (0.48)	5.51 (7.05)
Pyrene	0.0005–1 (0.03–1)	$y = (4 \times 10^6 x) + 30{,}813$	0.9997	67	0.0001	0.0003	0.32 (1.06)	3.89 (5.40)	0.48 (1.48)	7.22 (8.12)

[a] Precision was studied at standard concentration of 0.1 $\mu g \cdot mL^{-1}$; [b] the values in parentheses are gained from direct HPLC analysis.

3.2. Analytical Performance of the Proposed Method

The analytical performances of the proposed method for analysis of polycyclic aromatic hydrocarbons were validated by obtaining linear range, coefficient of determination (R^2), Limit of detections (LODs), limit of quantitations (LOQs,) and enrichment factors (EFs). Table 1 summarizes the analytical performance of the proposed method. LODs and LOQs ranged from 0.0001 to 0.0003 mg·L^{-1} and 0.0003 to 0.0005 mg·L^{-1}, respectively. The calibration curve exhibited linearity over the range of 0.0005–1 mg·L^{-1} with R^2 greater than 0.99. RSDs were in the range of 0.22–1.48% and 2.73–7.22% for retention time and peak area, respectively. High EFs (60–67) were also gained. Figure 6 depicts typical chromatogram comparing analyses of (a) a mixture standard of polycyclic aromatic hydrocarbons with direct injection by HPLC and (b) a mixture of standard of polycyclic aromatic hydrocarbons after magnetic stirring assisted demulsified dispersive liquid–liquid microextraction technique.

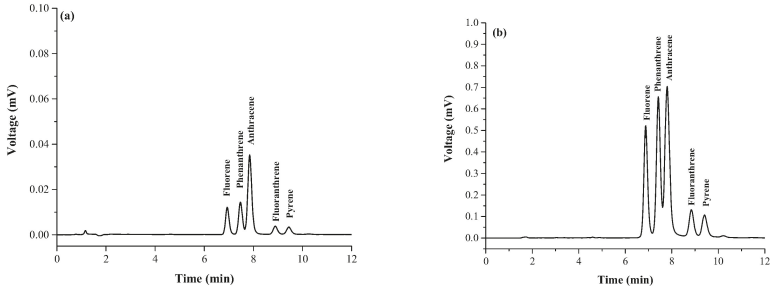

Figure 6. Chromatogram comparing analyses of (**a**) a mixture standard of polycyclic aromatic hydrocarbons with direct injection by HPLC and (**b**) a mixture of standard of polycyclic aromatic hydrocarbons after magnetic stirring assisted demulsified dispersive liquid–liquid microextraction technique: concentration of all standards was 100 ng·mL^{-1}.

3.3. Grilled Pork Analysis

To eliminate the matrix effect in real sample analysis, matrix-match calibration was used. A high degree of linearity was observed in the range of 0.01–0.5 mg·g^{-1} with R^2 greater than 0.995. LODs of the target analytes in real samples were studied with 3.3 × (SD y-Intercept/Average Slope) and LOQs were investigated using 10 × (SD y-Intercept/Average Slope) [24]. The obtained LODs and LOQs were in the range 0.001–0.005 mg·kg^{-1} and 0.004–0.010 mg·kg^{-1}, respectively. These are below the established EU maximum level (12 µg·kg^{-1}). For this analysis, all studied polycyclic aromatic hydrocarbons were found in the range of 0.30–1.00 mg·kg^{-1}. The results are summarized in Table 2. To test the accuracy of the proposed method, recovery was investigated by spiking standard solution at different levels (0.01, 0.05, 0.10 mg·kg^{-1}) before magnetic stirring assisted demulsified dispersive liquid–liquid microextraction. Figure 7 shows the chromatograms of grilled pork sample (untreated) (Figure 7a) as well as grilled pork samples spiked at concentrations of 0.01 mg·kg^{-1} (Figure 7b), 0.05 mg·kg^{-1} (Figure 7c), and 0.10 mg·kg^{-1} (Figure 7d). The chromatograms show that the sensitivity increases with increasing PAH concentration. The results (Table 3) show good analytical performance of the proposed method with the average recoveries for all studied analytes ranging from 82% to 99%, and good precision with relative standard deviation (RSD) less than 9%.

Table 2. The determination of polycyclic aromatic hydrocarbons in the studied grilled pork samples (n = 3).

Samples	Amount found ± SD, mg·kg^{-1}				
	Fluorene	**Phenanthrene**	**Anthracene**	**Fluoranthrene**	**Pyrene**
Grilled pork I (n = 3)	0.50 ± 0.10	1.00 ± 0.10	0.50 ± 0.10	0.80 ± 0.20	0.50 ± 0.01
Grilled pork II (n = 3)	-	0.70 ± 0.20	0.30 ± 0.20	0.50 ± 0.01	0.40 ± 0.01
Grilled pork III (n = 3)	0.30 ± 0.02	-	0.50 ± 0.20	0.70 ± 0.01	0.30 ± 0.20
Grilled pork IV (n = 3)	0.40 ± 0.10	0.40 ± 0.01	0.50 ± 0.10	0.60 ± 0.20	0.20 ± 0.10

-; not detected.

Table 3. Recovery obtained from the determination of polycyclic aromatic hydrocarbons in the studied grilled pork samples (n = 3).

Analytes	Spiked (mg·kg^{-1})	Grilled pork I		Grilled pork II		Grilled pork III		Grilled pork IV	
		RR (%)	**RSD (%)**	**RR (%)**	**RSD (%)**	**RR (%)**	**RSD (%)**	**RR (%)**	**RSD (%)**
Fluorene	0.01	83.45	1.52	87.64	4.73	95.73	6.63	91.57	8.76
	0.05	87.93	3.30	90.62	5.67	90.67	2.54	89.72	6.78
	0.10	84.63	6.35	96.87	3.79	85.33	2.63	98.74	6.87
Phenanthrene	0.01	82.93	5.36	92.39	4.33	87.73	3.87	89.30	7.45
	0.05	91.58	4.74	83.76	5.76	93.48	4.57	95.78	6.87
	0.10	98.74	6.79	90.78	7.63	90.63	7.86	90.87	7.86
Anthracene	0.01	82.97	4.53	87.33	3.63	92.78	6.75	90.63	3.67
	0.05	87.62	5.73	88.63	4.76	90.78	7.67	89.78	4.38
	0.10	90.73	8.63	93.74	6.78	91.87	1.56	89.90	3.78
Fluoranthrene	0.01	89.97	3.44	93.35	4.32	94.67	3.39	89.93	3.45
	0.05	91.56	4.67	89.90	6.32	91.75	4.13	83.63	6.78
	0.10	95.73	7.78	91.72	4.36	89.73	5.62	90.57	8.98
Pyrene	0.01	89.93	3.65	89.97	8.78	87.78	7.89	93.65	8.35
	0.05	90.67	4.78	91.63	5.67	88.98	6.78	91.32	5.56
	0.10	89.92	7.87	89.73	6.72	90.56	5.76	90.01	4.65

RR: Relative recovery; RSD: Relative standard deviation.

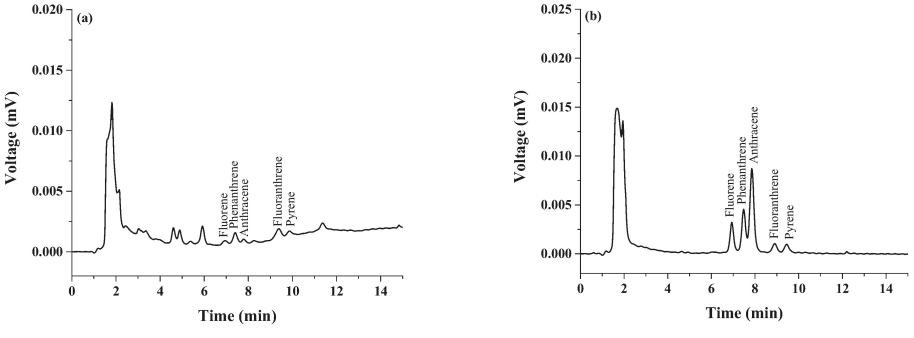

Figure 7. *Cont.*

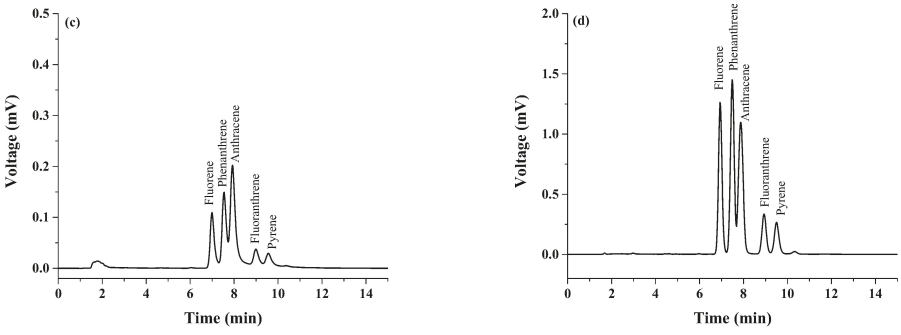

Figure 7. Chromatograms of (**a**) grilled pork sample, (**b**) grilled pork sample spiked with 0.01 mg·kg^{-1} of each polycyclic aromatic hydrocarbon, (**c**) grilled pork sample spiked with 0.05 mg·kg^{-1} of each polycyclic aromatic hydrocarbon, and (**d**) grilled pork sample spiked with 0.10 mg·kg^{-1} of each polycyclic aromatic hydrocarbon.

4. Conclusions

A fast and simple combining apparatus magnetic stirring assisted demulsified dispersive liquid–liquid microextraction has been investigated for extraction and preconcentration of polycyclic aromatic hydrocarbon residues coupled to high-performance liquid chromatographic analysis. In this procedure, a magnetic stirring-assisted process was used to accelerate the formation of fine droplets, which increased the extraction efficacy and decreased the extraction time. No centrifugation step was required. High enrichment factors, low LODs and good repeatability were obtained. The proposed method is both easy and rapid. As it uses less toxic organic solvent, it is also an environmentally friendly technique for the determination of polycyclic aromatic hydrocarbons in grilled pork samples.

Author Contributions: J.V., Y.S., and R.B. participated in designing the study. J J.V. and Y.S. conducted the study. Data was collected and analyzed by J.V. Manuscript was written by J.V., Y.S., R.B. and S.S.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Consumption of Minerals, Toxic Metals and Hydroxymethylfurfural: Analysis of Infant Foods and Formulae

Christian Vella [1] and Everaldo Attard [2,*]

[1] Department of Pharmacy, Faculty of Medicine and Surgery, University of Malta, Msida MSD 2080, Malta; christian.g.vella@gov.mt

[2] Division of Rural Sciences and Food Systems, Institute of Earth Systems, University of Malta, Msida MSD 2080, Malta

* Correspondence: everaldo.attard@um.edu.mt

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Abstract: Infant foods and formulae may contain toxic substances and elements which can be neo-formed contaminants or derived from raw materials or processing. The content of minerals, toxic elements, and hydroxymethylfurfural (HMF) in infant foods and formulae were evaluated. The effect of storage temperature on HMF formation in infant formulae and its potential as a quality parameter was also evaluated. Prune-based foods contained the highest HMF content. HMF significantly increased when the storage temperature was elevated to 30 °C for 21 days. All trace elements were present in adequate amounts, while the concentration of nickel was higher when compared to those of other studies. The study indicates that HMF can be used as a quality indicator for product shelf-life and that the concentrations of minerals and toxic elements vary greatly due to the diverse compositions of foods and formulae. Such contaminants need to be monitored as infants represent a vulnerable group compared to adults.

Keywords: infant formulae; infant foods; minerals; toxic metals; hydroxymethylfurfural; storage conditions; safety

1. Introduction

Infants are more sensitive than adults to food contaminants due to a higher rate of uptake by the gastrointestinal tract, an incompletely developed blood–brain barrier, an undeveloped detoxification system, and high food consumption relative to body mass [1]. Heavy metals are contaminants which can accumulate in infant foods through the food chain, during food processing or leakage from packaging materials [2]. Their effect on living organisms depends on the nature and concentration of the element concerned. Some elements are an essential part of the human diet, while others can be xenobiotic and highly toxic [3]. Maximum levels for heavy metals in infant foods and formulae are only defined for cadmium, lead, and tin through Regulation (CE) No. 1881/2006 and subsequent updates [1]. Contaminants can also be formed during the heating or preservation of foods and can pose harm to human health. These are termed neo-formed contaminants. Hydroxymethylfurfural (HMF) is a neo-formed contaminant in food, being an intermediate in the Maillard reaction which consists of a series of reactions, starting with a reaction between the carbonyl group of a reducing sugar with a free amino group, or it can result from the direct dehydration of sugars [4]. It is practically not present in fresh food but it is found in variable amounts in processed foods, such as jams, fruit juices, and syrups, as its synthesis depends on the temperature, pH, concentration of saccharides, presence of organic acids, and presence of divalent ions [5].

The aim of the study was to assess the content of minerals, toxic metals (Cr, Cu, Hg, Ni, Zn, Mn and Fe), and HMF in infant foods and formulae. This would provide an insight into the potential effects of undesirable substances within a vulnerable group.

2. Materials and Methods

2.1. Sample Collection

Thirty-two infant foods from four different manufacturers were randomly selected via convenience sampling from local pharmacies and supermarkets, and categorized as apple, pear, prune, fish, poultry, and ruminant-based foods. Six infant formulae from 3 different manufacturers were randomly collected from local pharmacies and were categorized as beginner infant formulae (0–6 months) and follow-on formulae (6–12 months).

2.2. Determination of pH

The pH of samples was measured with a Thermo scientific Orion Star A215 pH meter (Life Technologies Ltd., Paisley, UK). For infant foods, the pH was measured directly using a probe for viscous samples while for the powdered infant formulae, a reconstitution in de-ionized water at a ratio of 1:10 was carried out.

2.3. Determination of HMF

HMF content was determined according to a spectrophotometric method after White [6]. The determination of HMF content was based on the determination of UV absorbance of HMF at 284 nm (SpectroStar-Nano, BMG, Labtech, Ortenberg, Germany). The difference between the absorbance of a clear sample solution and the sample solution after the addition of 0.2% $NaHSO_3$ was determined to avoid the interference of other compounds at this wavelength. Five grams of each of the baby foods and infant formulae were tested for HMF content at a temperature of 18 °C. Furthermore, the infant formulae were incubated and maintained at 30 °C for 21 days in a water bath. The same HMF test procedure was used to determine the effect of temperature on HMF levels. Limits of detection (LOD) and limits of quantification (LOQ) for HMF were calculated as 3 s/m and 10 s/m, respectively, where s refers to the standard deviation of the intensity of blank samples and m refers to the slope of the calibration curve for HMF (Table 1).

Table 1. Hydroxymethylfurfural (HMF), mineral and toxic metal wavelength of detection, regression value (R^2), limits of detection (LOD) and limits of quantification (LOQ).

Method	Element	Wavelength (nm)	R^2	LOD (mg/kg)	LOQ (mg/kg)
White	HMF	284.000	0.99000	0.1122	0.3400
MP-AES	Cr	425.433	0.99999	0.0005	0.0014
MP-AES	Cu	324.754	1.00000	0.0007	0.0022
MP-AES	Hg	253.652	0.99990	0.0789	0.2391
MP-AES	Ni	352.454	0.99998	0.0056	0.0169
MP-AES	Mn	403.076	1.00000	0.0042	0.0127
MP-AES	Fe	259.940	0.99986	0.0037	0.0113
MP-AES	Zn	213.857	1.00000	0.0301	0.0912

2.4. Determination of Trace Elements

For mineral and toxic metal analysis, the samples were mineralized by digesting 1 g of the sample using 5 mL of 5% HNO_3 at 80 °C, followed by 2 mL of 34.5–36.5% H_2O_2 after the acid evaporated. Further mineralization of the sample was carried out by ashing at 500 °C in a muffle furnace (Wisetherm, Wisd, Laboratory Instruments, Germany) for 6 h. The ash was reconstituted in 5 mL of 5% HNO_3 and filtered. Deionized water was added up to 50 mL and the samples were quantitatively analyzed using

a Microwave Plasma-Atomic Emission Spectrometer (MP-AES 4100, Agilent Technologies Inc., Santa Clara, CA, USA). The method was validated according to Berg [7]. The LOD and LOQ for each heavy metal were calculated as 3 s/m and 10 s/m, respectively, with respect to the calibration curve for each element (Table 1).

2.5. Statistical Analysis

All measurements were conducted in triplicate and average results were reported. The statistical program Prism 5 (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis. The results for the heavy metal elements and hydroxymethylfurfural contents were analyzed by one-way ANOVA with the Bonferroni post hoc test to compare the statistical difference between means of the data sets and their mean difference. The same statistical test was carried out to compare the mean content of hydroxymethylfurfural between infant formulae stored at room temperature and infant formulae stored at 30 °C for 21 days. Principal component analysis and Pearson correlations were conducted on all samples, using XLSTAT v.2014.4.04 (Microsoft, version 19.4.46756, SAS Institute Inc., Marlow, Buckinghamshire, UK) to determine any clustering of minerals and toxic metals. A P value less than 0.05 was considered as statistically significant.

3. Results

A total of 38 samples were assessed for HMF content and selected heavy metal elements. The infant foods (n = 32) exhibited variable amounts of HMF, ranging from 0.89 mg/kg to 144 mg/kg, with the lowest content being present in poultry-based infant foods, while the highest content was present in prune-based products (Table 2). The HMF content in infant formulae (n = 6) ranged from 0.29 mg/kg to 7.87 mg/kg when examined at room temperature. The HMF content in all types of infant formulae significantly increased ($p \leq 0.05$) after being stored at 30 °C for 21 days and ranged from 1.80 mg/kg to 9.43 mg/kg (Figure 1). The mean heavy metal content of Cr, Cu, Hg, Ni, Fe, Mn, and Zn is shown in Table 3. The trace elements were detected in all infant food and formulae samples analyzed except for Hg, which was detected only in one sample from the pear-based infant food category (n = 6).

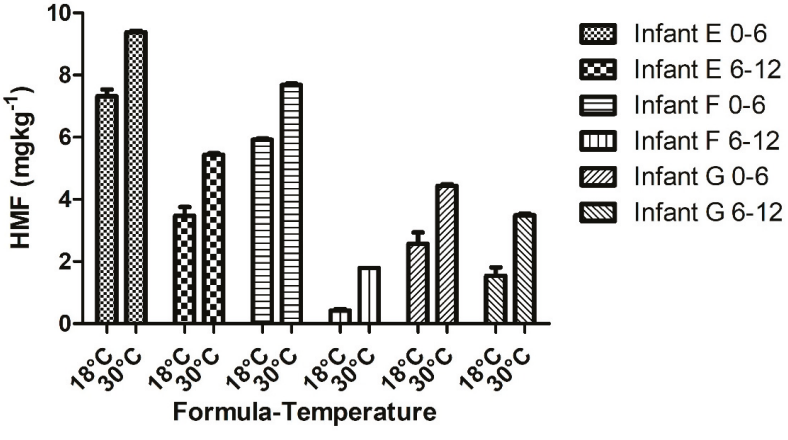


Figure 1. The HMF content in all types of infant formulae.

Table 2. HMF content (mg/kg) and pH of infant foods and formulae.

Mean HMF and pH Values	Prune-Based Food	Pear-Based Food	Apple-Based Food	Fish-Based Food	Poultry-Based Food	Ruminant Meat-Based Food	Formulae 0–6 Months	Formulae 6–12 Months
Mean HMF mg/kg (at 18 °C)	99.10 ± 11.45	6.327 ± 0.4945	9.674 ± 1.004	3.133 ± 0.2191	1.858 ± 0.1807	2.359 ± 0.1171	5.27 ± 1.40	1.81 ± 0.88
Mean HMF mg/kg (at 30 °C)	nd	nd	nd	nd	nd	nd	7.17 ± 1.44	3.57 ± 1.05
Mean pH	3.31 ± 0.05	3.558 ± 0.06	3.31 ± 0.04	5.64 ± 0.82	5.61± 0.16	5.17 ± 0.38	6.76 ± 0.17	6.66 ± 0.14

nd = not determined.

Table 3. Metal content (mg/kg) in infant foods and formulae.

Mean Metal Content (mg/kg)	Apple-Based (n = 6)	Pear-Based (n = 6)	Prune-Based (n = 4)	Fish-Based (n = 8)	Poultry-Based (n = 4)	Ruminant Meat-Based (n = 4)	Formulae 0–6 (n = 3)	Formulae 6–12 (n = 3)
Cr	0.21 ± 0.06	0.09 ± 0.03	0.18 ± 0.07	0.07 ± 0.02	0.04 ± 0.02	0.02 ± 0.01	0.29 ± 0.05	0.24 ± 0.03
Cu	0.65 ± 0.05	0.93 ± 0.11	0.66 ± 0.07	0.78 ± 0.07	0.68 ± 0.04	0.75 ± 0.07	3.33 ± 0.24	3.37 ± 0.21
Hg	nd	0.12 ± 0.12	nd	nd	nd	nd	nd	nd
Ni	0.63 ± 0.08	0.85 ± 0.03	0.86 ± 0.08	0.81 ± 0.06	1.07 ± 0.22	0.73 ± 0.06	0.76 ± 0.00	0.82 ± 0.06
Fe	0.86 ± 0.08	1.18 ± 0.26	1.67 ± 0.4	1.55 ± 0.14	1.64 ± 0.07	1.67 ± 0.41	18.34 ± 2.51	18.87 ± 3.06
Mn	4.93 ± 0.36	3.54 ± 0.06	3.22 ± 0.12	2.90 ± 0.11	2.37 ± 0.04	3.25 ± 1	2.13 ± 0.41	2.05 ± 0.21
Zn	1.07 ± 0.78	8.54 ± 8.05	1.03 ± 0.34	2.61 ± 1.46	3.19 ± 0.72	5.76 ± 0.69	27.24 ± 2.77	33.00 ± 0.95

nd = not detected.

4. Discussion

Toxic substances may be either present in the raw materials or evolve during the processing of the raw materials into the finished products. Although the assurance of food quality is the responsibility of the producer and manufacturer, authorities worldwide do not control food products for safety. Several reports have shown that baby foods may contain contaminants, some of which include microorganisms [8,9], mycotoxins [10,11], aromatic compounds [12,13], furans [14,15], and metals [16–18].

The HMF content was determined at a temperature of 18°C for the baby foods, and at two temperatures (18 and 30 °C) for the infant formulae. Since baby foods in individual jars are consumed within one meal and the foods have undergone extensive processing in industry, the baby foods were not tested at a temperature of 30 °C for a 21-day period. It is more likely that for infant formulae, repeated quantities are consumed from the same can over a period of time. There is no limit for the HMF content in foods, apart for honey at 40 mg/kg in general environmental conditions, 80 mg/kg for honey produced in tropical climates, and 15 mg/kg for honey with low enzymatic activity [19]. This makes it difficult to ascertain whether acceptable or excessive levels of HMF are found in the studied foods. The results from studies carried out by Kalábová and Večerek [20], and Čížková and coworkers [21], for the determination of the HMF content in infant foods, reported ranges from 2.10 mg/kg to 9.80 mg/kg and 4.10 mg to 28.90 mg/kg, respectively. The current study showed a larger spread of values nearly fifteen times the upper limit, observed by Kalábová and Večerek [20], and seven times the upper limit, observed by Čížková and coworkers [21]. This variability could be related to the type of food tested, since this varied in the different studies. A significant difference in the HMF content of prune-based infant foods compared to other infant foods ($p \leq 0.05$) was observed and these were identified as a potential source of high HMF consumption in children. Products processed from prunes, such as pitted prunes and prune juices, have been reported to have an HMF content as high as 291 mg/kg and 528 mg/L, respectively. The higher HMF value in fruit-based foods is due to greater carbohydrate degradation as a consequence of the Maillard reaction, which is favored by a lower pH (Table 2). On the other hand, a higher furan content is present in vegetable-based foods compared to fruit-based foods. This is related to either a greater furfural content or a greater ascorbic acid degradation [14].

The HMF content in infant formulae observed in the study, ranging from 0.29 mg/kg to 7.87 mg/kg, was comparable with other studies, such as that by Michalak and coworkers [22], reporting an HMF content between 1.22 mg/kg and 8.20 mg/kg. With respect to the changes of HMF content during storage at 30 °C for 21 days, the HMF content in all formulae increased significantly after storage ($p \leq 0.05$). This temperature-dependent effect was shown in various studies, such as that by Chávez-Servín and coworkers [23], where they demonstrated a similar significance and proportional increase after 70 days of storage at 25 °C. However, the relationship between HMF concentration and pH in infant formulae was not significant ($p > 0.05$). Therefore, HMF synthesis was not dependent on the pH of infant formulae. In a study conducted earlier by Chávez-Servín and coworkers [24], it was observed that infant formulae at a neutral pH for a period of 12 months of storage exhibited insignificant formation of HMF.

There was a variation in the absorbance value with respect to the concentration of the heavy metal element, and, therefore, a strong positive linear relationship was present between the two parameters ($r = 0.9986$). The low LOD and LOQ values demonstrate that the MP-AES method for the analysis of heavy metal elements was highly sensitive (Table 1). The heavy metal content varied widely due to many factors, such as differences between food types, the characteristics of the manufacturing practices and processes, and possible contamination during processing. The present study demonstrated wide variations in the concentration of the most essential and toxic elements in infant formulae and foods (Table 3). In the infant formulae, the manufacturer's fortification of essential elements resulted in concentrations many times higher than those found in foods, especially Fe, Zn, and Cu. The concentration of nickel in the samples, ranging from 0.63 mg/kg to 1.07 mg/kg, exceeded the reference value of 5 µg/kg bw/day set by the Food and Agriculture Organization/World Health Organization

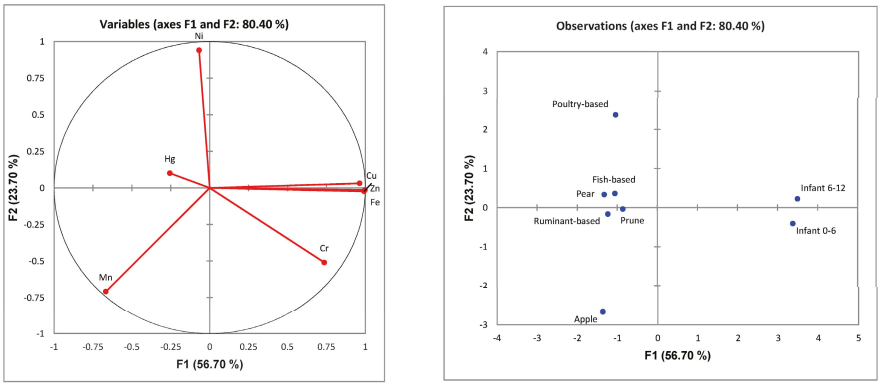
(FAO/WHO) Joint Expert Committee on Food Additives (JECFA) [25], as the daily intake of Ni through infant formulae ranged from 7 µg/kg bw day to 19 µg/kg bw day. Mehrnia and Bashti [26] reported daily intake values of nickel through infant formula more than tenfold the reference value set by the JECFA. Nickel toxicity is associated with immediate and delayed hypersensitivity reactions. It has the potential to cause immunological disturbances and act as an immunotoxic agent in humans [27]. Only one sample was contaminated with Hg at a concentration of 0.7 mg/kg. Since Hg was detected in a pear-based food, the presence of methylmercury is excluded, as this bioaccumulates in fish. Therefore, this value cannot be compared to the EFSA [28], which establishes a TWI reference value of 1.3 µg/kg bw for methylmercury. Cruz and coworkers [29] reported infant formulae testing positive for mercury, with levels of 0.63 mg/kg and 0.83 mg/kg.

Factor analysis using principal components was used to identify latent traits within the data. Pearson correlation (Table 4) revealed that there were several correlations between the minerals and toxic metals. There were positive correlations between Cr and Cu, Fe, Zn (r = 0.718, 0.725 and 0.631), Cu and Fe, Zn (r = 0.996 and 0.984), and Fe with Zn (r = 0.974). There were negative correlations between Cu and Mn (r = −0.636), Mn with Fe, and Zn (r = −0.654 and −0.641). Two latent factors had an eigenvalue greater than 1, which together explained 80.04% of the total variance (Figure 2a). The factor loadings demonstrated the different groups of variables. For the first factor, the factor loadings of Cr, Cu, Fe, and Zn, and the second factor, weighed heavily on Hg, Ni, and Mn. Figure 2b demonstrates the factor scores of the two latent factors. Factor 1, on the horizontal axis, demonstrates the clustering of baby foods on the left hand side of the scatter plot, while the infant formulae scattered more on the right hand side. This demonstrates the distinction of the foods and formulae characteristics with respect to mineral and toxic metal values.

Table 4. Minerals and toxic metals found in the baby foods and infant formulae.

Variables	Cu	Hg	Ni	Fe	Mn	Zn
Cr	**0.718**	−0.217	−0.393	**0.725**	−0.090	**0.631**
Cu		−0.159	−0.108	**0.996**	**−0.636**	**0.984**
Hg			0.106	−0.236	0.211	−0.062
Ni				−0.091	−0.574	−0.073
Fe					**−0.654**	**0.974**
Mn						**−0.641**

Bold values represent significant correlations.



(**a**) (**b**)

Figure 2. Principal component analysis (PCA) analysis of baby foods and infant formulae characteristics with respect to mineral and toxic metal contents (**a**) the factor loading plot demonstrating the different groups of variables; (**b**) the factor scores of the two latent factors.

5. Conclusions

Opinions on the cytotoxicity, carcinogenic, and genotoxic potential of hydroxymethylfurfural vary, while certain minerals and toxic metals are known to be deleterious if consumed in large quantities. However, the concentrations of such metals vary depending on the food type used. Infant foods and formulae contained varying amounts of HMF and metals, thus, the total daily intake of these contaminants is affected by individual feeding patterns. Notably, a high HMF content was observed in prune-based infant foods. On the other hand, with regard to the metal contents, it was observed that infant foods contained Mn, Zn, Fe, Cu, and Cr, while infant formulae contained Zn, Fe, Cu, Mn, and Cr in decreasing order. There was a low presence of Ni and negligible quantities of Hg. Infants are within a vulnerable age group and have a restricted diet compared to other age groups, therefore, it is recommended that foods are monitored to ensure safe use. The setting up of limits with respect to this vulnerable group should be considered through further studies, using a greater diversification of samples that are subjected under varying conditions.

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Conflicts of Interest: The authors declare no conflict of interest.

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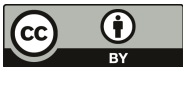

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Article

Assessment of Antibiotic and Pesticides Residues in Breast Milk of Syrian Refugee Lactating Mothers

Nadia Smadi [1,†], Adla Jammoul [2,†] and Nada El Darra [1,*]

1 Faculty of Heath Sciences, Beirut Arab University, Tarik El Jedidah–Beirut, P.O.Box: 115020, Riad EL Solh, Beirut 1107 2809, Lebanon
2 Food Department, Lebanese Agricultural Research Institute, Fanar P.O. Box 2611, Beirut 1107 2809, Lebanon
* Correspondence: n.aldarra@bau.edu.lb
† These authors contributed equally.

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Abstract: Occupational exposures and current diet are both sources of environmental contaminants that can be transferred in the mother's body. These chemicals can definitely penetrate to the developing foetus and the nursing infant from contaminated breast milk during the lactation period. Nowadays, one of the special interests is the exposure of new-borns to toxic chemicals such as pesticides and antibiotics reported in human milk due to their potential harms, especially developmental deficits in early childhood. The aim of our current study was to assess the occurrence of pesticide residues and antibiotic residues contamination in breast milk collected from Syrian refugee lactating mothers residing in North Lebanon Camps. A total of 120 breast milk samples (40 in triplicate) were collected from camps in Akkar, North Lebanon using an electrical pump. A survey was administrated to determine socio-demographic characteristics, dietary and smoking habits and medical history of participating lactating mothers. The milk samples were analysed for the presence of antibiotic residues and pesticide residues using liquid and gas chromatography tandem mass spectroscopy (LC-MS/MS) and Gas Chromatography-Tandem Mass Spectrometry (GC-MS/MS). This study reported the absence of antibiotic residues in 96.66% of our samples (n = 120) and the presence of pesticides residues in only 5% of our total breast milk sample. Our results considered the breast milk collected from Syrian refugee lactating mothers as safe from chemical contamination. It is worth conducting more studies on other Syrian refugee camps to test the effect of camp living conditions on breast milk safety.

Keywords: breast milk; antibiotic residues; pesticide residues; LC-MS/MS; GC-MS/MS

1. Introduction

Breastfeeding is admitted to present numerous beneficial health effects and it is vastly considered as the best suitable food for the baby [1]. The World Health Organization recommends to exclusively breastfeed for six months after birth [2]. However, the European recommendation varies among countries between four and six of exclusive breastfeeding [3].

In Lebanon and Syria, two Middle Eastern Arab countries sharing almost the same socio-demographic characteristics including religion and language, a percentage of 10% was shown for an exclusive breastfeeding rate at six months [4]. According to the United Nations Children Fund [5], the average of breastfed Syrian infants under six months of age was found to be 43%. The absence of support for breastfeeding by social workers and health care providers were considered the main reasons for low prevalence of exclusive breastfeeding. It could be also due to psychological reasons such as mother's emotional stress and the perceived breast-milk inadequacy [6].

Breast milk is an important source of energy and nutrients for children's health [7]. However, many studies have been conducted to assess the chemical contaminants in breast milk associated with its health effects on the infant and the mother. This contamination could occur due the exposure of

the nursing mother on a daily basis to chemical pollution of the environment. These environmental chemicals are released from several basic activities and different sources such as water, air, food and manufactured products [8].

Few research studies have reported the presence of antibiotic and pesticide residues in breast milk. It is worth mentioning that studies screening antibiotic residues are rare on the international scale. The latest study conducted in Turkey [9], detected quinolone and beta-lactam residues in human milk samples, with an occurrence of 8/34 (23.5%) and 29/34 (85.2%), respectively. It is to be noted that the lactating mothers enrolled in this study do not have antibiotic history. Antibiotic residues in human milk were thought to derive from the mothers' food such as chicken, meat and dairy products [10].

Additionally, some studies have screened the presence of pesticide residues in breast milk especially organochlorine pesticides [11–15]. The most important study was carried out in China where a wide range of concentrations for 23 organochlorine pesticide (OCP) compounds were detected in 24 pooled samples of breast milk. The dichlorodiphenyltrichloroethane (DDT) contamination in breast milk was the highest with a mean value 582.8 ± 362.7 ng/g. The explanation for such high contamination was mainly from the mother's dietary intake since positive correlation was observed between concentration of DDT in human milk and consumption of animal origin food in the Chinese population [16].

However, to our knowledge no studies have been conducted on Syrian refugee lactating mothers residing in camps especially where the living and environmental conditions are very poor. Moreover, our study is the first conduced in Lebanon, screening antibiotics and pesticide residues in breast milk. Thus, the aim of this study was to assess the occurrence of antibiotic and pesticide residues in the breast milk of Syrian refugee lactating women residing in Lebanon camps. It investigated as well the socio-demographic and nutritional factors associated with milk contamination.

2. Materials and Methods

2.1. Study Population and Area

This cross-sectional study was conducted in Syrian refugee camps located in Lebanon, North Lebanon city—Akkar camps. The participants in this study were lactating Syrian refugee mothers residing in camps. In total, 40 women were randomly recruited and invited to participate in the study by providing mature breast milk samples.

2.2. Ethical Approval

The study protocol and ethics of this study were approved by the institutional review board (IRB) code: 2019H-0087-HS-M-0325 (17 January 2019), of Beirut Arab University prior to approaching the participants and a written, signed informed consent was obtained from all participants. All women who volunteered to participate in this study were informed about the purpose of the study and agreed to be part of it.

2.3. Questionnaire

A questionnaire was used to collect data about the socio-demographic characteristics of participating lactating mothers. The questionnaire was adopted from different studies [14,16–20]. It consisted of eight subsections collected from different sources, addressing general information about the lactating mothers and the new-borns, dietary habits of lactating mothers, smoking status, geographic characteristics, pesticides spraying, medical history and supplements intake as well as the drinking water intake.

The questionnaire was translated into Arabic to ensure accuracy of the translation, back translation from Arabic to English was conducted by another translator. Both English versions were the same. The Arabic version of the questionnaire was pilot-tested on a sample of 30 mothers. The questionnaire was administered twice within a period of two weeks between each sitting. To determine test–retest

reliability, a paired *t*-test was performed to compare mean scores at T_1 (before) and T_2 (after two weeks). Pearson's correlation was calculated between T_1 and T_2, with a consistency interclass correlation (ICC) [21]. The values of ICC were considered excellent if >0.75 [22].

2.4. Sample Collection

During December 2018 and February 2019, 50 nursing mothers were contacted but only 45 agreed to answer the survey and participated in our study. The 45 nursing mothers that were visited for the survey were contacted again and only 40 of them agreed to donate breast milk by triplicate over three weeks.

Breast milk collection was done in the morning an hour after the previous breastfeeding. Samples (25–40 mL) were collected using a sterile, single-use electrical pump (Chico, Mod.06836, China). A polypropylene pump container was sterilized with 70% alcohol solution to prevent cross contamination between samples. Each sample was placed into a separate 100 mL dark glass (to preserve the samples from light), numbered then transported on ice (at 4 °C) to the laboratory, where they were transferred into urine cups, numbered and stored at −20 °C until analysis.

2.5. Chemicals and Reagents

The reagents used were of analytical grade. The antibiotic standards belonged to three different families: sulphonamides (sulfamethazine), tetracyclines (tetracycline, oxytetracycline) and beta-lactam antibiotics (ampicillin); in addition, 161 kinds of pesticide were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standards used present high purity grades (>99%). Individual stock solutions were prepared at 1000 µg/L in acetonitrile and stored at −20 °C.

The working standard solutions of a concentration of 10 µg/L each were prepared as dilution of the stock solution in water/methanol (50:50, *v*/*v*). The working standard solutions were stored at −20 °C. HPLC-grade water, HPLC-grade acetonitrile, and magnesium sulphate ($MgSO_4$) were also supplied by Sigma-Aldrich.

2.6. Sample Extraction for Antibiotics Residues Analysis

Using liquid chromatography–mass spectrometry, a multi-class method was developed for identifying and quantifying three antibiotics belonging to two different chemical classes (tetracyclines and beta-lactams). Afterwards, this method was optimized for the detection of antibiotics in breast milk.

100 µL from 1 % formic acid was added to a 2 mL of breast milk then vortexed for 60 s and kept in the dark for 10 min. Then 500 µL of EDTA (0.5%) was added and shaken vigorously for 1 min. Next, 8 mL TCA (5%) was added then shaken for 10 min before subjecting the tubes to centrifugation at 5000 rpm at 4 °C for 10 min. The supernatant obtained was then subjected to filtration through a 0.45 µm polyvinylidene fluoride (PVDF) filter for further liquid chromatography tandem mass spectroscopy (LC-MS/MS) analysis.

2.7. Sample Extraction for Pesticide Residues Analysis: QuEChERS Extraction

To ensure a quick and easy sample treatment, a modified QuEChERS (quick, easy, cheap, effective, rugged and safe) approach, originally developed as a powerful sample preparation, was used to analyse hundreds of pesticides in different kinds of food.

Acetonitrile (10 mL) was added to 10 mL of breast milk and shaken vigorously before adding magnesium sulphate $MgSO_4$ (4 g), sodium chloride NaCl (1 g), sodium dibasic citrate (1 g) and sodium tribasic citrate (0.5 g) and then shaken for 3 min. The tubes were then subjected to centrifugation at 5000 rpm at 4 °C for 5 min.

The aqueous supernatant (5 mL) was mixed with dispersive Enhanced Matrix Removal (EMR) dissolved in 5 mL water then shaken vigorously for 5 min. Another centrifugation was conducted at 5000 rpm at 4 °C for 5 min.

The aqueous supernatant (5 mL) obtained was transferred in a new Polish EMR Lipid (MgSO4 and NaCl tube, then shaken vigorously for 3 min before being centrifuged at 5000 rpm at 4 °C for 5 min. Afterwards, a filtration was made through a 0.22 µm polyvinylidene fluoride (PVDF) filter for further LC-MS/MS and GC/MSMS analysis.

2.8. LC-MS/MS Equipment

LC-MS/MS analyses was conducted using an Agilent 6430 LC/MS (Agilent technologies, Santa Clara, USA) for antibiotic and pesticide residues detection, and LC-MS/MS analyses were performed on LC-NexeraX2 Shimadzu 8045 LC/MS (Kyoto, Japan). The mass spectrometer was operated with an electron spray ionisation (ESI) in multiple reaction monitoring (MRM) mode, ionspray voltage 4 kV, nitrogen for desolvation and dried gas 11 L/min.

The quantification of the three antibiotics families and 161 kinds of pesticide in 120 breast milk samples was done through measuring peak areas in the multiple reaction monitoring chromatogram, and comparing them with the relevant matrix-matched calibration curves. A calibration curve ranging between 1 µg/L and 500 µg/L was carried out to verify linearity.

The performance of the analytical method was evaluated by checking the identification criteria for the presence of two transitions at the same retention time, the signal to noise ratio ≥ 10, the relative retention time of the analyte within a tolerance of 2.5% and the relative ion intensities ratio within a tolerance defined by the EU commission decision 2002/657/EC. The MRM parameters are shown in Table 1.

Table 1. Multiple reactions monitoring (MRM) acquisition condition for each antibiotic used.

Antibiotic Family	Antibiotic	Precursor Ion (*m*/*z*)	Collision Energy for Precursor Ion (eV)	Product Ion (*m*/*z*)	Collision Energy for Production Ion (eV)	Cone Voltage (V)	Retention Time (min)
Sulphonamides	Sulfamethazine	279.0 > 186.1	17	279.0 > 184.0	23	Default	5.012
Tetracyclines	Tetracycline	445.1 > 410.25	20	445.1 > 427.15	12		4.886
	Oxytetracycline	461.1 > 443.3	14	461.1 > 426.15	18		4.934
Beta-Lactam	Ampicillin	350.3 > 106.05	20	350.3 > 159.9	13		4.944

2.9. LC/MS/MS Parameters

The separation of the pesticide residues was performed using a C18 analytical column (zorbax 2.1 mm inner diameter × 150 mm length, 3.5 µm particle size; Agilent technologies, Santa Clara, CA, USA) and a C18 analytical column (Shim-pack GIST 2.1 mm inner diameter × 100 mm length, 3 µm particle size; Japan) for antibiotic residues separation. The separation of pesticides was accomplished at 40 °C. The flow rate and injection volume were 0.4 mL/min and 10 µL, respectively. The mobile phases used were (A) 5 mM ammonium acetate and 0.1% formic acid in water, and (B) 5 mM ammonium acetate in methanol. The gradient elution program started with 100% A for 5 min, then decreased to 50% for 13 min, then to 0% for 2 min and returned back to the initial conditions for 5 min. The final run time of the method was 29 min.

The separation of sulfamides, tetracyclines and beta-lactam was accomplished at 40 °C. The flow rate and injection volume were 0.3 mL/min and 10 µL, respectively. The mobile phases used were (A) 5 mM ammonium acetate and 0.1% formic acid in water, and (B) 5 mM ammonium acetate in methanol. The gradient elution program was as follow: B (40%) (2 min), B (90%) (9 min), B (10%) (9 min and 1 s), the final run time of the method was 13 min.

2.10. GC/MSMS Parameters

GC-MS/MS analyses were carried out with an Agilent 7890A GC equipped with 7693 Agilent auto-sampler and 7000C triple quadrupole GC/MS system. The separation was performed on a HP-5MS Agilent technologies (Santa Clara, CA, USA), 0.25 mm × 30 m, 0.25 µm, and helium (purity 99.996%)

was used as a carrier gas at a constant pressure of 11 psi. The inlet temperature was set at 250 °C; the mode of inlet was splitless; the injection volume was 1 µL. The column temperature program was as follows: the initial temperature was maintained at 70 °C for 1 min, increased to 160 °C at a rate of 50 °C/min, raised to 200 °C at 2 °C/min, and then at 16 °C/min up to 280 °C, and held there for 7.2 min. The total run time was 35 min. The mass spectrometer was operated with an electron impact (EI) source in multiple reaction monitoring (MRM) mode. The electron energy was 70 eV, and the source, transfer line and quadrupole temperatures were set at 280 °C and 150 °C, respectively. In order to prevent instrument damage, the solvent delay was set at 4.5 min.

2.11. Recovery Test

In-house validation was performed by fortifying the blank matrix at a level of 50 µg/L. The extraction was performed by the methods described in the Section 2.6 and 2.7. The spiked and blank samples were then analysed by LCMS/MS. Recovery was calculated by comparing the analysed concentrations with spiked concentrations.

3. Results and Discussion

3.1. Validation of the Arabic Version of the Questionnaire

The results of test–retest reliability are presented in Table 2. The paired *t*-test showed that the mean scores of the Arabic questionnaire did not vary significantly between T_1 and T_2. A statistically significant inter-item correlation, $p < 0.05$, was noted between T_1 and T_2. An excellent consistency was also noted, with an ICC varying between 0.998 and 1.00 [22].

Table 2. Mean and standard deviations, Pearson's correlation coefficients and intraclass correlation for the scores of the Arabic and Arabic versions ($n = 30$).

Scale	Mean Scores			Correlation between Scores at T1 and T2	Intraclass Correlation (ICC)	
	Arabic T_1	Arabic T_2	Paired *t*-Test			
	Mean ± SD		*p*-Value	Correlation Coefficient	ICC	95% CI
Dietary Habits of Lactating Mothers	71.2 ± 10.21	71.43 ± 10.23	0.032	0.998	0.998	0.996–0.999
Smoking Status	5.16 ± 0.94	5.14 ± 0.92	0.000	0.999	0.999	0.998–1.000
Demographic Characteristics	3.33 ± 0.711	3.32 ± 0.743	0.001	1.000	1.000	1.000
Pesticides	2.866 ± 0.860	2.867 ± 0.862	0.000	1.000	1.000	1.000
Medical History and Supplements Intake	6.566 ± 1.381	6.565 ± 1.380	0.000	0.999	0.999	0.998–1.000

3.2. Survey Results

The results of the questionnaire filled by 40 Syrian refugee nursing mothers is presented in Table 3. All the participants had an age below 30 years. The number of years of residency in North Lebanon camps for all lactating mothers was between one year and five years. All the mothers (100%) had an educational degree below secondary and they were not employed.

While assessing the smoking habits, it was shown that 100% of nursing mothers had never smoked before or after pregnancy since Syrian culture inhibits women from smoking, but they were randomly exposed to second hand smoking from their husbands. Lactating mothers were also asked about their supplement intake, 100% indicated that they were taking vitamins and iron supplements during their pregnancy period, however they stopped consuming these supplements after their child's birth in order to save money.

Table 3. Survey results of participating mothers.

Characteristics	Frequency	Percent
Residence years in Tripoli Lebanon camps (1–5 years)	40	100
Age (<30 years)	40	100
Gestational Age (= 9 months)	40	100
Lactation time (>= 120 days)	40	100
Level of education (Below secondary)	40	100
Occupation Not employed (Housekeeper)	40	100
Number of newborn (=1 child)	40	100
Infant Gender		
Male	16	40
Female	24	60
Age of newborn		
=< 5months	11	27.5
>5 months	29	72.5
Birth weight (g)		
=< 3.5 kg	39	97.5
> 3.5kg	1	2.5
Irregular newborn Sleep pattern	40	100
Colic Crying of newborn	40	100
Fish intake		
Never	40	100
Sea food intake		
Never	40	100
Cereals intake		
Twice a week	39	97.5
Daily	1	2.5
Potatoes intake		
Once a week	7	17.5
Twice a week	2	5.0
More than twice a week	18	45
Daily	13	32.5
Fresh Vegetables intake		
Once a week	26	65
Twice a week	14	35
Milk intake		
Never	39	97.5
Daily	1	2.5
Dairy Product intake		
Never	11	27.5
Once a week	19	47.5
> once a week	4	10
Twice a week	6	15
Pasta intake		
Once a week	15	37.5
More than Once a week	5.0	5.0
Twice a week	37.5	37.5
More than Twice a week	20.0	20.0
Rice intake		
More than Twice a week	10	25.0
Daily	30	75.0

Table 3. *Cont.*

Characteristics	Frequency	Percent
Grains		
Once a week	4	10.0
Twice a week	13	32.5
More than Twice a week	23	57.5
Soft Drink intake		
Never	12	30.0
Once a week	20	50.0
More than Once a week	2	5.0
Twice a week	6	15.0
Jam intake		
Never	27	67.5
Once a week	13	32.5
Coffee intake		
Never	23	57.5
Once a week	9	22.5
Twice a week	8	20.0
Tea intake		
Once a week	1	2.5
More than Once a week	1	2.5
Twice a week	11	27.5
More than Twice a week	12	30.0
Daily	15	37.5
Fruits intake		
Once a week	22	55.0
Twice a week	18	45.0
Salty Snack intake		
Never	19	47.5
Once a week	21	52.5
Chocolate intake		
Never	21	52.5
Once a week	17	42.5
Twice a week	2	5.0
Meat and Poultry intake		
Never	40	100
Eggs intake		
Never	16	40.0
Once a week	13	32.5
More than Once a week	6	15.0
Twice a week	5	12.5
Beverages		
Never	11	27.5
Once a week	17	42.5
More than Once a week	3	7.5
Twice a week	9	22.5
Smoking status		
Before pregnancy	40	100
No	40	100
During pregnancy	40	100
No		
Random smoke exposure		
Yes		

Table 3. *Cont.*

Characteristics	Frequency	Percent
Nearby Waste Disposal Yes	40	100
Nearby Cultivation Activity Yes	40	100
Spray indoor to prevent mosquitoes Yes	40	100
Spray outdoor with pesticides Yes	40	100
Vitamin supplement in pregnancy Yes	40	100
Iron supplement in pregnancy Yes	40	100
Vitamin_supplement_in_postpartum_2_months No	40	100
Iron_supplement_in_postpartum_2_months No	40	100
Antibiotic intake in pregnancy No	40	100
Antibiotic intake after pregnancy No	40	100
Anemia at any time Yes	40	100
Drinking water Well artesian water	40	100
Water bottles intake 1–2 bottle	40	100

When questioned on their dietary habits, it was clearly noticed that 100% of the participants never consumed fish and sea food due to poverty. The consumption of meat and poultry was also negligible for the same reason. With respect to their location of residence, 100% were living near a waste disposal site and cultivation activity since the camp was located in an agricultural area.

3.3. Assessment of Antibiotic Residues in Breast Milk

3.3.1. LC-MS/MS Method Performance for Antibiotic Residues

An in-house method was verified as per the criteria specified in EU commission decision 2002/675/EC to quantify antibiotics residues. The validation parameters were fixed by spiking blank powdered milk at a level of 50 ng/g. The measured parameters were specificity, linear range, repeatability, reproducibility, accuracy and limit of quantification (LOQ).

The antibiotic residue chromatograms of the reference standards used as well as the calibration curves are shown in Figure 1. The calibration curves were created from six matrix-calibration standards which were injected in each batch in the range of 1 to 50 ng/g. The calibration curves showed good linearity, characterized by a high correlation coefficient ($R^2 > 0.99$).

The precision of the method was determined using the spiked standards at the 50 µg/L level. The results for repeatability ranged from 5 and 18.8% (Table 4). The limit of quantification is considered as the lowest quantified level with S/N ≥ 10 in presence of the two transitions at the same retention time. The LOQ was calculated to be 2 µg/kg for all tested antibiotics. The mean recoveries of the

residues for the spiked samples ranged between 97% and 108% (Table 4). These values presented high mean recoveries within the acceptable range (80%–120%) except for ampicillin, which presented a low mean recovery, although it was still higher than 50%. These values were within the acceptable ranges (50%–120%) recommended by AOAC, 2002 [23].

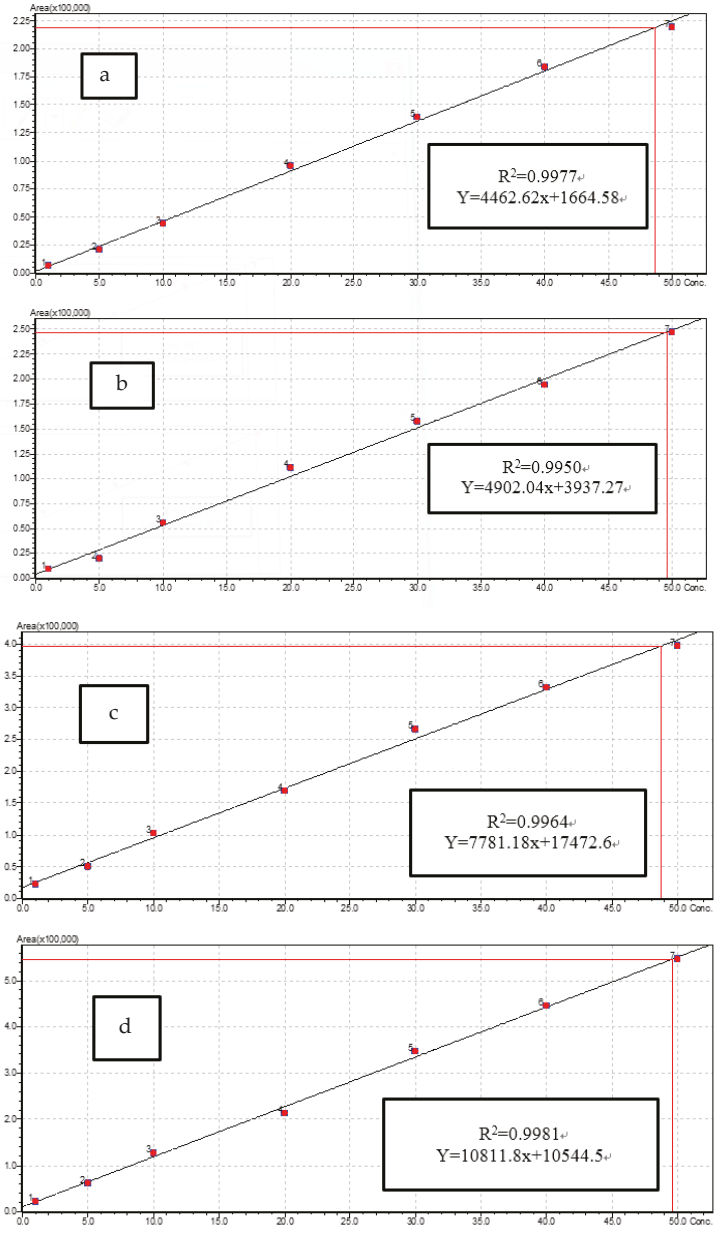

Figure 1. Calibration curves for (**a**) oxytetracycline, (**b**) tetracycline, (**c**) ampicillin and (**d**) sulfamethazine 1, 5, 10, 20, 30, 40 and 50 μg/L.

Table 4. Results of in-house verification of the LC-MS/MS method for the antibiotics considered in this study, with the standard deviation (STDEV), relative standard deviation of repeatability (RSD) and limits of quantification (LOQ).

Antibiotic Family	Antibiotic	Spiking Level (50 µg/L)	Mean Recoveries (%)	STDEV	RSD (%)	LOQ (µg/L)
Sulphonamides	Sulfamethazine	49.075	98.15	5.72	5.82	2 µg/L
Tetracyclines	Tetracycline	53.85	107.71	6.96	6.46	
	Oxytetracycline	48.664	97.328	18.22	18.72	
Beta-lactam	Ampicillin	26.475	52.95	0.627	1.18	

3.3.2. Occurrence of Antibiotic Residues in Breast Milk Samples

The method developed was applied to the determination of four antibiotics from three different chemical classes (sulphonamides, tetracyclines, and beta-lactams) in 120 breast milk samples collected from 40 lactating mothers residing in Lebanon's camp. In order to validate the results, an internal quality control was carried out on every batch of samples. Moreover, the retention time, quantification and confirmation transitions and relative ion intensities of the detected ion of the human milk samples were compared to those of the corresponding calibration standards in the same batch to identify the detected analytes.

While assessing the antibiotic residues in the breast milk samples (Table 5), the results showed that only 3.33% of the samples were contaminated with antibiotic residues. It is to be noted that none of the samples contained more than one antibiotic residues. The antibiotic residue contaminating the four samples was oxytetracycline. Of the samples, 96.67% presented a total absence of antibiotic residues. This result is well correlated with the survey, that shows that all the mothers did not receive antibiotics during pregnancy nor lactation period. Additionally, the survey results showed that 100% of the nursing mothers do not consume meat and chicken, that are known to be sources of antibiotic residues, due to the misuse of antibiotics in farms [10,24].

Table 5. Occurrence of sulphonamides, tetracyclines, and β-lactams in the 120 breast milk samples.

Breast Milk Samples (n = 120)	Sulphonamides	Tetracyclines		β-Lactams
	Sulfamethazine	Tetracycline	Oxytetracycline	Ampicillin
Mean * (µg/L)	0	0	5.04	0
min (µg/L)	0	0	0	0
max (µg/L)	0	0	6	0
n positive	0	0	3	0
% positive	0	0	2.5	0

* mean value of four contaminated samples.

3.3.3. Mean Concentrations of Antibiotic Residues for the Different Families in Breast Milk Samples

Table 5 represents the summary of multi-antibiotic residues occurrence in 120 human milk samples. The assessment of the sulphonamides, beta-lactam families and tetracyclines showed that three antibiotic residues were not detected in all milk samples, i.e., tetracyclines, sulfamethazine and ampicillin. However, as mentioned before, oxytetracycline was detected in four samples (3.3%). These four samples belong to three mothers; one of them reported one antibiotic residue concentration between the LOQ (= 2 µg/L) and LOD (= LOQ/3 = 2/3 = 0.66µg/L) followed by a second concentration higher than the LOQ in the week after. The other two samples exceeded the LOQ.

Hence, the percentage of positive samples (>LOQ = 2 µg/L) is 2.5%, since the fourth sample presented a mean of 0.64 µg/L which is lower than LOQ and LOD.

The mother that reported the highest value of oxytetracycline in her breast milk (6 µg/L) was the only mother that consumed milk on a daily basis. The mean value of oxytetracycline (5.04 µg/L) is

2.5 times higher than the LOQ (2 μg/L). The low oxytetracycline concentration in only three breast milk samples is derived from the nursing mother's food since they did not use any kind of antibiotics after pregnancy. However antibiotic residues can be found in dairy products since antibiotics are usually used in farms of dairy cattle for the treatment of diseases such as mastitis [25]. Probably the source of oxytetracycline is from nursing mothers' dairy product intake because our survey findings indicate the contaminated breast milk samples belong to 15% of mothers consuming dairy products twice per week. The presence of oxytetracycline in dairy products, could be due to the misuse of this antibiotic in farms and lack of abidance to the recommended withdrawal times [26].

3.4. Assessment of Pesticide Residues in Breast Milk

3.4.1. LC-MS/MS Method Performance for Pesticide Residuess

The pesticide residue chromatograms of the reference standards used as well as the calibration curves are shown in Figure 2. The calibration curves were created from nine matrix-calibration standards which were injected in each batch in the range from 1 to 500 ng/mL. The calibration curves showed good linearity, characterized by a high correlation coefficient ($R^2 > 0.99$).

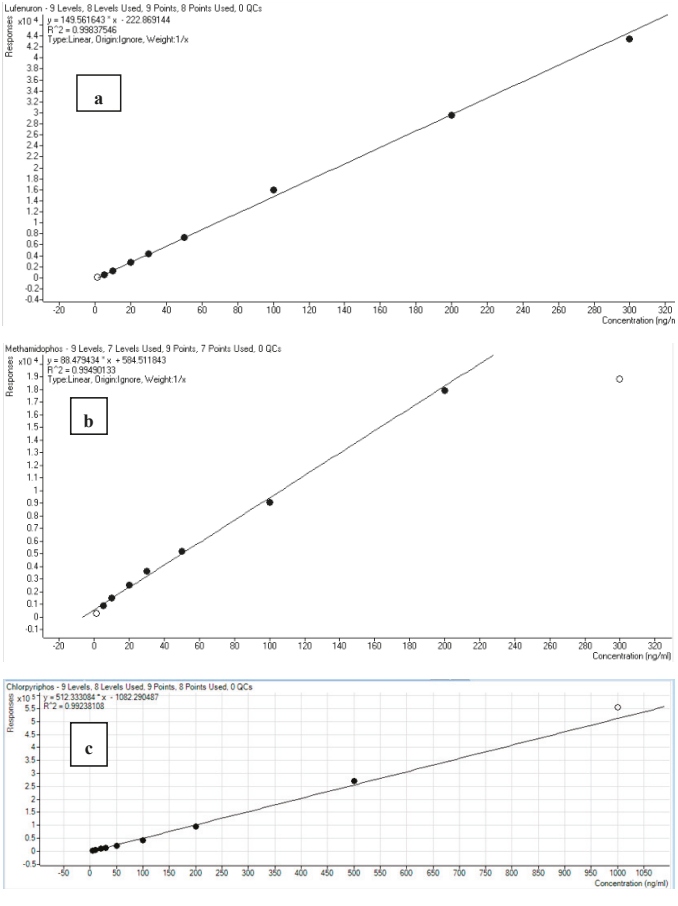

Figure 2. LC standard calibration curves for (**a**) lufeneron, (**b**) methamidophos and (**c**) chlorpyriphos at 5, 10, 20, 30, 50, 100, 200, 500 μg/L.

The precision of the method was determined using two spiked levels of 0.05 and 0.1 mg/kg. The limit of quantification was considered as the lowest quantified level with S/N ≥ 3 in presence of the two transitions at the same retention time. The LOQ was calculated to be 5 μg/kg for all tested pesticides. The mean recoveries of the residues for the spiked samples was between the acceptable range (80%–120%).

3.4.2. Occurrence of Pesticide Residues in Breast Milk Samples

While assessing the pesticide residues in the breast milk samples, the results showed that only 5% of the samples were contaminated with pesticide residues. The samples 95% were non-contaminated. It is to be noted that none of the samples contained more than one pesticide residue.

3.4.3. Mean Concentrations of Pesticide Residues in Breast Milk Samples

Table 6 represents the summary of multi-pesticide residues occurrence in 120 human milk samples. The assessment of the 161 pesticides showed that only three pesticide residues were detected in six samples, lufeneron, methamidophos and chlorpyriphos. Hence, the percentage of positive samples (>LOQ = 5 μg/L) was 4.16%. Only four samples were contaminated with lufeneron, the fifth sample was contaminated with methamidophos and the final sample was contaminated with chlorpyriphos with a concentration of 12.32 μg/L. The recovery tests results were at one level (10 μg/L) for methamidophos (86.5%), chlorpyriphos (103.6%) and lufeneron (105.2%).

Table 6. Occurrence in the 120 breast milk samples of lufeneron, methamidophos and chlorpyriphos.

Breast Milk Samples (*n* = 120)	Lufeneron	Methamidophos	Chlorpyriphos
Mean * (μg/L)	5.8754	2.198	2.05
min (μg/L)	5.1208	0	0
max (μg/L)	12.0447	13.1927	12.32
n positive	4	1	1
% positive	3.33	0.83	0.83

* mean values of six contaminated samples.

While assessing the pesticide residues in breast milk, the percentage of positive samples was only 5% in which methamidophos presented the highest concentration (13.1927 μg/L) in one sample only. The only mean value that exceeded the LOQ (5 μg/L) was reported for lufeneron pesticide (5.874 μg/L). The present results can be explained by nursing mother exposure to pesticides from fruit and vegetable intake [27], since the percentage of mothers consuming fruit and vegetables twice per week were 55% and 35% respectively, to the contrary of other studies conducted in several countries where the percentage of pesticide contamination in breast milk is high [14].

4. Conclusions

The contamination of breast milk is a critical problem since it affects the health of both the mother and her infant. In the last few decades, numerous studies reported chemical contamination in breast milk, especially pesticide residues, but antibiotic residue studies on breast milk remain rare. These contaminations are usually associated with socio-demographic status and dietary habits of nursing mothers. This study conducted for the first time, in Syrian refugee camps, presented an absence of antibiotic residues in the majority of the samples and the presence of pesticide residues in only 5% of our total breast milk sample. These findings consider the breast milk collected from Syrian refugee lactating mothers safe from the chemical contaminants screened. This could be due to the poor living and environmental conditions in the camp. It is worth conducting more studies on other Syrian refugee camps to test the effect of the camp living conditions on breast milk safety.

Author Contributions: N.S. declares that she is a Msc student. She contributed in the sampling, running the experiments and the data acquisition and data analysis. A.J. contributed in the design and the conception of the work, interpretation and the analysis of the data as the manuscript writing and submission. N.E.D. declares that she is the supervisor of the Msc student. She contributed in the design and the conception of the work, interpretation and the analysis of the data as well as the manuscript writing and submission. She is the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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Multi-Analyte MS Based Investigation in Relation to the Illicit Treatment of Fish Products with Hydrogen Peroxide

Federica Dal Bello [1,*], Riccardo Aigotti [1], Michael Zorzi [1], Valerio Giaccone [2] and Claudio Medana [1]

[1] Molecular Biotechnology and Health Sciences Dept. Università degli Studi di Torino, Via Pietro Giuria 5, 10125 Torino, Italy; riccardo.aigotti@unito.it (R.A.); michael.zorzi@unito.it (M.Z.); claudio.medana@unito.it (C.M.)

[2] Department of Animal Medicine, Productions and Health, Università degli Studi di Padova, Viale dell'Università 16, 35020 Legnaro, PD, Italy; valerio.giaccone@unipd.it

* Correspondence: federica.dalbello@unito.it

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Abstract: Fishery products are perishable due to the action of many enzymes, both endogenous and exogenous. The latter are produced by bacteria that may contaminate the products. When fishes age, there is a massive bacteria growth that causes the appearance of *off-flavor*. In order to obtain "false" freshness of fishery products, an illicit treatment with hydrogen peroxide is reported to be used. Residues of hydrogen peroxide in food may be of toxicology concern. We developed two mass spectrometry based methodologies to identify and quantify molecules related to the treatment of fishes with hydrogen peroxide. With ultra-high-performance liquid chromatography–mass spectrometry (UHPLC-MS) we evaluated the concentration of trimethylamine-N-oxide (TMAO), trimethylamine (TMA), dimethylamine (DMA), and cadaverine (CAD) in fish products. After evaluating LOQ, we measured and validated the lower limits of quantification (LLOQs as first levels of calibration curves) values of 50 (TMAO), 70 (TMA), 45 (DMA), and 40 (CAD) ng/mL. A high ratio between TMAO and TMA species indicated the freshness of the food. With a GC-MS method we confirmed the illicit treatment measuring the levels of H_2O_2 after an analytical reaction with anisole to give 2-hydroxyanisole as a marker. This latter product was detected in the headspace of the homogenized sample with simplification of the work-up. A LLOQ of 50 ng/mL was checked and validated. When fish products were whitened and refreshed with hydrogen peroxide, the detected amount of the product 2-hydroxyanisole could be very important, (larger than 100 mg/kg). The developed analytical methods were suitable to detect the illicit management of fishery products with hydrogen peroxide; they resulted as sensitive, selective, and robust.

Keywords: mass spectrometry methods; fishery product; hydrogen peroxide; illicit treatment

1. Introduction

Fishery products are defined by Regulation (EC) N 853/2004 of the European Parliament and the Council as "all seawater or freshwater animals (except for live bivalve mollusks, live echinoderms, live tunicates and live marine gastropods, and all mammals, reptiles and frogs) whether wild or farmed and including all edible forms, parts and products of such animals" [1]. The description includes all fishes (Osteichthyes, bony fishes, and Chondrichthyes, cartilaginous fishes), shellfish, and clams.

The fishery products are classified in four commercial categories: Fresh (no manipulation), prepared (any operations that affect the anatomical wholeness of the animal), frozen, and processed (any operation that transform the product, such as smoking, marinating, salting). The freshness of

a fish product is the most important commercial quality factor for the consumer, because the safety is an essential prerequisite without which food cannot be placed on the market or further transformed. The Regulation (EC) N 2406/96 of the European Parliament and the Council defines four categories for fresh fish products (extra, a, b, and c); fishery products grouped in the last class must be judged as not suitable for human consumption and must be removed from the market [2].

Fishery products are perishable due to the action of many enzymes, both endogenous, located in the fish muscles, and exogenous, produced by intrinsic bacteria that are present and can contaminate in the products [3]. Consequently, the exponential growth of bacteria that triggers oxidative chemical reactions causes fading and opacification of the product, with appearance of *off-flavor*, that is an unpleased flavor caused by chemical lipid oxidation or non-protein nitrogen (NPN) degradation [3,4]. NPN in fishery products are distinguished in two structural categories: Volatile and nonvolatile compounds. Nonvolatile NPN compounds are mostly represented by heterocyclic metabolites while volatile ones own low molecular weight and are represented in fishery products mainly by ammonia, trimethylamine (TMA, C_3H_9N), and dimethylamine (DMA, C_2H_7N). TMA is an endogenous compound abundant in fishery products: It is a post-mortem product, deriving from trimethylamine-N-oxide (TMAO, C_3H_9NO) by an enzymatic activity [5–8].

TMAO is an amine oxide, less volatile, and less basic with respect to TMA, due to its oxidation. Under enzymatic activity, TMAO could generate several chemical compounds: DMA and formaldehyde (FA) from endogenous (aquatic environment) muscle bacteria (*Pseudomonas* and *Alteromonas*) activity, and TMA from exogenous (which accumulates in fish products after capture and is typical of the terrestrial environment) bacteria (*Salmonella*, *Vibrio*) activity [9]. Only a limited population of bacteria can cause deterioration of fishery products: They are called specific spoiling micro-organism (SSO) and are mostly Gram-negative micro-organisms. After fishing, SSO can contaminate fishery products on the surface and grow even at low temperatures [10–13]. DMA, FA, and TMA are products of enzymatic degradation by intrinsic bacteria together with biogenic amines (histamine, tyramine, phenylethylamine, putrescine, and cadaverine), thiols and hydrogen sulfide (H_2S). Moreover, in mollusks such as squid, the SSO enzymes can produce hypoxanthine and acetic acids salts, that contribute to the appearance of *off-flavor* [14,15].

The organo-nitrogen compounds derived from degradation of NPN and protein are quantifiable as a total volatile basic nitrogen TVB-N; for fresh fish products the TVB-N amount should be minor of 10 mg/kg, for aged ones the quantity ranges normally between 300–350 mg/kg (Regulation (EC) N 853/2004) [16–18]. Due to the unhealthiness, the *off-flavor* appearance, and color changes, aged fishery products must be retired from the market. However, some illicit treatments on these products might simulate a "false" freshness and one of these treatments is the use of hydrogen peroxide (H_2O_2). The illicit treatment with 0.5%–0.8% hydrogen peroxide water solutions is known and was reported in the literature [19]. Residues of hydrogen peroxide in food may be of toxicology concern.

Hydrogen peroxide is both an oxidant in aqueous solution with acidic pH and a reductant in alkaline water solution. When it is used as an illicit treatment for fishery products, the oxidizing properties are exploited. H_2O_2 can indeed convert TMA, a degradation product, to TMAO, the amine oxide naturally present in living fishes. TMAO is odorless and it has oxidizing properties giving to the fishery substrate high redox potential. This great redox potential is typical of muscle tissue of fresh fishery products, and decreases rapidly when TMAO is reduced to TMA by enzymatic activity [20–22].

If the TMAO amount is increased by H_2O_2 treatment, the proteins are stabilized [23]. For example, the amount of mucins, the main glycoproteins of mucus, is reduced on the fish skin after H_2O_2 treatment because of chemical degradation. The decrease in mucins concentration reduces viscosity and slows down the appearance of *off-flavor* [24].

Finally, the illicit treatment with H_2O_2 can cause a whitening and "re-freshing" effect on fishery products due to its oxidative properties (peroxidation of double bonds present in chromophores) [25,26].

The aim of the present study was the development of mass spectrometry (MS) based methods to evaluate the concentration of different compounds related to the illicit treatment of fish food based on the

use of hydrogen peroxide (whitening and "re-freshing"). For this purpose, both liquid chromatography and gas chromatography hyphenated mass spectrometers were used. Mass spectrometry is recognized as one of the powerful and sensitive analytical techniques to identify, characterize, and quantify small molecules, such as amines and ethers. The use of this kind of MS based techniques is worthwhile in food analysis requiring complementary approaches to the detection of chemicals with different physical–chemical properties such as in the present work [27–29].

Two analytical methods were developed: The first one was a direct LC-MS/MS method for the determination of various amines and trimethylamine-oxide (TMAO); the second one was an indirect SPME-GC-MS method for the determination of residues of H_2O_2 on different fishery products matrices, by the hydroxylation reaction of anisole to 2-hydroxyanisole (guaiacol).

Another aim of this work was the application of the developed methods to investigate about H_2O_2 fish products treatment. The consequent alteration of the concentration ratio of TMAO and trimethylamine (TMA) which is a known fish product freshness parameter was evaluated.

2. Materials and Methods

All solvents and analytical standards of dimethylamine (DMA), trimethylamine (TMA), trimethylamine-N-oxide (TMAO), cadaverine (CAD), hydrogen peroxide solution, anisole, guaiacol, and p-xylene-d10 were purchased from Sigma Merck (Merck, Milan, MI, Italy). High-performance liquid chromatography (HPLC)-grade water was obtained from a MilliQ Academic water purification system (Millipore, Milan, Italy). Before use, solvents were filtered through a 0.45 μm filter and degassed for 10 min in an ultrasonic bath.

Fishery products samples were: Atlantic bonito (*Sarda sarda*) mackerel-like fish of the family Scombridae; European squid (*Loligo vulgaris*) belonging to the family Loliginidae. Atlantic bonito and European squid samples were purchased in a local market or caught fresh in Tyrrhenian Sea and brought in the laboratory no later than 5 h after fishing, in ice.

2.1. UHPLC-Tandem Mass Analysis of NPN

A Nexera (Shimadzu, Milan, MI, Italy) UHPLC (Ultra-high-performance liquid chromatography) coupled through an ESI source with a QTRAP-5500 tandem mass analyzer (Sciex, Milan, MI, Italy) was used to quantify the NPN in fishery samples.

The chromatographic separation was achieved using a RP C18 column (Kinetex EVO, 5 μm, 150 × 2.1 mm, Phenomenex, Castel Maggiore, BO, Italy) and heptafluoro-butanoic acid 10 mM in water (eluent A) and in methanol (eluent B). The gradient run started with 1% B, increased to 35% B in 8 min, up to 100% in 3 min, followed by reconditioning time. Flow rate and injection volume were 200 μL min^{-1} and 5 μL, respectively.

The triple quadrupole was used in a MRM positive ion mode with the following source parameters: Curtain gas (arbitrary unit, arb), 25; spray voltage (V), 5500; gas1 (arb), 35; gas2 (arb), 40; capillary temperature (°C), 400. Nitrogen was used as curtain gas, gas 1, and gas 2. The MRM transitions, potentials, and collision energies were listed in Table 1.

Table 1. MRM (multiple reaction monitoring) parameters and voltages for tandem mass analysis in a positive ion mode of dimethylamine (DMA), trimethylamine (TMA), trimethylamine-N-oxide (TMAO), and cadaverine (CAD). DP: Declustering potential; EP: Entrance potential; CE: Collision energy; CXP: Collision exit potential.

Compound	Q1 *m/z*	Q3 *m/z*	DP (V)	EP (V)	CE (V)	CXP (V)
DMA	46	30	96	10	37	13.5
TMA	60	44	90	10	24	11
TMAO	76	58	90	12	22	15
CAD	103	86	60	10	13	23

The developed analytical method was validated evaluating selectivity, linearity, accuracy and precision, and lower limit of quantitation following the FDA guidelines [30].

2.2. Gas Chromatography–Mass Spectrometry Analysis of H_2O_2 Residue

For the GC-MS analysis we used a Varian Saturn 3900 (Agilent, Milan, MI, Italy) system, equipped with a 1177 injector. The separation column was a Zebron ZB-624 30 m, i.d. 0.25 mm (Phenomenex, Castel Maggiore, BO, Italy) applying a temperature gradient from 40 to 240 °C in 16 min. Injector temperature was 240 °C; split mode was employed and helium gas flow was 1.2 mL min^{-1}.

The mass spectrometry was a Varian Saturn 2100 T ion trap analyzer (Agilent, Milan, MI, Italy) equipped with an EI (electron ionization) source. The full mass acquisition range was from 40 to 500 *m/z*.

The developed methodology was based on the paper of Tanaka et al. [31] calibrating the modified procedure in order to measure H_2O_2 concentration in the range 0.05 to 1.00 μg/mL.

2.3. Sample Preparation for UHPLC/GC-MS Analysis of NPN

Samples of fish products muscles were weighted, minced, and extracted with pH 2.5, 0.1 M phosphate buffer: 40 mL of buffer were used for 8 g of fish sample; the suspensions were then centrifuged (2300 *g* for 10 min) and filtered (0.45 μm).

Some samples were treated with hydrogen peroxide to simulate the illicit treatment: After a total immersion of 8 g of fish products in H_2O_2 solution (0.8%) for 2 min, the liquid was removed and samples were rinsed with fresh water. Then, treated fishery products samples were extracted as just described.

For UHPLC-MS, 1 mL of extracted solution was diluted using the starting eluent mixture, placed in a vial, and analyzed in a MRM mode using the triple quadrupole in a positive ion mode.

For GC-MS, 2 mL of extracted solution were placed in a vial for headspace solid-phase microextraction (HS SPME) and added with 100 μL of potassium ferricyanide ($K_8Fe(CN)_6$) as catalyzer and 2 μL of anisole. The solution was heated at 60 °C in an oil bath for 1 h; the fiber for head space analysis was exposed for 30 min. A Supelco 75 μm Carboxen™-PDMS (polydimethylsiloxane) (Merck, Milan, MI, Italy) fiber was used. The extraction recovery of anisole was checked to be 80% by the use of p-xylene-d10 as an internal standard for 30 min fiber exposition.

3. Results

With the developed MS based methodologies we were able to quantify the amount of the amines dimethylamine (DMA), trimethylamine (TMA), trimethylamine-N-oxide (TMAO), and cadaverine (CAD) with the UHPLC-tandem mass analysis. The concentration of H_2O_2 residues was measured with the use of a HS SPME-GC-MS method.

3.1. Results of UHPLC-Tandem Mass Analysis of NPN

The chromatographic separation of the analyzed amines is shown in Figure 1. The ion pairing effect of the heptafluoro-butanoic acid present in the mobile phase allowed a valuable retention to obtain a satisfying separation of the analytes.

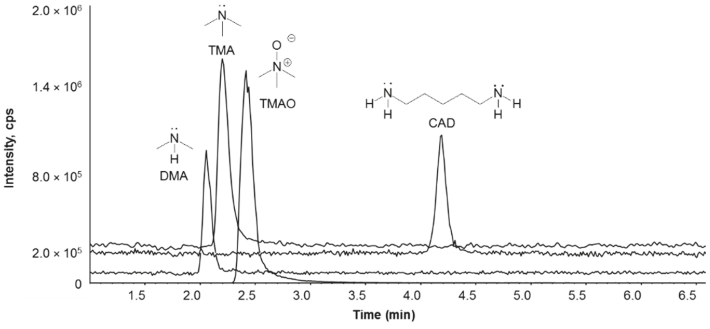


Figure 1. Chromatographic separation of dimethylamine (DMA, R_t 2.2 min), trimethylamine (TMA, R_t 2.25 min), trimethylamine-N-oxide (TMAO, R_t 2.5 min), and cadaverine (CAD R_t 4.3 min).

In order to quantify the amines in fresh fishery products samples or in fishery products subjected to an illicit treatment with H_2O_2, three calibration curves were prepared: (I) In pure pH 2.5, 0.1 M phosphate buffer, (II) In the extraction buffer fresh samples of Atlantic bonito, and (III) In the extraction buffer of fresh samples of European squid.

Each matrix material was weighted and extracted as previously described; since the matrix has a basal amount of amines, it was mandatory to prepare a matrix-blank without the addition of analyte (DMA, TMA, TMAO, and CAD) standards. Once obtained, the matrix-blank and the calibration curves were obtained by adding increasing amounts of amines as follows: 50, 100, 200, 400, 600, 800 ng/mL. A standard addition curve of TMAO in a real sample of European squid is shown in Figure 2. The curve had a positive intercept value because of the basal amounts of the analyte in fish.

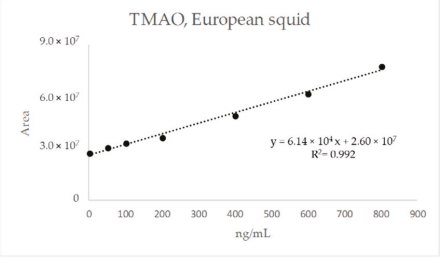


Figure 2. A standard addition curve of TMAO in a real sample of fresh European squid. The basal concentration of TMAO was 1150 ng/mL.

A full validation of the UHPLC-MS method for amines determination was performed. We followed the Food and Drug Administration (FDA) guidelines to evaluate the protocol [30] and used our previous work as an example for validation parameter definition [32]. Validation parameters were listed in Table 2 for all the UHPLC-MS analytes. LOD and LOQ were evaluated on the standard calibration curve by the signal to noise values of 3 and 10, respectively. LLOQ is the lower limit of quantification determined on the basis of a simple LOQ. To obtain LLOQs, LOQ standard solutions were prepared, used as the first level of each calibration curve, and validated by comparison of blank solutions. Validation parameters definition is given in the following paragraph.

To test the possible interferences at the analyte of interest's known retention time and *m/z*, a standard solution that had every analyte at a known concentration except for the one of interest, was analyzed. The selectivity % (Sel% = (Area at analyte retention time/Average area analyte in LLOQ) × 100) had to be ≤30%. The statistical parameter related to the linearity of calibration curve is the percentage difference (Diff% = (slope − average slope)/ average slope) × 100). Diff% had to be ≤25%.

Inaccuracy of lower limit of quantitation (LLOQ) had to be ≤20% and relative standard deviation % of accuracy of LLOQ ≤15%. Finally, to test recovery, matrices were spiked with a combined standard solution of amines at known concentration and processed as described in the Sample preparation section. The recovery (Rec = (Area at analyte retention time in spiked matrix/Average analyte area in spiked water solution) × 100) had to be between 85% and 120%. The validation parameters were respected in all cases.

Table 2. Validation parameters for calibration curves in (I) pH 2.5, 0.1 M phosphate buffer, (II) Atlantic bonito extraction solution, (III) European squid extraction solution. Diff% slope: Difference % of the slope of the calibration curve; RSD% LLOQ%: Relative standard deviation % of accuracy of lower limit of quantitation; BIAS% of LLOQ: Inaccuracy of lower limit of quantitation; LLOQ: Lower limit of quantitation.

Compound	Parameter	Matrices		
		(I)	(II)	(III)
DMA	Selectivity %	1.80	2.50	3.42
	Diff% slope	4.74	6.53	7.84
	RSD% LLOQ	14.7	15.0	14.9
	BIAS% LLOQ	19.0	20.3	19.5
	LLOQ (ng/mL)	25.0	45.0	45.0
	Recovery %	95.2	87.6	85.1
TMA	Selectivity %	0.50	1.32	1.97
	Diff% slope	3.60	5.19	6.41
	RSD% LLOQ	2.16	7.86	4.36
	BIAS% LLOQ	20.0	19.8	19.0
	LLOQ (ng/mL)	30.0	70.0	70.0
	Recovery %	93.6	89.3	86.0
TMAO	Selectivity %	0.05	0.90	0.85
	Diff% slope	4.07	14.6	8.2
	RSD% LLOQ	8.60	10.6	9.63
	BIAS% LLOQ	9.50	12.7	18.6
	LLOQ (ng/mL)	30.0	50.0	50.0
	Recovery %	102.5	91.4	90.7
CAD	Selectivity %	0.03	0.40	0.60
	Diff% slope	6.85	8.97	12.3
	RSD% LLOQ	9.80	15.3	14.3
	BIAS% LLOQ	16.0	17.6	19.0
	LLOQ (ng/mL)	20.0	40.0	40.0
	Recovery %	99.8	94.1	85.8

The UHPLC-tandem mass method was then applied to real fishery product samples of Atlantic bonito (*Sarda sarda)* and European squid (*Loligo vulgaris*): A) Freshly caught; B) freshly purchased in a local market; C) aged (four days at room temperature); and D) H_2O_2 treated (as described before).

The obtained results are summarized in Table 3. In freshly caught and freshly purchased samples the measured amount of amine was similar; in Table 3 the quantity of amines in freshly caught samples only was reported. High levels of TMAO were found in freshly caught Atlantic bonito (1700 ± 238 mg/kg) and European squid (1200 ± 336 mg/kg) samples. Conversely, in these samples the TMA amount was low, 170 ± 24 and 210 ± 29 mg/kg, respectively.

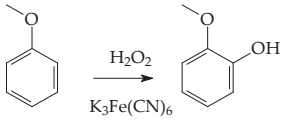
When fishery products initiated to degrade due to temperature (4 h at room temperature) or bacterial activities, the TMAO and TMA amounts reversed. Finally, when hydrogen peroxide was used as an illicit treatment as whitening and refreshing agents, exploiting its oxidant properties, the balance was shifted again towards a higher level of TMAO. In *Sarda sarda* the values after H_2O_2 treatment were TMAO 410 ± 57, TMA 720 ± 180 mg/kg; and in *Loligo vulgaris* were TMAO 850 ± 212, TMA 200 ± 28 mg/kg.

Table 3. Concentration values of trimethylamine-N-oxide (TMAO) and trimethylamine (TMA) in real fish samples of Atlantic bonito (*Sarda sarda*) and European squid (*Loligo vulgaris*). The amount is expressed in mg/kg. Fresh referred to freshly caught samples; aged to samples left at room temperature for 4 h; H_2O_2 treatment to samples treated with hydrogen peroxide (see Material and Methods).

Sample	Compound	Fresh (mg/kg)	Aged (mg/kg)	H_2O_2 Treatment (mg/kg)
Sarda sarda	TMAO	1700 ± 238	170 ± 24	410 ± 57
	TMA	170 ± 24	1250 ± 350	720 ± 180
Loligo vulgaris	TMAO	1200 ± 336	140 ± 20	850 ± 212
	TMA	210 ± 29	970 ± 242	200 ± 28

3.2. Results of GC-MS Analysis of H_2O_2 Residues

To confirm the illicit management of fishery products with hydrogen peroxide, we implemented a previously published GC-ECD assay based on peroxide detection by indirect oxidation of anisole to guaiacol (2-hydroxyanisole) (Scheme 1) [31].



anisol guaiacol

Scheme 1. Oxidation reaction with hydroxyl peroxide of anisole to guaiacol (2-hydroxyanisole) catalyzed by potassium ferricyanide.

We developed a headspace solid-phase microextraction (HS SPME) GC-MS methodology to improve sensitivity, easiness of operation, and reliability.

To quantify the residues of H_2O_2 we prepared a calibration curve with the standard addition method using fresh fish product samples of European squid and Atlantic bonito as matrices. The extracted solution of fresh fishery products was added with aliquots of hydrogen peroxide to give amounts of 0.0, 0.05, 0.1, 0.25, 0.5, and 1.0 µg/mL of H_2O_2. Then, 100 µL of potassium ferricyanide ($K_8Fe(CN)_6$) and 2 µL of anisole were added to the obtained solutions before fiber exposing for head space analysis. We measured the 2-hydroxyanisole peak area which was related to the H_2O_2 addition. Figure 3 shows a standard addition calibration curve of H_2O_2 in European squid samples. The obtained LLOQ was 50 ng/mL. When the concentration of hydrogen peroxide was zero, no 2-hydroxyanisole was detected.

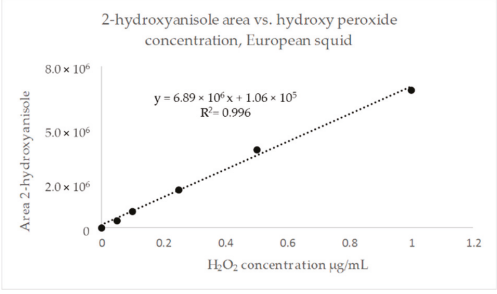


Figure 3. A standard addition curve of H_2O_2 in a fresh sample of European squid.

We then applied the developed HS SPME-GCMS method to five real fishery products samples, in particular squid which were the subject of illicit treatment with hydrogen peroxide due to its properties of whitening agent.

QC and real samples were prepared as described in the Sample Preparation section: The amount of H_2O_2 added for the redox reaction of anisole to guaiacol was set at 0.5 μg/mL. In these conditions we tested a blank solution without fishery products (no *Sarda sarda* or *Loligo vulgaris*), fresh (blank matrix), fresh and treated in controlled conditions (QC) and illicit treated fishery products samples of Atlantic bonito and European squid. All of the five samples coming from legal controls showed H_2O_2 values higher than 100 ppm. Table 4 shows the results. The peak area of 2-hydroxyanisole in the buffer without matrices confirmed the added amount of H_2O_2 of 0.5 ± 0.07 ppb. In the presence of matrices, the peak area decreased due to the matrix effect. In the case of fresh fish product samples, this effect was quantifiable in a loss of 12% of H_2O_2 amount (0.44 ± 0.11 μg/mL for Atlantic bonito and 0.43 ± 0.10 μg/mL for European squid). In fresh samples treated with hydrogen peroxide in controlled conditions (complete immersion of samples in a 0.8% H_2O_2 solution followed by rinsing with fresh water in laboratory) the amount of H_2O_2 was much higher than 1 ppm, the highest calibration curve point. However, extrapolating the results, it seemed larger than 100 ppm. The same was for the samples, especially squid, illegally treated with hydrogen peroxide.

Table 4. Quantitation of H_2O_2 in a 0.1 M phosphate buffer pH 2.5 solution and in fishery products samples. QC were quality control samples treated with hydrogen peroxide in controlled conditions (0.5 μg/mL of H_2O_2); five real samples were illegally treated with peroxide.

Matrix	Samples	H_2O_2 (μg/mL)
Buffer		0.5 ± 0.07
Sarda sarda *Loligo vulgaris*	fresh	0.44 ± 0.11 0.43 ± 0.10
Sarda sarda *Loligo vulgaris*	QC	>100
Sarda sarda *Loligo vulgaris*	real	>100

4. Discussion

The developed mass spectrometry based analytical methods were adequately selective to quantify the amount of peculiar molecules related to the illicit treatment of fishery products with hydrogen peroxide. In particular, to quantify trimethylamine, trimethylamine-N-oxide, dimethylamine, and 2-hydroxyanisole.

Many other analytical methods were proposed to measure the analytes [33–41]. The following paragraph gives a summary about was presented.

Bilgin's and Chung's research groups performed a quantitation of some amines in a large number of fish samples using HPLC coupled to a photodiode array detector [33] or other detectors such as chemiluminescent nitrogen, SPME-GC-MS, and spectrophotometric ones [34]. The first study regarded the determination of histamine, cadaverine, and tyramine after derivatization with dansyl chloride in 63 fish samples. The declared limit of quantitation was in the range between 0.010 and 0.100 μg/mL. Chung et al. determined TMAO, TMA, DMA, and FA in 266 fish samples. They were used respectively for TMAO, HPLC coupled to a chemiluminescent detector; for the TMA and DMA SPME-GC-MS method with a carboxen/divinylbenzen/polydimethylsiloxane fiber; and LC-visible analyzer for FA after derivatization with 2,4-dinitrophenylhydrazine. The stated LOQ were 25, 10, 10, 5 mg/kg for TMAO-DMA-TMA-FA, respectively [34]. Moreover, Soncin [40] and Chan et al. [35] in their studies used as analytical methodologies the headspace solid-phase extraction coupled with GC-MS. The first performed an identification and quantitation of markers of spoilage in fish and found as indicators

four molecules. They defined the technique suitable for analyzing the volatile compounds [40]. Chan's study monitored the concentration of DMA and TMA in fish and the achieved limit of detection was 100 ppb [35].

Heude in his paper published on Food Analytical Methods in 2015 used a ^{1}H high resolution magic angle spinning NMR spectroscopy to determine the *K*-value [36] and trimethylamine nitrogen content as parameters of freshness and quality of fish products. They studied four species of fish and highlighted as great advantages the possibility to not process fish samples, no extraction was required for NMR analysis [36]. Feng Li et al. in their research used ion chromatography coupled to an unsuppressed conductivity detector to measure the amount of DMA, TMA, and TMAO in aquatic products. They obtained a good lower limit of detection (60, 80, 100 ng/mL for DMA-TMA-TMAO) and quantified the analytes in three real samples [38].

Finally, the remaining methods developed [37,39,41] were based on mass spectrometry techniques in order to determine the amines of interest. Le's group using a C18-PFP column and triple quadrupole quantify TMAO in human plasma samples as a potential indicator of cardiovascular health. The determined LOD and LOQ were 1 and 6 ng/mL [37]. Romero-Gonzáles et al. measured cadaverine and TMA with UHPLC-MS with a LLOQ of 25 and 60 ppb, respectively [39]. Finally, using a hydrophilic interaction liquid chromatography coupled to mass spectrometry Wu et al. quantified in fish meals five amines with satisfactory LOD and LOQ [41].

The present method is based on ultra-high liquid chromatography coupled to mass spectrometry and demonstrates to be sufficiently sensitive to quantify the four studied amines in fishery products in 5 min only. As previously indicated, the lower limits of quantification in matrices for DMA-TMA-TMAO and CAD were 45, 70, 50, and 40 ng/mL, respectively. The selectivity of the method was remarkable in spite of the low mass to the charge value of molecular protonated ions of the analytes. This region of low *m/z* ratio ($[M+H]^+$ 46, 60, 76, and 103 for DMA-TMA-TMAO, and CAD) is normally affected by a high background noise, but using tandem mass spectrometry the signal to noise ratio was reasonably good.

To highlight the illicit treatment of fishery products with hydrogen peroxide, we focused our attention on the ratio between TMAO and TMA. As shown in Table 3, subsequently to the prohibited washing with H_2O_2, the ratio between the measured compounds was shifted again towards a higher concentration of TMAO, one of the endogenous molecular marker of freshness. This ratio was not completely reversed because hydrogen peroxide acted only on the skin of the fishery products, besides the alkylamine is present in higher concentration in muscles. However, the treatment could make the product look younger and fresher.

As explained in the results section, the GC-MS method was an upgrade of the Tanaka et al. GC-ECD methodology for the quantitation of hydrogen peroxide in Chinese foods. The quantitation is possible thanks to the indirect measurement of 2-hydroxyanisole generated by a potassium ferricyanide catalyzed redox reaction of anisole with H_2O_2 [31]. In that paper, Tanaka et al. performed a deep investigation about reaction environment (pH range), catalyzer, and hydrogen peroxide concentrations in order to obtain the highest possible yield of redox reaction. For ECD quantitation, pentafluoro-benzoyl chloride was used as a derivatizing agent prior to analysis of the product of the reaction, 2-hydroxyanisole. The LLOQ by Tanaka et al. was 0.10 μg/mL.

With the present method based on mass spectrometry we aimed to improve the literature method both for sample preparation and sensitivity. It is well known that SPME followed by gas chromatography coupled to mass spectrometry is a highly sensitive method and the electron ionization source (EI) owns a high fragmentation repeatability.

The sample preparation herein described is free from the necessity of derivatization to detect the redox reaction product 2-hydroxyanisole. The HS SPME with carboxen/polydimethylsiloxane fiber was improved by testing various pH buffers, catalyzer concentrations, reaction times and fiber exposure times, stirring of solution.

We found that the best conditions to obtain the highest amount of 2-hydroxyanisole were: Solution buffering at pH 2.5, 0.1 M catalyzer concentration, 1 h at 60 °C time reaction, time of 30 min of exposure of the fiber without stirring. In these conditions a LLOQ value of 0.05 μg/mL was obtained.

With this HS SPME-GC-MS method we measured the amount of H_2O_2 in fishery products, comparing fresh and illicit treated samples. In the latter case, the concentration should be hypothesized by extrapolating over the calibration curve range because the signal was too abundant, and it was calculated larger than 100 ppm, as listed in Table 4.

5. Conclusions

In conclusion, the developed mass spectrometry based analytical methods show to be suitable to notice the illicit treatment of fishery products with hydrogen peroxide. Both the UHPLC-MS method and the HS SPME-GC-MS method are applicable to detect molecules related to the use of hydrogen peroxide solution to whiten and refresh aged fish food.

By LC-MS, low molecular mass amines were detected with high selectivity and good sensitivity. The TMAO and TMA ratio was shown to be reversed by the illicit treatment, simulating an unreal apparent freshness of fishery products foods.

The use of hydrogen peroxide on fish products was confirmed by the measurement of 2-hydroxyanisole with HS SPME-GC-MS after a redox reaction between anisole and residual H_2O_2 in the extracted solution, by exploiting potassium ferricyanide catalysis. After the optimization of sample preparation for headspace solid-phase microextraction and redox reaction parameters, the method was suitable to quantify the H_2O_2 residues in fish food matrices. When fishery products, especially squid, were whitened with hydrogen peroxide, its amount was shown to be easily detectable.

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