Università di Foggia
Dipartimento di Scienze Mediche

PhD course in translational medicine and food: innovation, safety and management

XXXIII cycle

Development of new analytical methods for the monitoring of emerging contaminants in foodstuffs

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A thesis presented for the degree
of Doctor of Philosophy

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Index

1. INTRODUCTION

1.1 Food emerging contaminants

Food manufacturing comprises several phases from the cultivation in field to the packaging. In every single step, foods may be contaminated by chemical and biological substances. The substances that may unintentionally contaminate foods are called contaminants (Council Regulation 1993/315/EEC). Sources of contamination are varied and include agriculture (therapeutic, prophylactic, metaphylactic and growth-promoting antibiotics), the aquatic environment, disinfection residues, by-products, food production and storage materials. Biological contamination includes bacterial, fungal, viral and protozoan microbial species. Moreover, classes of chemicals often associated with food pollution include metals (mercury, lead and arsenic), persistent organic pollutants (dioxin, DDT, chlordane), pesticides (permethrin, endosulfan) and agrochemicals (hexacyclohexanes, toxaphene) and food packaging chemicals (Bisphenol A (BPA), melamine) (Garvey 2019).

Contaminants in foods may cause severe damages to human health. For example, pathogenic microorganisms present in foodstuffs have been associated with more than 200 disease conditions including gastroenteritis and cancer (Garvey 2019). Furthermore, chemically contaminated foods have serious implications on the health of individuals that may range from mild gastroenteritis to fatal cases of hepatic, renal and neurological syndromes. For instance, heavy metals can cause intrauterine growth retardation and an increase of gastrointestinal diseases. Moreover, some pesticides can lead to neural and kidney damage, congenital disabilities, reproductive problems, and can prove to be carcinogenic (Rather et al. 2017).

The risks for human health that result from a newly identified hazard to which a significant exposure may occur or from an unexpected new or increased significant exposure and/or susceptibility to a known hazard are defined as emerging risks (EFSA/SC/415 Final 2007). The identification of these emerging risks is crucial in order to implement measures aimed at protecting consumers' health (EFSA et al. 2018). Food contaminants that may represent an emerging risk for human health are called "food emerging contaminants" (FECs) (Farré and Barceló 2012; Kantiani et al. 2010). Neonicotinoids, bisphenols and alkylphenols are examples of substances that have been already considered FECs or that would be declared to be FECs in the near future because a significant exposure may occur due to an increase in their use (Farré and Barceló 2013; Careghini et al. 2015).

1.1.1 Neonicotinoids

Pesticides have been used intensively during the last century in order to significantly increase crop yields and food production. These substances include several groups of compounds such as organochlorine, organophosphate, carbamate, pyrethroids, growth regulators, neonicotinoids (NNs), and now biopesticides, which have been developed one after the other. However, soon after pesticides became widespread, it was observed that their application was causing contamination of the ecosystem and foods, both at local and global scale. The dispersion of pesticide residues in the environment may cause mass killings of nonhuman biota, such as bees, birds, amphibians, fish, and small mammals. Consequently, considerable efforts have been made to design new chemicals, improve pesticides formulations, application devices, and chemical delivery mechanisms in an attempt to reduce exposure of biota and environmental contamination (Carvalho 2017).

Neonicotinoids are broad-spectrum, systemic compounds that exhibit activity against sucking insects, several species of flies and moths and may represent an alternative to traditional pesticides. They were developed in the 1980s, and the first commercially available compound, imidacloprid, has been in use since the early 1990s. They have become one of the fastest growing classes of pesticides used in agriculture because they possess lower mammalian toxicity, less resurgence problems, environmental protection, pest management selectivity and less toxicity towards natural enemies. Their common names are acetamiprid, clothianidin, dinotefuran, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam (Kundoo et al. 2018).

However, in the last few years it has been observed that the exposure to NNs may cause oxidative stress, adverse reproductive and developmental effects, hepatotoxicity and endocrine disruption effects (Thompson et al. 2020). Thus, NNs can be considered FECs, as already stated by Farré et. al in 2012 (Farré and Barceló 2012), because they have been widely used in the last few years, it has been proved that they may be dangerous for the human health and they can be found in foods (Chen et al. 2014).

The European Commission, taking into account the human health hazard associated with the consumption of foods contaminated with NNs, has set maximum residue limits (MRLs) for these substances in foodstuffs (see table 1).

1.1.2 Bisphenols and alkylphenols

Residues of bisphenols have been found in both packaged and unpackaged food such as packaged cereals, meat, fish, spices, ready to eat food, snacks, ice cream and unpackaged meat and fish. Most likely, the direct contamination of fish may be caused by the leach of plastics in oceans, while the contamination in meat is the result of the contact with material containing BPA, for example during post-mortem processing. As regards packaged foods, the packaging has a key role in ensuring food preservation by mainly acting as a barrier against the substances and the microorganisms that are responsible for chemical, physical and microbiological deterioration (Vilarinho F. et al. 2019). Nowadays, food packaging are made mostly with plastic polymers, which characteristics in terms of flexibility, workability, and extensibility may be properly modulated by adding plasticizers, such as bisphenols (BPs) (Selke and Culter 2016). BPs have been largely used by industries in order to produce polycarbonate and epoxy resins, designed to be in direct contact with foodstuffs (Russo et al. 2019). However, BPSs may migrate from plastic food packaging to foods after the manufacturing process and hydrolysis of the polymer (Vilarinho F. et al. 2019).

Table 1. Maximum residue limits (MRLs) for neonicotinoids in foodstuffs.

Neonicotinoid	MRLs (mg kg^{-1})	Bibliographic references
Imidacloprid	10 – 0.1	EFSA et al. 2019
Acetamiprid	3 – 0.02	Commission regulation 2019/88/EU
Dinotefuran	8 – 0.02	Commission regulation 2014/491/EU
Thiamethoxam	20 – 0.02	Commission regulation 2017/671/EU
Clothianidin	1.5 – 0.02	Commission regulation 2017/671/EU

Bisphenol A (BPA) has been the most widely used and studied among BPs (Farris 2014). However, in 2017 BPA was declared an endocrine disruptor (ECHA/PR/17/12, 2017). In order to substitute BPA in the production of food plastic packaging, other bisphenols are beginning to be used, such as for example bisphenol B (BPB) and bisphenol F (BPF) (Rochester and Bolden 2015; Wang et al. 2021; Liu et al. 2017; Deceuninck et al. 2015). However, the safety of BPA substitutes has not been fully verified. Many studies indicate that these molecules, that have structural similarities with BPA, have shown both endocrine disruption effects and others adverse health effects (Moon 2019). Instead, bisphenol A diglycidyl ether (BPADGE) has been widely used in the production of coatings and adhesives for food-contact materials (Petersen et al. 2008). Toxicological information on BPADGE are scarce and further studies are needed. However, it has been demonstrate that

BPADGE can disrupt placenta cells lipidome and it may be an endocrine disruptor (Marqueño et al. 2019).

Furthermore, also antifogging agents such as nonylphenols may be added to plastic food packaging materials (Selke and Culter 2016). The term nonylphenols refers to a complex mixture of mainly nonyl-substitued phenols, among which the 4-nonylphenol (4-NP) has been the most used, although many studies have reported its endocrine disruption activity (Fernandes et al. 2008). BPA, BPADGE and 4-NP negative effects on human health can be considered a newly identified hazard with a significant exposure. Therefore, they can be considered contaminants of emerging interest, as already stated by Carenghini et al. referring to BPA (Careghini et al. 2015). As concerns BPA substitutes, they may be considered newly identified hazards to which a significant exposure may occur. As a result, these molecules may be also considered as FECs.

The European Commission, taking into account the declared hazard for human health of bisphenols, bisphenol derivatives and nonylphenols, has set a specific migration limit (SML) for some of these compounds. Specifically, SMLs of 0.05 $mg \cdot kg^{-1}$ (Commission regulation 2018/213/EU) and 9 $mg \cdot kg^{-1}$ (Commission regulation 2005/1895/EC) have been established for BPA and BPADGE (as sum of BPADGE, BPADGE·H_2O and BPADGE·$2H_2O$), respectively. At the moment, no limitations have been reported for BPF, BPB, bisphenol AF (BPAF) and 4-NP.

1.2 Analytical methods currently used for some FECs in foodstuffs

The most currently used analytical methods for some FECs in foods are shown in table 2. The most used FECs extraction technique, among all the mentioned methods, is the solid phase extraction (SPE). This method is used to extract analytes from liquid and solid matrices in order to clean-up a sample before its chromatographic separation and detection for confirmation and quantification purposes. Solid samples including the food ones have to be properly pre-treated to obtain an efficient extraction of the analytes. Then, the resulting liquid extract is loaded onto a cartridge containing a stationary phase, which in almost all cases requires to be conditioned before its use. This step is performed by performing several steps of washing and elution in order to purify the extract from the interferences and recovering the analytes (Żwir-Ferenc and Biziuk 2006). However, this technique has several drawbacks: i) the sample has to undergo proper pre-treatments and must be available in considerable quantity (up to 5 g or 20 mL); ii) the washing and elution steps are usually performed by using large volumes of organic solvents (in the range of 3 – 30 mL); iii) the

cartridge is disposable and has to be conditioned before every single use; iv) the technique is time-consuming.

Table 2. Analytical methods currently used for some FECs in foodstuffs.

n° of analytes	Food sample	Extraction/ clean-up	Separation	Detection	Reference
		Bisphenols			
1	Milk	SPME	LC	FD	Liu et al. 2008
1	Canned fatty foods	CME	LC	FL	Bendito et al. 2009
2	Canned food	SPME	GC	MS	Rastkari et al. 2010
5	Milk	SPE	LC	FD	Grumetto et al. 2013
8	Milk	SPE	LC	UV	Sun et al. 2014
8	Soft drinks	SPE	LC	FD	Russo et al., 2016
5	Canned energy drinks	SPE	LC	FD	Gallo et al. 2017
5	Water, beverages	SPE	GC	MS	Cao and Popovic 2018
2	Sacked mouse foods	SPE	LC	MS	Xie et al. 2018
2	Royal jelly	SULLE	LC	FD	Tu et al. 2019
8	Eggs	SPE	LC	MS	Xiao et al. 2020
1	Canned Food	SPE	LC	MS	Maragou et al. 2020
		Neonicotinoids			
5	Honey	SPE-DLLME	LC	MS	Campillo et al. 2013
7	Cucumber, Eggplant	SPE	LC	UV	Watanabe et al. 2015
7	Honey	DLLME	LC	UV	Jovanov et al. 2015
5	Wine	SPE	LC	MS	Rodríguez-Cabo et al. 2016
8	Tea	QuEChERS-SPE	LC	MS	Jiao et al. 2016
4	Sunflower seeds	SPE	LC	MS	Shi et al. 2017
7	Honey	SPE, QuEChERS	LC	MS	Valverde et al. 2018
5	Milk	LLE	LC	MS	Lachat and Glauser 2018
10	Honey, Royal-jelly	SPE	LC	MS	Hou et al. 2019
14	Tea	QuEChERS	LC	MS	Zhang et al. 2020
7	Grains	QuEChERS-DLLME	LC	MS	Ma et al. 2020

Alkylphenols					
5	Bottled water	SPE	LC	MS	Pernica et al. 2015
10	Fruit juices	SPME	LC	MS	Viñas et al. 2016
6	Baby foods	SPE	GC	MS	Pastor-Belda et al. 2017

SPME: solid phase micro extraction; CME: coacervative microextraction; SPE: solid phase extraction; SULLE: sugaring-out assisted liquid-liquid extraction; DLLME: dispersive liquid−liquid microextraction; QuEChERS: quick, easy, cheap, effective, rugged, and safe method; LLE: liquid–liquid extraction; LC: liquid chromatography; GC: gas chromatography; FD: fluorescence; FL: fluorimetry; MS: mass spectrometry; UV: ultraviolet.

Besides SPE, also QuEChERS (quick, easy, cheap, effective, rugged, and safe) and DLLME (dispersive liquid−liquid microextraction) techniques have been widely used in order to extract some FECs in foodstuffs. The QuEChERS procedure includes three main steps, namely sample pre-treatment, extraction and clean up. Usually, solid samples have to be homogenised before performing the subsequent steps. Typically, the extraction step is carried out by adding to the homogenised solid sample an organic solvent and a salt in order to promote a partitioning based on the salting-out phenomenon. This first step allows the extraction of both target analytes, but also of interfering compounds. Therefore, it is essential to perform a clean-up step that is usually made by using a dispersive solid-phase extraction. QuEChERS fundamentals, relevant improvements and applications have been widely discussed by Perestrelo et al. in 2019 (Perestrelo et al. 2019). The main drawback of this technique is the need for further clean-up processes when it is used to extract substances from complex samples. For example, Jiao et al. in 2016 and Ma et al. in 2020 developed two methods to extract and purify neonicotinoids from tea and grains by using QuEChERS combined with SPE or DLLME (Jiao et al. 2016, Ma et al. 2020).

In DLLME, the target analytes are extracted from liquid samples by adding a mixture of an extraction solvent and a dispersing solvent. This addition results in the formation of a dispersion that has to be removed by centrifugation. Then, the extracting solvent containing analytes is withdrawn with a microsyringe (Zgoła-Grześkowiak and Grześkowiak 2011). A wide discussion on DLLME procedure and application has been reported by Leong at al. in 2014 (Leong et al 2014). DLLME is an appropriate tool for the analysis of samples with a relatively simple matrix, such as water samples. The main drawback may be the need to perform complex and time consuming sample pre-treatments for the analysis of food samples (Viñas et al. 2014).

Regardless of what extraction method has been used, solutions containing the analytes subsequently have been separated by liquid or gas chromatography and detected by using different techniques (see table 2).

Some FCEs, such as BPs and NNs, have been detected also by using electrochemical techniques. Pesticides have been detected by using immunosensors, chemically modified sensors, DNA based aptasensors and graphene oxide based sensors (Rapini et al. 2016, Urbanová et al. 2017, Reynoso et al. 2019, Ganesamurthi et al. 2020). Indeed, the electrochemical detection of bisphenols has been performed mainly by using aptasensors, enzyme based electrochemical sensors, molecularly imprinted sensors, and nanomaterials based sensors (Ballesteros-Gómez et al. 2009, Zhang et al. 2021).

A chemical sensor is a device that transforms chemical information, such as the concentration, into a useful analytical signal. The signal may originate from a chemical reaction of the analyte or from a physical property of the system investigated (Hulanicki et al. 1991). Electrochemical sensors are a category of chemical sensor that transforms the analytical information in a measurable electrical signal (Kreysa et al. 2014). The electrochemical sensors are usually composed by two components, namely a system that allows the recognition of the chemical information and a physicochemical transducer that converts the chemical response into the electrical signal (Faridbod et al. 2011). There are many types of electrochemical sensors depending on the nature of these two components. Biosensors are electrochemical sensors in which the chemical information is recognized by the interaction between the analyte and a bioelement, such as enzymes, antibodies or living cells (Mohanty and Kougianos 2006). Immunosensors are biosensors in which the used bioelements are antibodies, aptamers and microRNA (Cristea et al. 2015).

However, these devices have several drawbacks. The need for complex and time-consuming pre-treatments, the need to use biological elements, they are typically used to detect only one analyte at time, and the occurrence of electrode fouling with a consequent time dependent deterioration of response. For example, the device used by Rapini et al. in 2016 to detect only the acetamiprid was previously prepared by co-electrodeposition of polyaniline film and gold nanoparticles via cyclic voltammetry, before immobilizing the thiol-tethered DNA (Rapini et al. 2016). In 2021 Zhang et al. published a review on electrochemical detection of BPs in food. This review confirms the problems and limitations affecting the electrochemical devices, in particular the detection of one bisphenol at time.

Multianalyte detection can be performed by using post-column amperometric techniques as the electrochemical detection methods, in which a known potential is applied to an electrode in order to record a current signal that is proportional to the analyte concentration. Amperometric detection at solid electrodes under constant applied potential (DC) can be used for the detection of a wide variety of compounds also after a chromatographic

separation. However, some analytes cannot be detected under constant applied potentials due to the electrode fouling, caused by the adsorption of the electrochemical formed reaction products with a consequent time dependent deterioration of the response. Therefore, to perform an accurate detection, the electrode must be continuously reactivated, or "cleaned", on-line. Among all the possible cleaning pre-treatments, only the electrochemical reactivation can be performed efficiently on-line by applying a sequence of potential steps to perform detection and reactivation in the same experiment. This kind of detection technique is called pulsed electrochemical detection (PED) typically used with solid metal electrodes, and a wide discussion of its use as the detector system in high-performance liquid chromatography was reported by LaCourse (LaCourse 1997). In 2015, it have been presented the potential of pulsed amperometric detection (PAD) at glassy carbon electrodes as a sensitive and reproducible determination of electroactive compounds (Nardiello et al. 2015). This technique has been successfully used for the detection of β-Agonists (Mentana et al. 2021), nitrosamines (Mentana et al. 2021) and phenolic compounds (Natale et al. 2015).

Fouling species, which are typically adsorbed on carbonaceous materials produced during a short detection potential step, can be desorbed quite efficiently from the electrodes by the application of a large positive-potential pulse to generate a surface cleaning. After the anodic potential pulse, the electrode need to be reactivated by a negative-potential pulse. By using this three-step potential waveform, a reproducible detection can be achieved. In particular, the detection potential in the potential-time waveform is chosen to be appropriate for the electrochemical reaction of the analyte, and the electrode current is integrated during a short period of time (t_{int}) after a delay time (t_{del}). T_{del} and t_{int} constitute the detection period of time (t_{det}). Following the detection step, adsorbed species are desorbed by applying an oxidation potential step (E_{ox}) for a short period of time (t_{ox}). The "clean" but less active electrode surface is then regenerated by a subsequent application of a negative-potential step (E_{red}) of short duration (t_{red}). Other electrochemical techniques based on sequences of potential steps (i.e. multiple pulse amperometry detection) involve the sampling of the current response instead of the integration (PalmSens 2019).

1.3 Natural Deep Eutectic Solvents (NADESs) for the extraction of some FECs from foodstuffs

An interesting alternative to the most used extraction of FECs, namely SPE, may be represented by the extraction/clean-up based on NADESs. The term Deep Eutectic Solvents (DESs) was coined in 2004 to describe mixtures of quaternary ammonium salts with hydrogen bond donors, although the systems were described the year before, but not named

(Häkkinen and Abbott 2021). DESs are mixtures of compounds that have a much lower melting point than that of any of its individual components, mainly due to the generation of intermolecular hydrogen bonds (Dai et al. 2013). The charge delocalization occurring through hydrogen bonding between DESs components is responsible for the decrease in the melting point of the mixture relative to the melting points of the individual components (Smith et al. 2014). Several definition have been proposed in order to specify when a decrease of the melting point should be considered "deep". In 2009 Martins at al., on the basis of several publications on DESs, suggested to use the word "deep" for mixtures of two or more pure compounds for which the eutectic point temperature is far below that of an ideal liquid mixture and that are liquid at operating temperature (Martins et al 2019). They exhibit several interesting properties namely a low vapour pressure, non-flammability, ease of preparation, easy availability from relatively inexpensive components, the purification is not necessary, are less volatile than organic solvents (Zhang et al. 2012; Smith et al. 2014). Moreover, the physical properties of DESs can be customized by a proper choice of the chemical compounds used for their preparation. In general, DESs are obtained by mixing, with a moderate heating, two or three components, namely, hydrogen bond acceptors (HBA) and hydrogen bond donors (HBD), which can be associated with each other by means of hydrogen bond interactions (Pena-Pereira and Namieśnik 2014; Makoś et al. 2020; Smith et al. 2014). DESs can be dived in hydrophilic and hydrophobic (HDESs). Hydrophilic DESs have been the first prepared, and currently in most of them choline chloride is used due to its low cost, biodegradability and low toxicity (Zhang et al. 2012). HDESs are the most recent, they are a result for the need of increasing solvents interaction with less polar or apolar organic molecules (Dwamena 2019). NADESs are DESs prepared by using natural products, such as organic acids, amino acids, sugars, urea or choline derivatives for their preparation (Dai et al. 2013; Paiva et al. 2014). The phenomenon of solubility of substances in DESs is complex and still under study. It has been demonstrated that solute dissolving in DESs depends on enthalpy, entropy, solute size (Abbott et al. 2017), formation of hydrogen bonds (Häkkinen and Abbott 2019; Alshammari et al. 2021), π-π interactions (Chen et al. 2019) and polarity (Tang and Row 2020).

Currently, DESs have been already used to extract several compounds, such as dyes (Zhang et al. 2020; Ozak e Yılmaz 2020; Ge et al. 2021; Faraji 2019; Liu et al. 2019), phenolic compounds (Ozturk et al. 2018; Barbieri et al. 2020; Ruesgas-Ramón et al. 2017), curcumin (Altunay et al. 2020) and flavonoids (Bajkacz and Adamek 2018) from food samples. Moreover, DESs have been used to extract BPs from liquid samples, such as water (Florindo

et al. 2020; An and Row 2021), beverages (Shishov et al. 2018; Baute-Pérez et al. 2021; Yang et al. 2020), edible oils (Xie et al. 2020) and tea infusions (Zhang et al. 2021). To the best of our knowledge, there is only one extraction method in which DESs have been used to extract BPs from solid food samples (canned tuna and marine fish tissues) (Noori and Ghanemi 2019). However, this method has several drawbacks. It requires the execution of many time–consuming steps for the sample pre-treatment, including a liquid-liquid partition with an organic solvent and a further purification step on magnesium silicate mini-column, which has to be previously activated. Additionally, this method allows only the detection of BPA and 4-nonylphenol, but the blank samples seem to not be bisphenol A-free.

DESs have been also used to extract pesticides from foodstuffs. Most of time they have been used in the development of dispersive liquid liquid micro-extraction (DLLME) methods (Carbonell-Rozas et al. 2021; Kachangoon et al. 2020; Monajemzadeh et al. 2021; Jouyban et al. 2020; Farajzadeh et al. 2019) or dispersive micro solid-phase extraction (DMSPE) techniques (Khosrowshahi et al. 2021; Song et al. 2019). However, in the development of these methods, DESs have been used as dispersive or adsorbent solvents in liquid solutions.

2. THESIS STATEMENT

The need for safeguarding human health has always been a matter of great interest. There are several sources of hazard for humans. One of them is the consumption of foods contaminated with chemical substances that may cause severe damages to human health. The contamination may occur in every single step of food production, from the field to the packaging. Among all the contaminants, those representing an emerging risk for human health are known as "food emerging contaminants (FECs)" and constitute a very hot topic. The identification of these emerging risk as a key role in implementing measures to protect humans' health. To date, numerous analytical methods have been developed to monitor contaminants in foods. The extraction of the FECs has been mostly performed by using methods that have several drawbacks. For example, they may be time consuming, complex and may require the use of large volumes of organic solvents. Typically after the extraction samples are separated by using chromatographic techniques. Ultimately, the analytes are detected mostly by mass spectrometry. However, some FECs have been also detected by using electrochemical techniques that in most cases require complex and time-consuming pre-treatments, the use of biological elements and that may have a time dependent response.

Within this framework, the aim of my research has been the development of new alternative analytical methods for the monitoring of some FECs, such as bisphenols and neonicotinoids, in solid foodstuffs. In particular, a new easy, quick and green extraction method has been developed by using homemade natural deep eutectic solvents (NADESs). Several experiments has been performed in order to study how some factors, such as the mass of the components, may influence both the formation of the NADESs and their stability in time. The extraction method has been thoroughly studied in order to understand what are the key factors affecting the recovery of analytes. The potential of the proposed new extraction/cleanup method based on NADESs has been also demonstrated by the analysis of different solid foodstuff matrices, by using LC-Fluorescence, and by the determination of important validation parameters, such as recoveries, LOD, LOQ, and repeatability, following the recommendations of EC regulations and decisions.

In addition, a protocol based on microextraction by packed sorbent (MEPS) has been developed in order to make NADES extracts compatible with some separation/detection methods. Moreover, several studies have been made in order to assess the electrochemical behaviour of some FECs at different solid electrodes with the aim of developing a reproducible, easy, in-flow method for their detection.

3. MATERIALS AND METHODS

3.1 Chemicals

Ammonium acetate (HPLC grade), D-(-)-fructose and sodium hydroxide solution 50% in water were purchased from J.T.Baker™ (Deventer, Netherlands). Acetic acid glacial was obtained from Carlo Erba Reagents (Milano, Italia). Water of HPLC MS/MS grade were purchased from Honeywell (New Jersey, United States). Acetonitrile (≥ 99.9%), Methanol (≥ 99.9%), D-(+)-glucose (99.5% GC), xylitol (≥ 99.9%), choline chloride (≥ 98.9%), L-Menthol (≥ 99%), Camphor (95%), bisphenol A (BPA) (97%), bisphenol A diglycidyl ether (BPADGE) (analytical standard), bisphenol AF (BPAF) (analytical standard), bisphenol F (BPF) (analytical standard), bisphenol E (BPE) (analytical standard), bisphenol B (BPB) (analytical standard),bisphenol S (BPS) (analytical standard), 4-nonylphenol (4-NP) (analytical standard), acetamiprid (ACE) (analytical standard) and thiametoxam (analytical standard) were purchased from Sigma-Aldrich (Stenheim, Germany). Cetylstearyl alcohol (50% Cetyl alcohol and 50% Stearyl alcohol) and stearic acid were obtained from Zenstore (Pagani, Italy).

3.2 Food samples

Real samples, namely: raw ham, tuna in olive oil and packed in glass jar, dried apples, dried tomatoes and dried blueberries, were purchased from local markets. 50g of each food sample have been weighted and then grounded by a high speed blender in an aluminium container for about one minute. The grounded samples were stored at -21°C when not used immediately.

3.3 Electrochemical studies

Electrochemical measurements were performed by using a PalmSens1 electrochemical analyser connected with a PSTrace software (Ver. 5.7) (AW Utrecht, The Netherlands). Cyclic voltammetry studies were performed using a conventional three-electrode cell with different combination of electrodes, see table 3.

Table 3. Electrodes used to perform cyclic voltammetries.

Electrode type	Material	Specifications
Working	Glassy carbon	Diameter 3.0 mm
Working	Gold	Diameter 2.0 mm
Working	Platinum	Diameter 2.0 mm
Refererence	Ag|AgCl	Saturated
Counter	Platinum	Wire

Ag|: silver/silver chloride.

In the cyclic voltammetry experiments several different mobile phases were used as the supporting electrolyte (SE), see table 4.

Table 4. Supporting electrolyte solutions used to perform cyclic voltammetries.

n	Mobile phase composition
1	ACN/Acetic acid 0.06% (20/80 V/V)
2	ACN/Acetic acid 0.06% (40/60 V/V)
3	ACN/Acetic acid 0.06% (50/50 V/V)
4	ACN/Acetic acid 0.06% (60/40 V/V)
5	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)

n: mobile phase number; ACN: acetonitrile.

Standard solutions at 5 mM have been prepared by dissolving a known amount of analyte powder directly in the supporting electrolyte solution. Prior to use, the electrode surfaces were polished with alumina powder, sonicated in ultrapure water for 10 min, and cycled 10 times in the relevant supporting electrolyte.

Flow analysis have been performed by using an apparatus consisting of a Minipuls 3 peristaltic pump (Gilson, Villiers Le Bel, France), a six-way low pressure injection valve (Rheodyne mod. 5020, Cotati, CA, USA) with a 110 µL injection loop, and a conventional thin-layer electrochemical cell (EG&G Princeton Applied Research, Princeton NJ, USA) with a dual GCE working electrode (3 mm diameter, 1 mm electrode gap), and a thin-layer flow cell gasket of 255 mm thickness. All potentials were referred to a saturated Ag|AgCl reference electrode. The carrier supporting electrolyte consisted of ACN/Acetic acid 0.06% 40/60 V/V. Acquisition and data processing were performed by PalmSens1 electrochemical analyser connected with a PSTrace software (Ver. 5.7) (AW Utrecht, The Netherlands) by using the Multiple Pulse Amperometry Detection (MPAD) and the DC chronoamperometry techniques. Standard solutions at 5 mg L^{-1} have been prepared by dissolving a known amount of analyte powder directly in pure acetonitrile.

3.4 High performance liquid chromatography (HPLC) with fluorescence diode-array detector (FL-DAD)

Chromatographic analyses were performed by a HPLC consisted of a degasser system with nitrogen, a binary pump and a fluorescence detector (Agilent-1100 Series, Palo Alto, CA, USA), equipped with an Eclipse XDB-C18 (4.6 × 150 mm, 5 µm particle size) analytical column (Agilent, United States). Signals were recorded by a ChemStation computer software (Agilent, Palo Alto, CA, USA). HPLC separations were performed by a two-step program, using the mobile phase A consisting of water/acetonitrile 65/35 V/V and the mobile

phase B composed of water/acetonitrile 50/50 V/V. The mobile phase A was run for 7 minutes and then the mobile phase was step to B and run for the following 20 minutes. Hence, the mobile phase returned to A for an equilibration time of 20 minutes before the successive injection. The excitation wavelength and the emission wavelength were respectively 230 nm and 312 nm. The flow rate was 1 mL/min. BPs standard mix solutions were prepared at concentrations of 2000, 100, 75, 50, 25, 10, 5, 2, 1 $\mu g \cdot kg^{-1}$.

3.5 Solvents and solutions used in the preliminary experiments

In HPLC-FD experiments standard solutions at 125µM have been prepared by dissolving 125 µL of a solution 1 mM of 4 BPs (BPA, BPAF, BPADGE, 4-NP) in 1000µL of a solution 60/40 ACN/Acetate buffer at pH 6.00 ± 0.02 V/V. The solutions of four three (BPA, BPAF, BPADGE) at 70 µg L^{-1} in NADES1 have been prepared by dissolving 10 µL of a solution 20 mg L^{-1} of the three bisphenols in 1500µL of NADES1. The mixture has been placed in an ultrasound bath at 60°C for 20 minutes. Then it has been diluted 1:1 V:V with a solution of 80/20 MeOH/H2O V/V. The solutions of three bisphenols (BPA, BPAF, BPADGE) at 0.1µM have been prepared by dissolving 50 µL of a solution 1 µM of the three bisphenols in 500µL of pure ACN. The solution at 70 µg L^{-1} in ACN/H_2O of five bisphenols (BPF, BPE, BPA, BPB, BPAF, BPADGE) have been prepared by dissolving 10 µL of a solution of the five bisphenols at 20 mg L^{-1} in 1500µL of 55/45 ACN/H_2O V/V. Then 500µL of the obtained solution have been diluted 1:1 V:V with pure water. The mixtures have been filtered with PTFE-filters. The solution of four bisphenols (BPF, BPA, BPB, BPAF) at 10 µg L^{-1} has been prepared by dissolving 10 µL of a solution 20 mg L^{-1} in 2000µL of 50/50 H_2O/MeOH V/V. The solution has been filtered with PTFE-filters.

In the selection of the most appropriate filtration approach the solutions of three bisphenols (BPA, BPAF, BPADGE) at 65 µg L^{-1} have been prepared by dissolving 500µL of a solution of the three bisphenols at 130 µg L^{-1} in 500 µL of ACN/Acetate buffer 60/40 V/V at pH 6.00 ± 0.02 or in 500 µL of pure water.

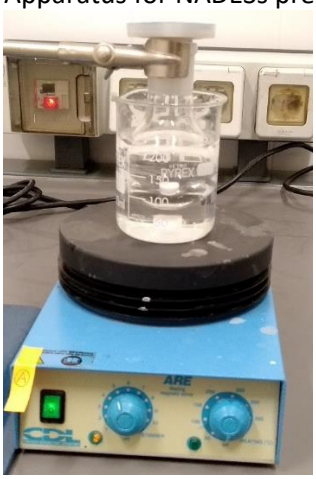
In the selection of NADESs for the development of the extraction method standard solutions of five bisphenols (BPF, BPE, BPA, BPB, BPAF) at 70 µg L^{-1} have been prepared by dissolving 10 µL of a solution the five bisphenols at 20 mg L^{-1} in 3000µL of pure ACN. Blank solutions of NADES3 have been prepared by adding 500µL of a solution made by 80/20 MeOH/ H_2O V/V to 500µL of NADES3. The solutions of three bisphenols (BPA, BPAF, BPADGE) at 0.07 mg L^{-1} have been prepared by adding 10 µL of a solution 20 mg L^{-1} of the three bisphenols to 1500µL of NADES1. The mixture has been placed in an ultrasound bath at 60°C for 20 minutes. Then it has been diluted 1:1 V:V with solutions

of (i) 50/50 MeOH/H_2O V/V, (ii) 60/40 MeOH/H_2O V/V, (iii) 70/30 MeOH/H_2O V/V; (iv) 80/20 MeOH/H_2O V/V, (v) pure MeOH. After the dilution, the solutions have been filtered with PTFE-filters.

3.6 NADES preparation and application to solid food samples extraction

Know amount of NADES components were weighted in a flask and heated in a water bath at 70°C. (figure 1). Each mixture was continuously shaken with a stirring bar. FECs extraction from food samples by a NADES based protocol was carried out as follow: 0.4 mg of each grounded food sample were weighted directly in a polypropylene (PP) centrifuge tube. Food samples were spiked at 10 µg kg^{-1} by adding 10 µL of a solution of four BPs (BPF, BPA, BPB, BPAF) at 2 mg L^{-1}. Then 1000µL of NADES1 (Glucose : Choline Chloride 1:1 m:m with 9% of water) preheated at 50°C were added to the sample. The mixture was mixed with a vortex for 25 s, and then placed in an ultrasound bath at 60° for 20 minutes. Afterwards, 1000µL of pure methanol were added and the mixture was mixed for 25 s by a vortex, centrifuged at 5000 rpm for 5 minutes, and then filtered with 0.22µm polytetrafluoroethylene filters.

Figure 1. Apparatus for NADESs preparation.



3.7 Performances of NADES based extraction method

Method performance was evaluated by determining limit of detection (LOD), limit of quantification (LOQ), specificity, repeatability and recovery on three replicates for each food sample.

3.8 MEPS protocol for clean-up of NADES extracts

MEPS was obtained from SGE Analytical Science (Milton Keynes, UK). MEPS barrel insert and needle assembly (BIN) volume was 8 µL (silica-C18, particle size of 45 µm and pore size of 60 Å) and the syringe volume was of 100 µL. The MEPS device was used with an

eVol® autosampler that was employed for the clean-up of the sample extracts and was operated in the "draw-eject" mode where the liquid sample is pulled and pushed through the syringe N-times at a pumping rate in the order of $\mu L \cdot s^{-1}$. At the first use, the MEPS was conditioned by flushing methanol (200 µL) 2 times and then ultrapure water (200 µL) 2 times at a speed of 2 (selected by the autosampler and corresponding to about 7 $\mu L \cdot s^{-1}$). 250 µL of the diluted DES extract were processed 60 times at a speed of 2. Then, the MEPS barrel was washed with water (250 µL 10 times at a speed rate of 2), afterward the analytes adsorbed into the MEPS were eluted flushing 30 times 250 µL of methanol through the syringe at a speed rate of 2; this final solution was analysed after filtration trough polytetrafluoroethylene filters of 0.22 µm. Finally, MEPS syringe was washed with methanol (250 µL 10 times at a speed rate of 2) and conditioned with water (250 µL 10 times at a speed rate of 2). The latter steps eliminated any memory effects and operated as a conditioning step before the successive extraction, respectively. When not in use the C18 bin was stored in a methanol:water (60:40 v/v) solution. The whole microextraction procedure, which lasts about 56 min, was conducted at room temperature and it is summarized in table 5.

Table 5. NADES extract clean-up by MEPS protocol

STEP (N. Type)	**Sample**	**Sample volume** (µL)	**Number of cycles**	**Speed value on eVol® / Estimated flow rate** (N. / $\mu L \cdot s^{-1}$)	**Estimated time** (min)
1. Conditioning	Water	250	10	2 / 7.2	4.63
2. Extraction of analytes	Sample extract	250	60	2 / 7.2	27.77
3. Washing	Water	250	10	2 / 7.2	4.63
4. Elution of analytes	Methanol	250	30	2 / 7.2	13.88
5. Washing	Methanol	250	10	2 / 7.2	4.63

4. RESULTS AND DISCUSSION

4.1 Electrochemical studies on some food emerging contaminants (FECs)

The electrochemical behaviour of bisphenol A (BPA), bisphenol AF (BPAF), bisphenol S (BPS), bisphenol A diglycidyl ether (BPADGE), 4-nonylphenol (4-NP), acetamiprid (ACE), and thiametoxam (TMX) has been assessed by cyclic voltammetry, using three different working electrode materials, five different supporting electrolyte solutions and four potential ranges (table 6).

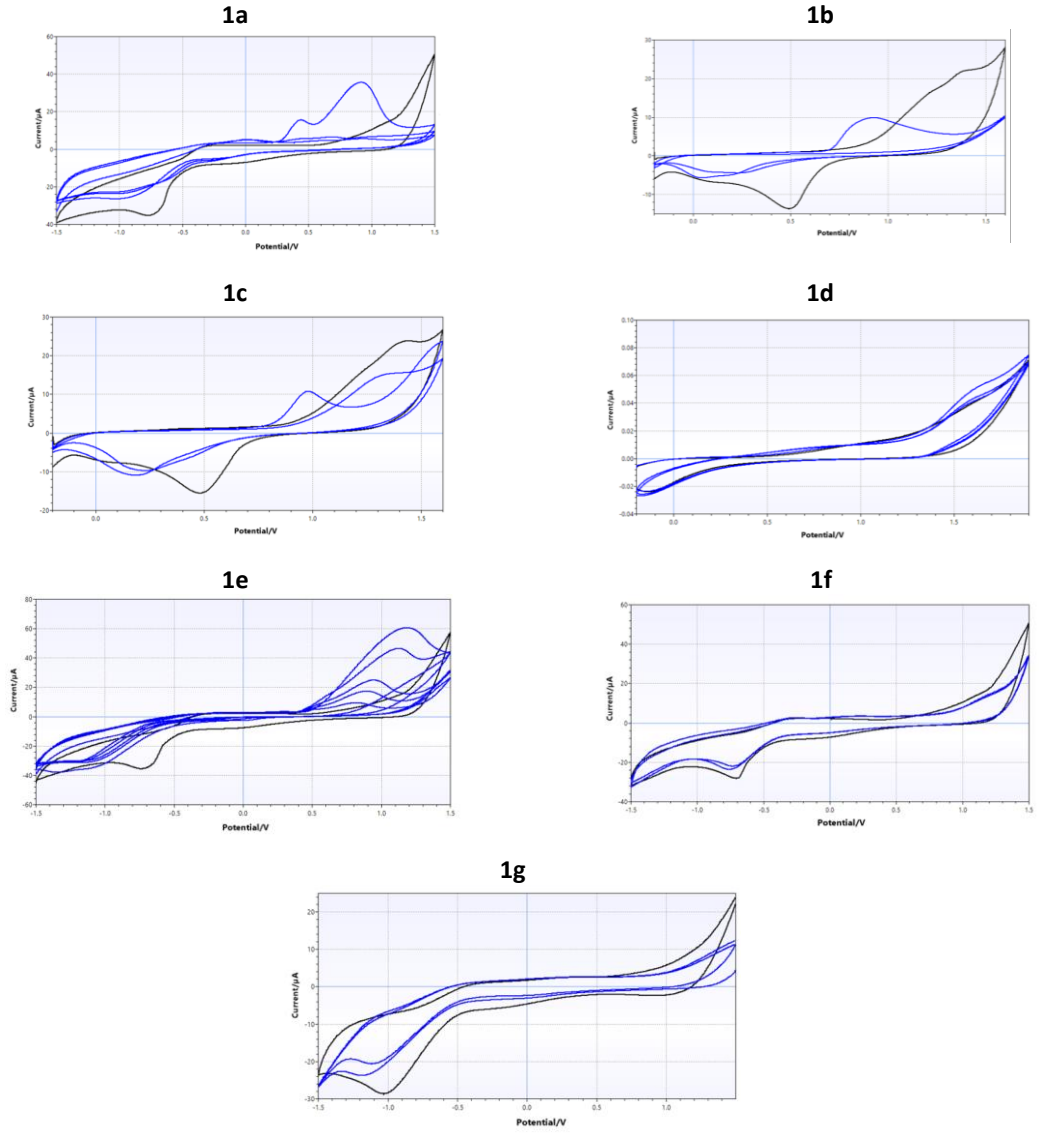
Table 6. Cyclic voltammetry experiments on some food emerging contaminants (FECs) at 5 mM.

n	FECs	Supporting electrolite solution	WE	PR (V)
1	BPA	ACN/Acetic acid 0.06% (40/60 V/V)	GCE	From -1.5 to 1.5
2	BPA	ACN/Acetic acid 0.06% (40/60 V/V)	GCE	From 0.0 to 1.5
3	BPA	ACN/Acetic acid 0.06% (20/80 V/V)	GCE	From -1.5 to 1.5
4	BPA	ACN/Acetic acid 0.06% (20/80 V/V)	GCE	From 0.0 to 1.5
5	BPA	ACN/Acetic acid 0.06% (60/40V/V)	GCE	From -1.5 to 1.5
6	BPAF	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Au	From -0.2 to 1.6
7	BPAF	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Pt	From -0.2 to 1.6
8	BPS	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Au	From -0.2 to 1.6
9	BPS	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Pt	From -0.2 to 1.6
10	BPADGE	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Au	From -0.2 to 1.6
11	BPADGE	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Pt	From -0.2 to 1.9
12	BPADGE	ACN/Acetic acid 0.06% (60/40 V/V)	GCE	From -1.5 to 1.5
13	4-NP	ACN/Acetic acid 0.06% (60/40 V/V)	GCE	From -1.5 to 1.5
14	4-NP	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Au	From -0.2 to 1.6
15	4-NP	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Pt	From -0.2 to 1.6
16	ACE	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Au	From -0.2 to 1.6
17	ACE	ACN/Acetic acid 0.06% (50/50 V/V)	GCE	From 0.0 to 1.5
18	ACE	ACN/Acetic acid 0.06% (50/50 V/V)	GCE	From -1.5 to 1.5
19	ACE	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Pt	From -0.2 to 1.6
20	TMX	ACN/Acetic acid 0.06% (50/50 V/V)	GCE	From -1.5 to 1.5
21	TMX	ACN/Acetic acid 0.06% (50/50 V/V)	GCE	From 0.0 to 1.5
22	TMX	ACN/Acetate buffer pH 6.00 ± 0.02 (30/70 V/V)	Au	From -0.2 to 1.6

WE: working electrode; PR: potential range; BPA: bisphenol A, BPAF: bisphenol AF; BPS: bisphenol S; BPADGE: bisphenol a diglycidyl ether; 4-NP: 4-nonylphenol; ACE: acetamiprid: TMX: thiametoxam; GCE: glassy carbon electrode; Au: gold electrode; Pt: platinum electrode.

The supporting electrolyte solutions have been selected considering that BPs, NNs and APs can be properly separated in reverse phase chromatography using a mix of acetonitrile and water or acidified water (Grumetto et al. 2013; Fattore et al. 2015; Staniszewska et al. 2014; Campillo et al. 2013; Rodríguez-Cabo et al. 2016). Furthermore, the percentage of the two components have been chosen considering the mobile phase composition needed to elute the analytes in the above-mentioned chromatographic separations. The current-potential (i-E) curves corresponding to some cyclic voltammetries of FCEs are shown in figure 2.

Figure 2. Cyclic voltammograms of bisphenol A (1a), bisphenol AF (1b), bisphenol S (1c), bisphenol a diglycidyl ether (1d), 4-nonylphenol (1e), acetamiprid (1f), thiametoxam (1g) at 5 mM.



Blue lines correspond to analyte response, 1st and 2nd cycle (1b, 1c, 1f, 1g), 1st, 2nd and 3rd cycle (1a, 1d), 1st, 2nd, 3rd, 4th and 5th cycle (1e). Black lines correspond to blank solution of supporting electrolyte: ACN/Acetic acid 0.06% 60/40 V/V (1a, 1e); ACN/Acetic acid 0.06% 50/50 V/V (1f, 1g); ACN/Acetate buffer (pH 6.00 ± 0.02) 30/70 V/V (1b, 1c, 1d). Cyclic voltammetry studies were performed using a 3.0 mm glassy carbon (1a, 1e, 1f, 1g), a 2.0 mm gold (1b, 1c), and a 2.0 mm platinum (1d) working electrode. Scan rate: 0.1 V/s

The results of the cyclic voltammetry experiments allowed gathering several information. Acetamiprid and thiametoxam did not show an electrochemical response regardless of the working electrode, the supporting electrolyte and the potential range. Instead, measurable responses have been detected in the first cycle for the other FECs. Nevertheless, apart from all the different conditions, it has been observed a marked signal decrease in the subsequent cycles (table 7).

Table 7. Decrease of current intensity of some food emerging contaminants (FECs) in cyclic voltammetry experiments.

n	FECs	WE	PR	R (%)	C
1	BPA	GCE	W	100	III
2	BPA	GCE	N	100	II
3	BPA	GCE	W	100	III
4	BPA	GCE	N	100	II
5	BPA	GCE	W	100	III
6	BPAF	Au	W	100	II
7	BPAF	Pt	W	100	II
8	BPS	Au	W	100	II
9	BPS	Pt	W	100	II
10	BPADGE	Au	W	57	V
11	BPADGE	Pt	W	93	III
12	BPADGE	GCE	W	n.r.	-
13	4-NP	GCE	W	93	V
14	4-NP	Au	W	63	V
15	4-NP	Pt	W	n.r.	-

n: number referring to table 6; WE: working electrode; PR: potential range; R: reduction; C: voltammetric cycle to which the reduction is referred; BPA: bisphenol A; BPAF: bisphenol AF; BPS: bisphenol S; BPADGE: bisphenol a diglycidyl ether; 4-NP: 4-nonylphenol; GCE: glassy carbon electrode; Au: gold electrode; Pt: platinum electrode; W: wide; N: narrow; n.r.: no response.

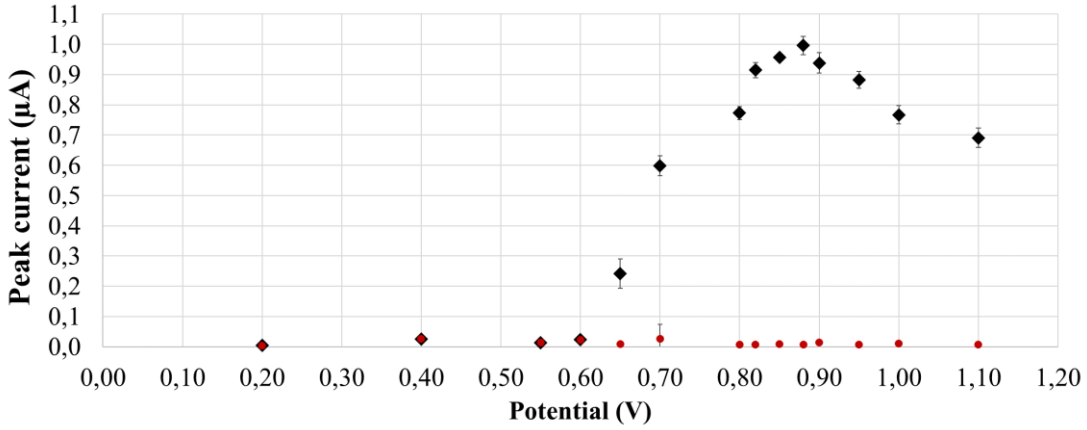
The remarkable current decrease suggests the occurrence of chemical reactions, which follow the oxidative electron transfer, with a consequent by-products production that cause the electrode fouling. It is therefore crucial to prevent the electrode fouling in order to perform repeated analyses, during detection after chromatographic separation. Typically, the electrode cleaning has been done by rubbing with alumina slurry or carrying out a pre-anodization at a fixed potential value (Kamau 1988). However, the just mentioned techniques are incompatible with the flow analysis since they have to be manually performed before every single injection. An alternative might be to perform an extensive electrochemical anodization of glassy carbon electrode. This might allow the

desorption/oxidation of the substances adsorbed onto the electrode surface as consequence of the formation of an oxygen-rich multilayer surface film (Nardiello et al. 2015). However, the application of an anodization might cause the formation of oxidized species on the electrode surface. Moreover, CV experiments shown that BPA, BPAF, BPS, BPADGE and 4-NP are oxidized in a potential region corresponding to a reduced electrode surface. This suggested that the electrode surface should be activated prior to apply the detection potential. This might be done by applying a reduction cathodic step to the electrode. Therefore, all the above-mentioned evidences suggested that the electrode surface should be firstly activated, than used for the detection and finally cleaned. These steps might be performed continuously by applying sequences of potentials, thus ensuring the compatibility with in-flow analysis (LaCourse 2011).

Sequences of potentials have been already successfully applied for a sensitive and reproducible determination of phenols, polyphenols and arylethanolaminic (Nardiello et al. 2015; Natale et al. 2015). Take into account the information gathered by cyclic voltammetric studies and the literature data, a waveform made by three steps of potential has been developed, namely detection, cleaning and reactivation by using the Multiple Pulse Amperometry Detection (MPAD) technique available on PSTrace software of the PalmSens1 potentiostat. The signal output is the current value sampled in the time window of the detection step. Each step is defined by a potential and a time value. Usually, the total time of the waveform should not exceed 1 s in order to guarantee a proper number of points sampled, and then a peak shape integrity. Several flow injection analysis have been performed in order to assess both the most adequate detection potential for BPA also taking into account the best signal-to-noise ratio. The best results have been obtained with a detection potential of +0.88 V (see figure 3).

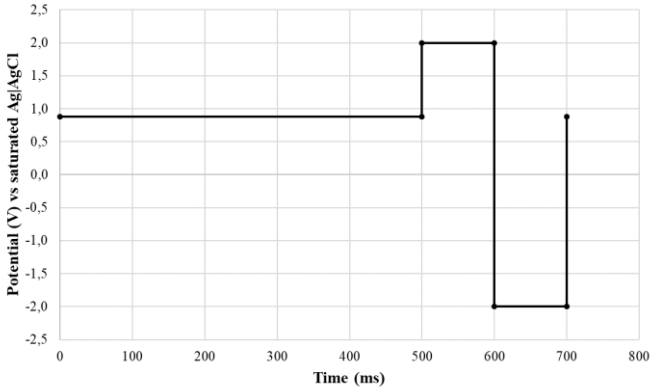
Once assessed the best detection potential, several experiments have been performed in order to assess the other waveform parameters, such as time and potential of the cleaning and the activating step. The optimized waveform has been composed by a detection step at + 0.8 V for 0.5 s, a cleaning step at + 2.0 V for 0.1s and an activating step at -2.0 V for 0.1s (see figure 4).

Figure 3. Optimization of the detection potential for bisphenol A (BPA) detection. Results refer to in flow injection analysis of BPA at 5 mg L^{-1}.



Black dots: mean of peak currents; red dots: mean of noise. The means were obtained by performing five repeated injections. Mobile phase: ACN/Acetic acid 0.06% 40/60 V/V. Flow rate: 0.5 ml min^{-1}. Injection volume: 110 µL.

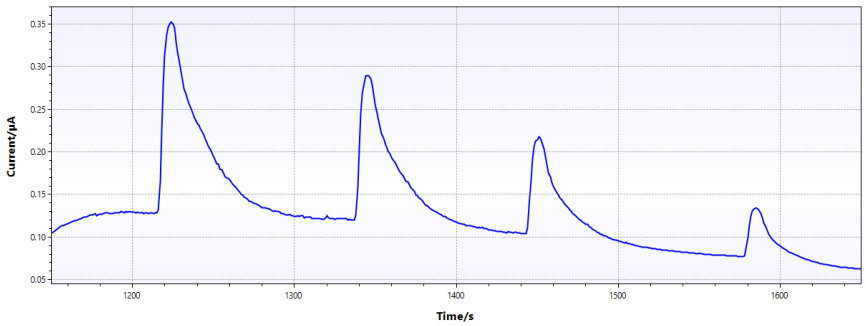
Figure 4. Optimized three-step potential waveform for the pulsed amperometric detection of BPA at a glassy carbon electrode.



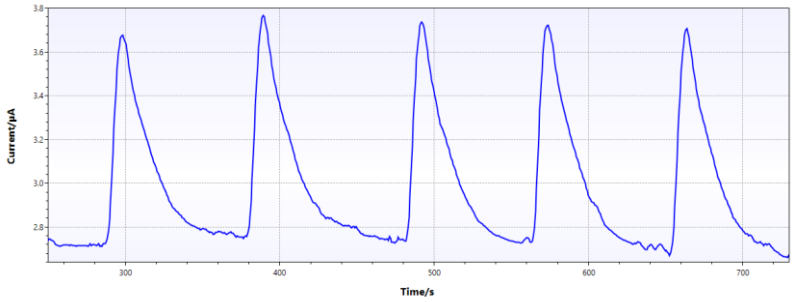
Subsequently, the optimized waveform has been applied in flow injection analysis for the determination of BPA. These results have been compared with detection at a constant potential of + 0.8 V (figure 5). Response sensitivity, reproducibility and stability have been greatly enhanced by using the optimized waveform to detect BPA. Peak heights recorded by applying the optimized waveform are pretty reproducible and stable in time. Conversely, currents shown in the detection at constant potential strongly decrease from the first to the last injection. The percentage decrease is equal to almost 74%. This demonstrates that the application of a proper potential waveform is essential to clean and reactivate the electrode surface in order to ensure a stable and reproducible response.

Figure 5. Flow injection responses for BPA 5 mg L^{-1}.

1a



1b



Mobile phase: ACN/Acetic acid 0.06% 40/60 V/V. Loop: 110 µL. Flow rate: 0.5 mL min^{-1}. 1a: detection at fixed potential of + 0.8 V; 1b: detection with the Multiple Pulse Amperometry optimized waveform.

4.2 NADESs preparation: theoretical and practical issues

NADESs have been prepared by using solid natural compounds in order to comply with the green chemistry principle, whose term "indicates the creation of chemical products and procedures that reduces the use and production of harmful materials" (Abdussalam-Mohammed et al. 2020). Usually two method were developed to prepare DESs, namely heating under stirring and freeze drying (Tang and Row 2013), and, in the present work has been used the first, whose key principle is to provide heat to NADESs components in order to promote their interaction, thus supporting the formation of the eutectic mixture. Ideally, the melted solution should be fluid and stable at room temperature (Zhang et al. 2012). However, melting the DESs components to obtain a colourless fluid may be tricky. Several combinations of compounds in different operating conditions have been tested in order to prepare NADESs. The natural compounds and their combinations have been chosen in order to obtain NADESs with different physical-chemical proprieties, suitable for the extraction of some FECs from solid foodstuffs, and particularly attempts have been made in order to prepare NADESs with different polarity properties. In table 8 are shown all the tested mixtures for the preparation of NADESs.

The performed tests have shown that the NADESs formation and the time needed for the mixture to become a eutectic solvent might depend on several factors, such as water content,

mass ratio, whole mass of the components, preliminary grounding and mixing steps of the components, stirring of the mixture and heating time.

Table 8. Mixture tested for the preparation of natural deep eutectic solvents (NADESs).

n°	Type	C_1	C_2	C_3	Water (%)	Mass ratio	Mass (g)	Grounding (Y/N)	Mixing (Y/N)	Time (min)	Formed (Y/N)	Remarks
1	P	Glu	ChCl		9	1:1	6	Y	Y	30	Y	
2	P	Glu	ChCl		0	1:1	6	Y	Y	60	n	
3	P	Glu	ChCl		9	1:1	6	Y	N	190	n	
4	P	Glu	ChCl		9	1:1	40	Y	Y	50	Y	
5	P	Glu	ChCl		9	1:1	40	N	Y	90	Y	
6	P	Glu	ChCl		9	1:1	40	N	Y	60	Y	
7	A	Can	Men		0	1:1	16	Y	Y	150	Y	
8	A	Can	Men		0	1:2	12	Y	Y	60	Y	Some crystals formed in the mixture after 12 h
9	A	Can	Men		0	1:1,5	7	Y	Y	10	Y	
10	A	Can	Men		0	1:1,5	17	Y	Y	30	Y	
11	A	Can	Men		0	1:1,5	30	N	Y	10	Y	
12	A	Can	Men		0	1:1,5	25	N	N	30	N	
13	P	Glu	Fru		20	1:2	40	Y	Y	40	Y	
14	P	Glu	Fru		9	1:1	6	Y	Y	80	Y	The mixture was not liquid after 12 hours
15	P	Xyl	Glu		10	1:1	6	Y	Y	135	Y	The mixture was not liquid after 12 hours
16	P	Xyl	Glu		20	1:1	6	Y	Y	135	Y	The mixture was not liquid after 12 hours
17	P	Xyl	Glu		30	1:1	6	Y	Y	135	Y	The mixture was not liquid after 12 hours
18	P	Xyl	Glu		40	1:1	6	Y	Y	135	Y	The mixture was not liquid after 12 hours
19	P	Xyl	ChCl		12	3:5	4	Y	Y	20	Y	The mixture become pink after 12 hours
20	A	Can	C-al	S-al	0	1:1:1	13	Y	Y	30	Y	The mixture was not liquid after 12 hours
21	A	S-ac	C-al	S-al	0	1:1	14	Y	Y	10	Y	The mixture was not liquid after 12 hours
22	M	Glu	C-al	S-al	0	1:1:1	15	Y	Y	50	N	
23	M	ChCl	S-ac		0	1:2	8	Y	Y	60	N	
24	M	ChCl	S-ac		0	1:3	8	Y	Y	60	N	

n°: mixture number; C_1: compound one, C_2: compound 2; C_3: compound 3; Y: yes; N: no, P: polar; A: apolar; M: mixed, apolar and polar; Glu: glucose, ChCl: choline chloride; Can: camphor; Men: menthol; Fru: Fructose; Xyl: xylitol; C-al: cetyl alcohol; S-al: stearyl alcohol; S-ac: stearic acid. All the mixture have been heated in a water bath at 70°C

The formation of the glucose-choline chloride based NADES seems to not occur without adding water to the mixture and without stirring. In addition, the time needed to prepare this NADES might depend on both performing a preliminary grounding and mixing step with a pestle in a ceramic mortar and on the total mass of the used compounds. For instance, to prepare 6g of NADES 30 minutes were needed, while the eutectic formation took 50 minutes to prepare 40g of NADES. Instead, the execution of the preliminary grounding and mixing step resulted in decreasing the time need to form NADES of ten minutes. This might depend on molecules start to interact in this preliminary phase due to the application of a mechanical action that may increase the energy provided to the system.

As regards the camphor-menthol based NADES several issues have been observed. The formation of a eutectic mixture, stable in the time, might be dependent on the mass ratio of components. All the tested mass ratios have led to the formation of the NADES, and the process took 150 minutes for a mass ratio of 1:1 and 60 minutes for a mass ratio of 1:2. Nevertheless, in the second case the NADES was not stable in the time. Also in this case, the formation of NADES seems to take more time for greater mass of components. Conversely, the time resulted drastically reduced if the grounding step was not performed. During the grounding step the mixture might absorb water vapour from air that might hinder the interaction between the apolar compounds. As concerns glucose-fructose based NADES, it seems to be faster to prepare and more stable in time if low percentage of water are added. Mixtures from 14 to 21 (see table 8) turns into eutectic mixtures but they are not stable in

the time, while those prepared with polar and apolar compounds never formed eutectics. An interesting observation has been done for the glucose-choline chloride based NADES: after 27 days at room temperature and in a closed flask the mixture of components became a NADES even in absence of added water. All these experiments showed how complex the preparation of NADESs can be and how many factors have to be considered. In addition, it seems that the same factor might act differently depending on the type of components used for NADES production. In 2022, Zhang et al. studied the preparation strategy and stability of deep eutectic solvents based on choline chloride-carboxylic acid (Zhang et al. 2022). Among all the studied factors, they observed an interesting effect of the molar ratio between the DES components on DESs formation.

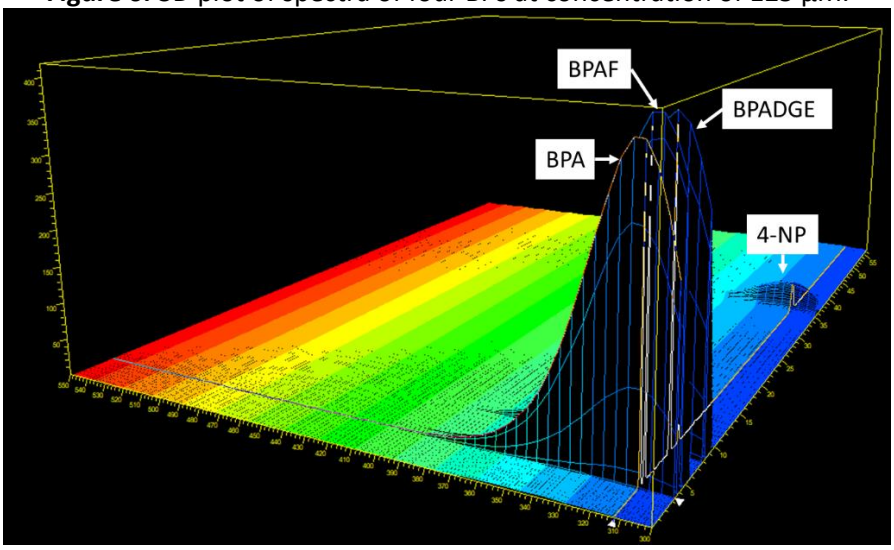
4.3 Development of an extraction method for solid food samples based on NADES

4.3.1 Preliminary experiments

4.3.1.1 Liquid chromatography with fluorescence detection (HPLC-FD)

Several experiments have been made in order to study the bisphenols behaviour in HPLC-FD. First, different chromatograms in multiemission and multiexitation mode have been performed in order to assess good values of excitation and emission wavelengths. The idea has been to find proper combinations of wavelengths in order to enhance the signals of the analytes and to reduce those of the interfering compounds. Figure 6 shows a 3D plot of spectra of four BPs (BPA, BPAF, BPADGE, 4-NP) at 125µM, separated by an isocratic elution with a 60/40 V/V ACN/Acetate buffer at pH 6.00 mobile phase.

Figure 6. 3D plot of spectra of four BPs at concentration of 125 µM.



Elution mode: isocratic. Mobile phase: ACN/Acetate buffer at pH 6.00 60/40 V/V. Flow rate: 1ml/min. Acquisition mode: multiemission (from 300 nm to 550 nm) and multiexcitation (from 230 nm to 500 nm). BPA: bisphenol A; BPAF: bisphenol AF; BPADGE: bisphenol a diglycidyl ether; 4-NP: 4-nonylphenol.

Eight mix of acetonitrile with acetate buffer (pH 6.00 ± 0.02) or pure water have been tested as mobile phases (table 9) for the bisphenols separation in HPLC-FD.

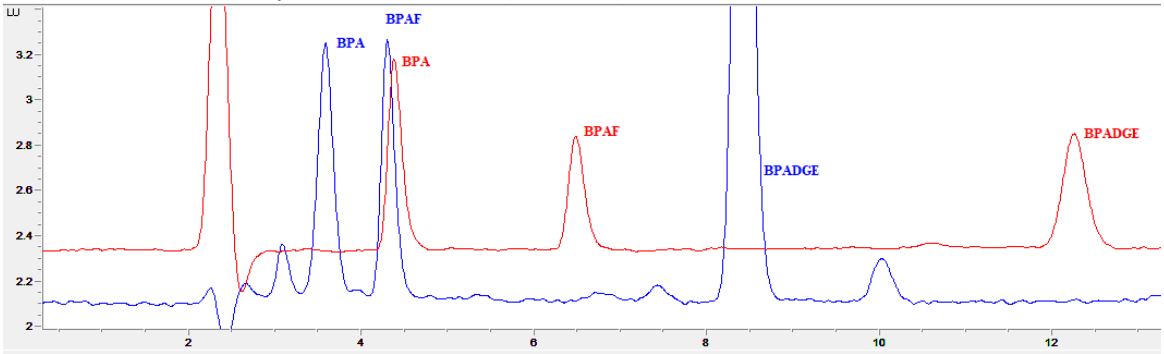
Table 9. Solution used as the mobile phases in HPLC-FD for the analysis of BPs.

Mobile phase components	V/V ratio
ACN/Acetate buffer (pH 6 ± 2)	55/45
ACN/Acetate buffer (pH 6 ± 2)	60/40
ACN/H_2O	35/65
ACN/ H_2O	40/60
ACN/ H_2O	45/55
ACN/ H_2O	50/50
ACN/ H_2O	55/45
MeOH/ H_2O	55/45

ACN: acetonitrile, MeOH: methanol.

Preliminary experiments have been performed by using mobile phases containing acetate buffer because it has been supposed that the pH might influence BPs separation and detection. Several experiments have been performed in order to study how different amounts of acetonitrile might affect the separation of BPs. As shown in figure 7, the dependence of the retention times on the polarity of mobile phase is very strong, and small additions of acetonitrile to the mobile phase cause an elution of the BPs too much close to the solvent front where the potential interfering matrix compounds can be eluted.

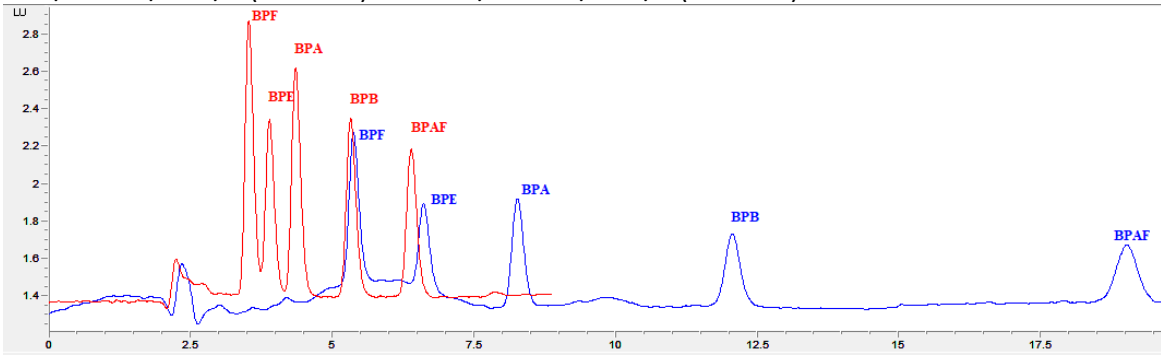
Figure 7. Chromatograms of BPs standard solutions at 70 µg/L (red line) in NADES1 and 0.1 µM in ACN (blue line). Mobile phases: ACN/Acetate buffer at pH 6.00 55/45 V/V (red line) and ACN/Acetate buffer at pH 6.00 60/40 V/V (blue line).



Elution mode: isocratic. Excitation wavelength: 230 nm. Emission wavelength: 312 nm. BPA: bisphenol A; BPAF: bisphenol AF; BPADGE: bisphenol A diglycidyl ether.

Good results in terms of separation have been also obtained without acetate buffer, with the same behaviour of the retention time dependence on the mobile phase polarity. Also in this case, the elution times of BPs decrease by the increase of the organic phase contained in the mobile phase (figure 8).

Figure 8. Chromatograms of BPs standard solutions at 70 µg/L in ACN/H_2O. Mobile phases: ACN/H_2O 40/50 V/V (blue line) and ACN/ H_2O 50/50 V/V (blue line).

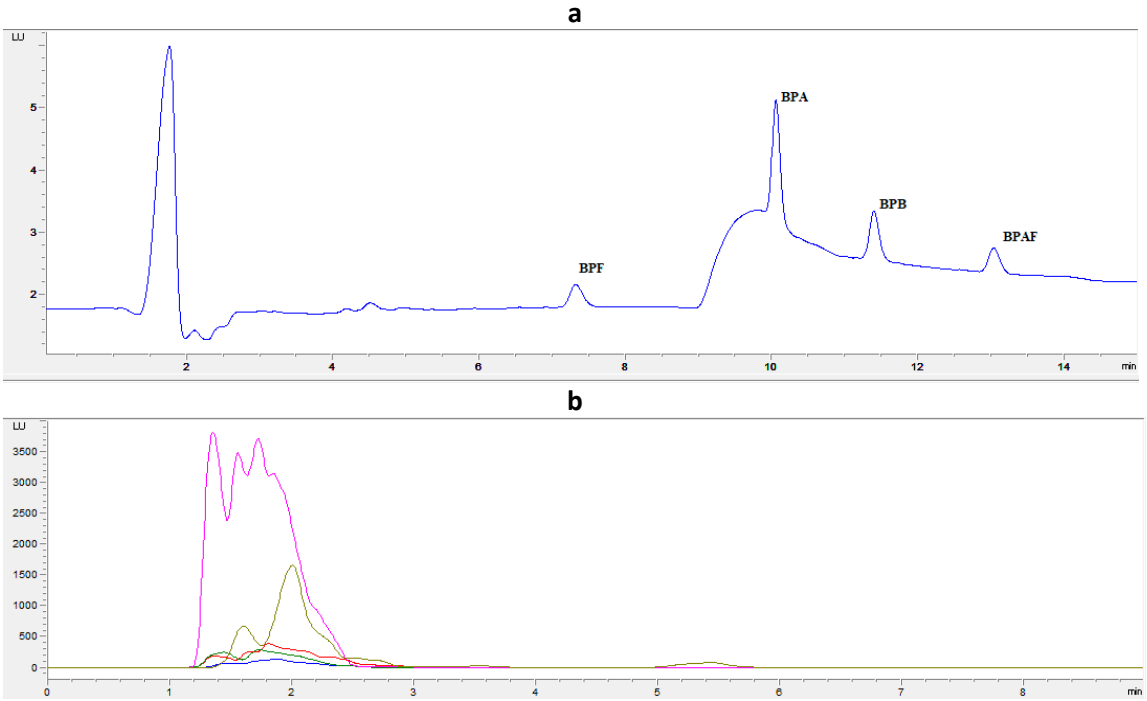


Elution mode: isocratic. Flow rate: 1mL/min. Excitation wavelength: 230 nm. Emission wavelength: 312 nm. BPF: bisphenol F; BPE: bisphenol E; BPA: bisphenol A; BPB: bisphenol B; BPAF: bisphenol AF; BPADGE: bisphenol a diglycidyl ether.

In chromatograms obtained by using as the mobile phase a solution composed by water and methanol, the peaks of the BPs had uneven shapes and there were a higher base line noise. Therefore, mobile phases made of acetonitrile and water have been chosen to separate the BPs. Several experiments have been performed in order to assess a good elution program for the analysis of the food extracts. It has been observed that real samples contain several polar interfering compounds. Thus, it has been supposed that BPs might be separated from the food interfering compounds by using a two step program with mobile phases of different polarity (amounts of acetonitrile). Good results have been obtained by using a program of a first step at ACN/Water 35/65 V/V for seven minutes and a second step at ACN/Water 50/50 V/V for twenty minutes. This program allowed to separate BPs from the food interfering compounds. Indeed, all the polar compounds have been eluted before the elution of the first bisphenol, namely bisphenol F (figure 9).

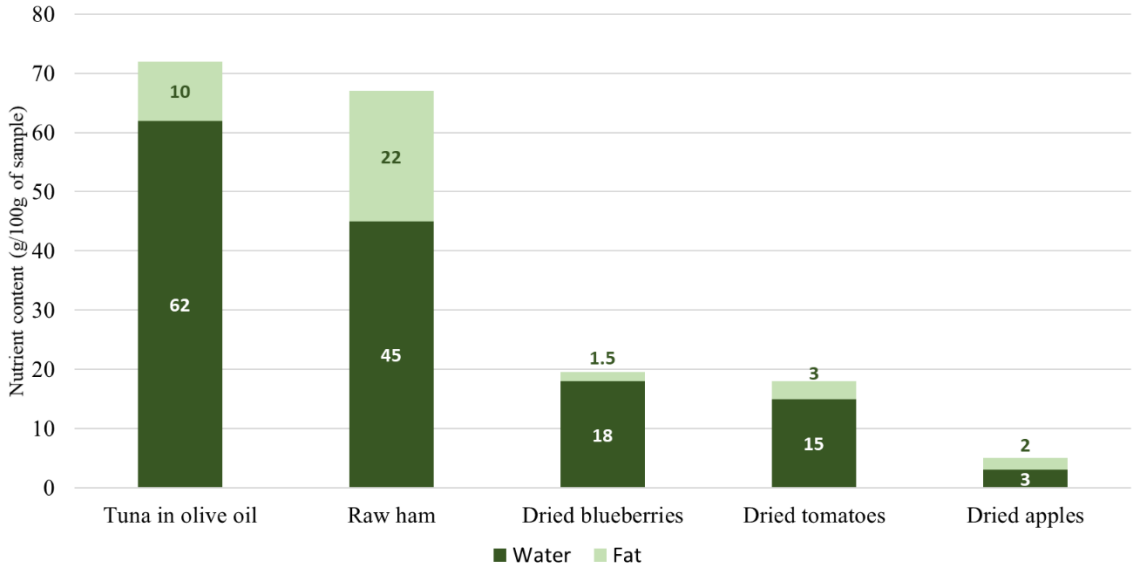
Blank samples of foodstuffs, namely dried blueberries (blue line), dried apples (red line), dried tomatoes (green line), raw ham (pink line) and tuna in olive oil (olive green), have been extracted by applying the developed method (see paragraph "3.6 NADES preparation and application to solid food samples extraction"). The selection of the foodstuffs has been based on several considerations. With regard to what discussed in the "introduction" paragraph, solid food samples usually packed in plastic packaging were selected. Moreover, since it has been supposed that water content in food may affect NADES1 stability, while fat may compete with BPs diffusion from food to NADES1, five solid foodstuffs of both animal and plant origin with a different water and fat content (obtained from the nutritional data available on food labels) have been selected (figure 10).

Figure 9. Chromatograms of a BPs standard solution at 10µg/L (a) and of blank samples of foodstuffs (b).



Elution program: mobile phase A (ACN/H_2O 35/65 V/V) for 7 minutes, mobile phase B (ACN/H_2O 50/50 V/V) for 20 minutes. Flow rate: 1 ml/min. Excitation wavelength: 230 nm. Emission wavelength: 312 nm. BPF: bisphenol F; BPA: bisphenol A; BPB: bisphenol B; BPAF: bisphenol AF.

Figure 10. Water and fat content in the selected foodstuffs.

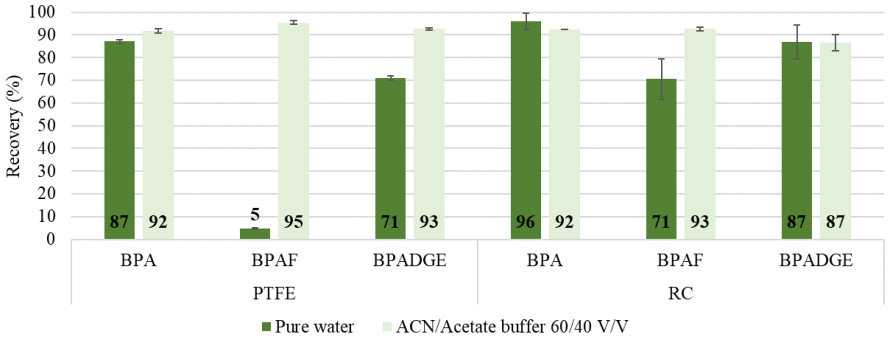


4.3.1.2 Selection of the most appropriate filtration approach

Several preliminary experiments showed that the recovery of the analytes after the filtration may depend on both the filter material and the solvent used. Therefore, different combination of filter materials and solvents have been tested. Three materials have been used in order to perform the experiments, namely nylon (N), regenerated cellulose (RC), and polytetrafluoroethylene (PTFE). The BPs were completely retained by the N-filters.

Recovery values for BPs after filtration with RC-filters and PTFE-filters are shown in figure 11.

Figure 11. Mean recoveries (n =3) of BPs standard solutions at 65 µg/L after filtration with PTFE-filters and RC-filters.



BPA: bisphenol A; BPAF: bisphenol AF; BPADGE: bisphenol A diglycidyl ether; PTFE: polytetrafluoroethylene; RC: regenerated cellulose.

Recovery values for BPs after the filtration step with PTFE-filters seem to increase by adding an organic solvent to the solution. As regards the filtration with RC-filters, recovery values seem to not depend univocally from the presence of an organic solvent in the solution to be filtered, but also on the analyte chemical-physical characteristics. Moreover, results obtained by using PTFE-filters were more reproducible than the ones obtained with RC-filters. Therefore, PTFE-filters have been selected for the development of the extraction method.

4.3.1.3 Selection of NADESs for the development of the extraction method

An exhaustive discussion on all the tests performed in order to prepare NADESs with different physical-chemical properties has been reported in the paragraph "4.2 NADESspreparation: theoretical and practical issues". Essentially, three NADESs, two polars and one apolar (see table 10), resulted suitable for the development of the extraction process of BPs from solid foodstuff real samples. NADES1 and NADES3 were prepared by weighted directly the components in a glass flask of 50 mL. Then these mixtures were directly placed in a water bath at 70°C under continuous mixing. Indeed, the components of the NADES2 were weighted and grounded with a pestle in a ceramic mortar and then placed in a water bath at 70°C under continuous mixing.

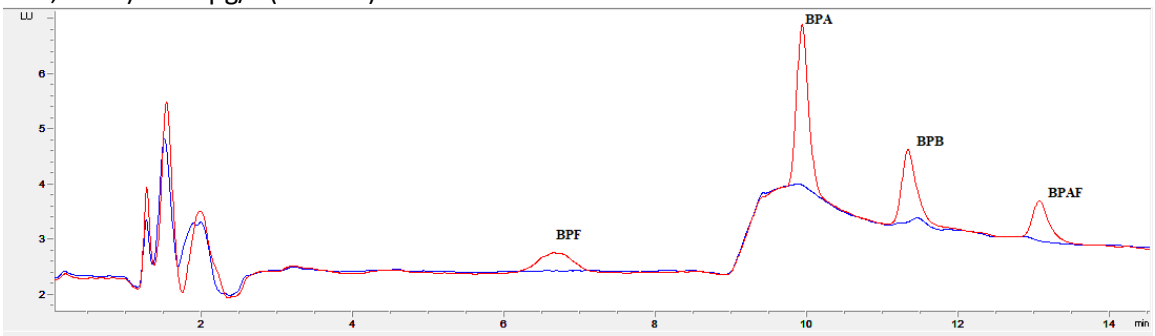
Table 10. Prepared NADESs.

	Type	C_1	C_2	Water (%)	Mass ratio
NADES1	P	Glu	ChCl	9	1:1
NADES2	P	Glu	Fru	20	1:2
NADES3	A	Can	Men	0	1:1.5

C_1: compound one, C_2: compound 2; P: polar; A: apolar; Glu: glucose, ChCl: choline chloride; Fru: Fructose; Can: camphor; Men: menthol.

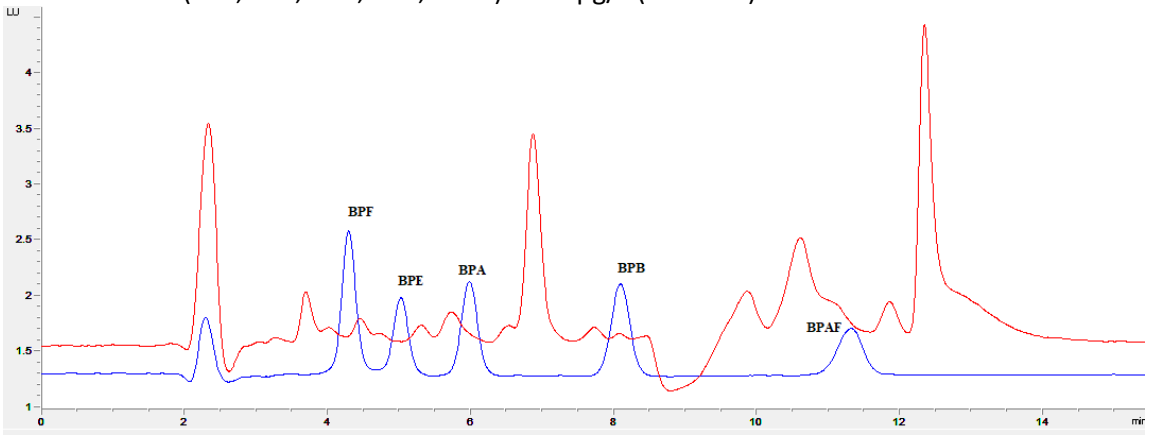
The selection of the NADESs to be used in the development of the extraction method has been done by considering several factors. For example, it was thought to select at least one polar and one apolar NADES. NADES3, consisting of camphor and menthol, was the only apolar NADES produced. Between the two polar NADESs, namely NADES1 and NADES2, the first has been chosen because a higher number of tests have been performed on its preparation and stability. Before using NADES1 and NADES3 for the development of the extraction method they have been tested by HLPC-FD in order to assess the absence of interfering compounds. NADES1 resulted to be free from interfering compound (see figure 12). Solutions of bisphenols and blanck NADES1 used to record the chromatograms in figure 1 have been obtained by applying the developed extraction method. Conversely, NADES3 has shown several peaks that may interfere with BPs detection (see figure 13). In consideration of the observations made, NADES1 has been selected to develop the extraction method.

Figure 12. Chromatograms of the NADES1 (blue line) and of a standard mix of four BPs (BPF, BPA, BPB, BPAF) at 10 µg/L (red line).



Elution program gradient step: mobile phase A (ACN/H_2O 35/65 V/V) for 7 minutes, mobile phase B (ACN/H_2O 50/50 V/V) for 20 minutes. Flow rate: 1 mL/min. Excitation wavelength: 230 nm. Emission wavelength: 312 nm. BPF: bisphenol F, BPA: bisphenol A, BPB: bisphenol B; BPAF: bisphenol AF.

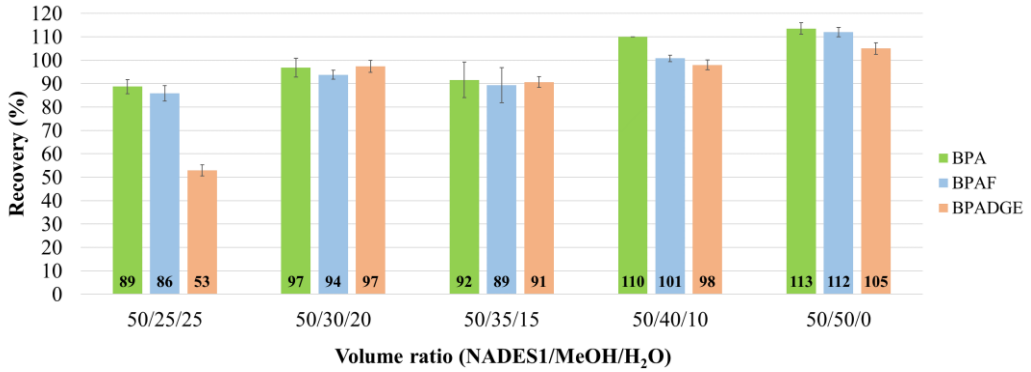
Figure 13. Chromatograms of NADES3 diluted 1:1 with MeOH/ H2O 80/20 V/V (red line) and of a mix of five BPs (BPF, BPE, BPA, BPB, BPAF) at 70 µg/L (blue line).



Mobile phase: ACN/H_2O 44/55 V/V. Flow rate: 1ml/min. Excitation wavelength: 230 nm. Emission wavelength: 312 nm. BPF: bisphenol F, BPE: bisphenol E, BPA: bisphenol A, BPB: bisphenol B; BPAF: bisphenol AF.

From previous tests, it has been highlighted the need to use an organic solvent in order to improve the performance of the filtration with PTFE-filters. Therefore, several experiments have been carried out in order to study how the filtration may influence the recovery of analytes in NADES1 diluted with organic solvent/water mix. Methanol was chosen as the organic phase because previous experiments showed that NADES1 possess a good mixability with this organic solvent. In fact, when NADES1 is mixed with ACN a phase separation occurs. In figure 14 are displayed the mean recoveries of BPs solubilised in NADES1 after a 1:1 dilution in different methanol/water mix and filtration by PTFE-filters. Good results have been obtained when NADES1 was diluted with solutions containing at least 60% of methanol. However, pure methanol has been selected to dilute NADES1 because it ensures good recovery values, a good mixability with NADES1 and a good reproducibility. Undiluted NADES1 has not been tested owing to its high viscosity at room temperature that makes the filtration a hard task.

Figure 14. Mean recoveries (n =3) of BPs at 0.07 mg L^{-1} in NADES1 after dilution and filtration with PTFE-filters.



Elution mode: isocratic. Mobile phase: ACN/Acetate buffer at pH 6.00 55/65 V/V. Flow rate: 1mL/min. Excitation wavelength: 230 nm. Emission wavelength: 312 nm. BPA: bisphenol A; BPAF: bisphenol AF; BPADGE: bisphenol A diglycidyl ether.

4.3.2 Key factors that may influence the extraction process

The proposed extraction method has been developed through the investigation of several theoretical, technical and practical issues. The aim has been the development of a green easy, quick, flexible and effective extraction procedure. In this context, on the basis of the physical-chemical characteristics of NADES, such as the viscosity, and occurrence of BPs in plastic material, particular attention has been devoted to both materials and operations commonly used in the extraction processes.

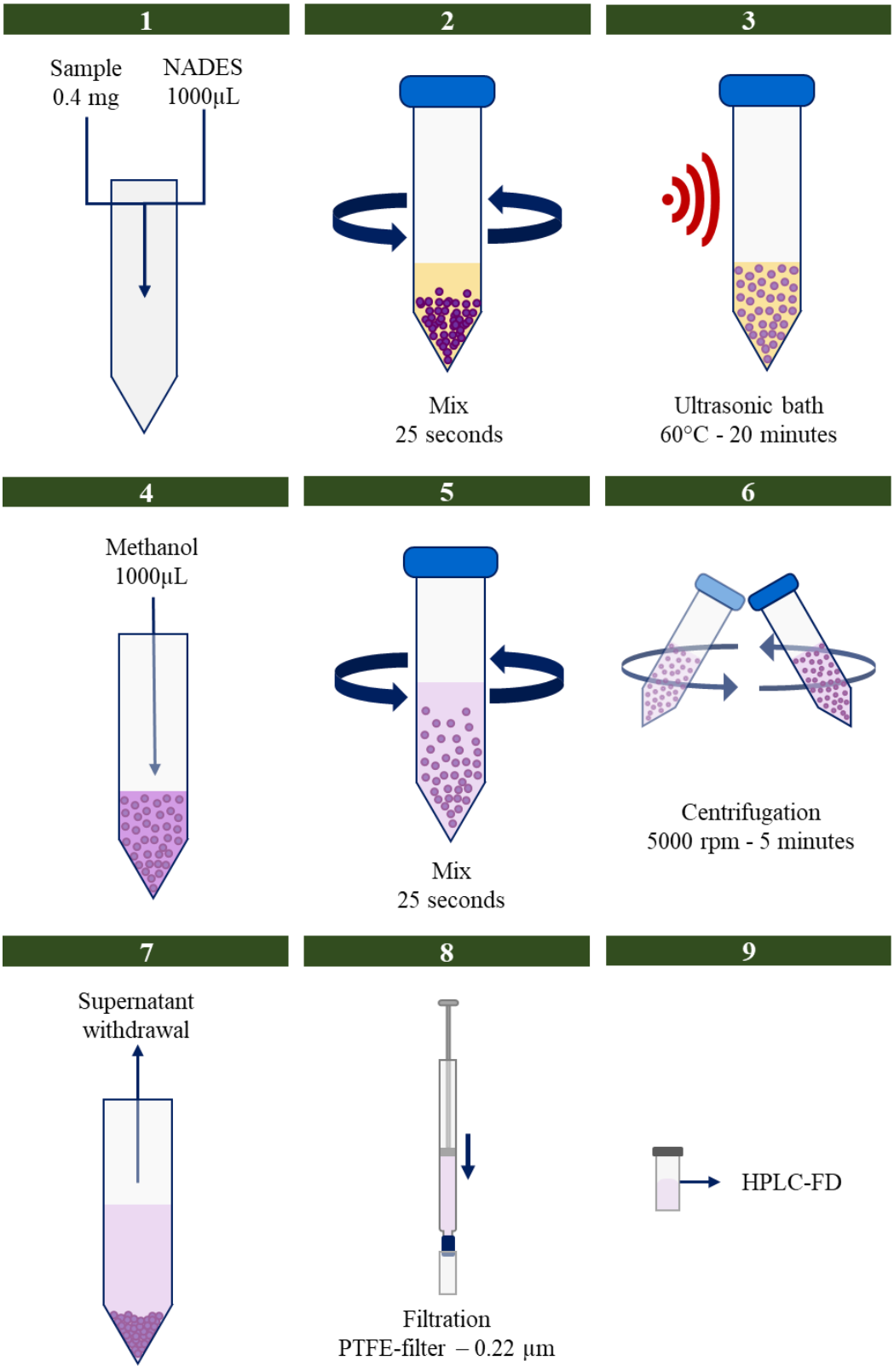
As above-mentioned the viscosity of NADES may cause several problems in handling and extraction procedures, as withdrawal and displacement by pipettes, mixing with solid samples, extraction of analytes (two-phase partitioning), centrifugation and final filtration

prior to analysis. The viscosity problem has been overcame by warming up the selected NADES1 at 50°C before its addition to solid food samples, and by using an ultrasound bath at 70°C to combine the action of acoustic waves and heath to improve the BPs extraction. Concerning the withdrawal and displacement of NADES1, in preliminary experiments graduated glass pipettes were used in order to avoid possible cross-contamination by BPs used in some plastic materials. However, this method was difficult to be carried out and the withdrawal resulted not reproducible owing to the high viscosity. Consequently, positive displacement pipettes with plastic tips have been used in order to perform an easy and accurate withdrawal of NADES1. Also for the extraction procedure and the following centrifugation, an appropriate selection of the most centrifuge tube materials has been done by considering several factors, as thermal and organic solvent stability and mechanical stability to the centrifugation speeds required. Polypropylene (PP) has been selected because it offers better physical properties in terms of resistance to mechanical or temperature gradients stresses. Before their use in the development of the extraction method PP tubes and pipette tips have been tested in order to assess the absence of interfering compounds (including BPs), by using blank samples submitted to the extraction process and to stressful conditions, and analyzed by HPLC-FLD.

The handling of NADES1 extract after the extraction process resulted to be difficult. Due to its high viscosity, both the centrifugation and the filtration steps resulted to be ineffective. Specifically, regardless of the speed and the time of the centrifugation the solid phase was not separated from the liquid one, thus making impossible or complicate the liquid withdrawal. In addition, when NADES1 was filtered, the filter tended to clog up very fast, thus making the filtration impossible. Consequently, it has been supposed that the addition of a dilution solvent may reduce the NADES1 viscosity thus improving the centrifugation and the filtration. The composition of the diluting solvent has been selected by considering both the preliminary experiments on PTFE-filters and the mixability with NADES1 (see paragraph “4.3.1.3 Selection of NADESs for the development of the extraction method”). The volume of the diluting solvent has been selected in order to reduce NADES1 viscosity, but keeping the dilution factor as low as possible. Several tests have been performed with volumes of pure methanol ranging from 300µL to 1000µL, and the best compromise has been reached by 1000µL of pure methanol added directly in the PP centrifuge tubes and by mixing the solution for about 25 seconds with a vortex before the centrifugation and filtration with PTFE-filters.

The developed protocol is shown in figure 15. The amount of solid sample / volume of NADES1 ratio (0.4g / 1 mL) has been selected taking into account the need of reducing the dilution factor and to ensure a good mixability with the solid food samples.

Figure 15. Developed extraction protocol for the extraction of bisphenols from solid food samples.



4.3.3 Method performances

Once the extraction method has been developed in consideration of all the theoretical, technical and practical issues, the main performance parameters (Commission Decision 2002/657/EC, Regulation 2017/625/EEA) have been evaluated in order to test method validity in generating reliable results. The limit of detection (LOD), the limit of quantification (LOQ) and the specificity have been assessed in order to study the performance of the chromatographic separation with fluorescence detection. Moreover, the performance of the extraction method have been evaluated by estimating the repeatability and the recovery.

4.3.3.1 Limits of detection (LOD) and quantification (LOQ)

LOD and LOQ have been determined by using the signal-to-noise ratio (S/N) method (Desimoni and Brunetti 2015). Firstly, the peak-to-peak noise around the retention time of the analytes has been measured by analysing the chromatograms of spiked food samples after the extraction process. Afterwards, LOD and LOQ values have been calculated by considering the values of concentration corresponding to a response equal to a S/N of three for LOD and of ten for LOQ. The estimated values for real food samples are shown in table 11. LOD and LOQ were in the range 0.18 – 1.6 µg/L and 0.61 – 5.3 µg/L, respectively. In particular LOD and LOQ values determined for BPA resulted well below the established specific migration limits of 50µg·kg^{-1} (Commission Regulation 2018/213/EU), also taking into account the whole dilution factor of 5 introduced by the proposed extraction/clean-up method.

Table 11. Means and standard deviations (n = 3) of limits of detection (LOD) and quantification (LOQ) for BPs at 10 µg kg^{-1} in real matrices.

	BPF	BPA	BPB	BPAF
			LOD ± s (µg L^{-1})	
Dried blueberries	0.62 ± 0.08	0.36 ± 0.04	0.32 ± 0.06	0.73 ± 0.09
Dried tomatoes	1.2 ± 0.04	0.18 ± 0.05	0.56 ± 0.06	0.42 ± 0.09
Raw ham	-	0.89 ± 0.04	1.4 ± 0.07	1.6 ± 0.03
Tuna in oil	1.1 ± 0.07	0.73 ± 0.08	1.0 ± 0.09	0.34 ± 0.04
			LOQ ± s (µg L^{-1})	
Dried blueberries	2.1 ± 0.27	1.2 ± 0.12	1.1 ± 0.20	2.4 ± 0.29
Dried tomatoes	4.2 ± 0.13	0.61 ± 0.17	1.9 ± 0.19	1.4 ± 0.31
Raw ham	-	3.0 ± 0.14	4.8 ± 0.24	5.3 ± 0.09
Tuna in oil	3.6 ± 0.25	2.4 ± 0.28	3.5 ± 0.31	1.1 ± 0.13

BPF: bisphenol F, BPA: bisphenol A, BPB: bisphenol B; BPAF: bisphenol AF, LOD: limit of detection; LOQ: limit of quantification; s : standard deviation

4.3.3.2 Specificity

For analytical methods, the power of discrimination between analytes and interfering compounds is crucial. The evaluation of method specificity can be done by analysing blank samples of foods (Araujo 2009). For real samples of dried blueberries, dried tomatoes and tuna in oil no interfering compounds have been detected in the retention time window of analytes. In raw ham samples it has been assessed some issues in the detection of BPF and BPB (see figure 16 and 17). BPF could not be determined. Instead, BPB signals in extract samples has been estimated by using a peaks deconvolution with a Gauss function of the OriginPro 2022 SR1 9.9.0.225 software (see figure 18).

Figure 16. Chromatograms of a standard solution of bisphenol F (BPF) at 10 µg/L in NADES1 (red line) and an extract of raw ham spiked at 10 µg/kg (blu line).

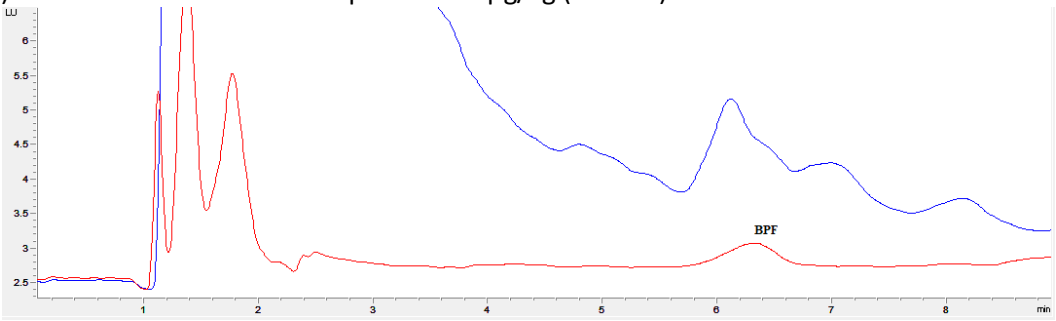
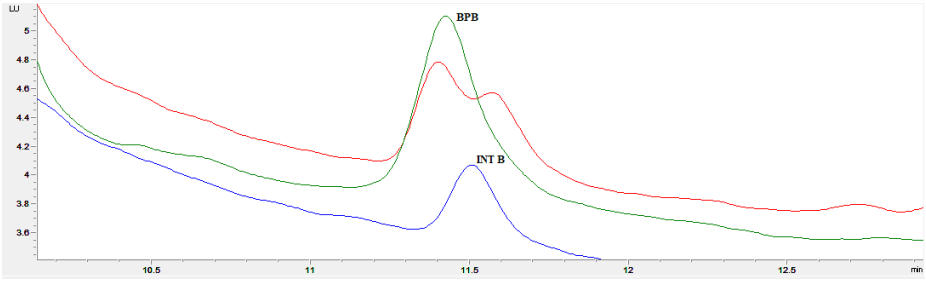
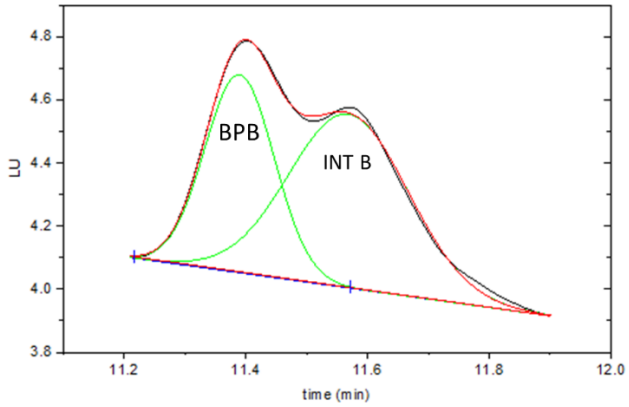


Figure 17. Chromatograms of a standard solution of bisphenol B (BPB) at 10 µg/L in NADES1 (green line), extract of raw ham spiked at 10 µg/kg (red line) and extract of blank raw ham (blue line).



INTB: interfering compound for bisphenol B.

Figure 18. Deconvolution of bisphenol B in the chromatogram of a raw ham extract.

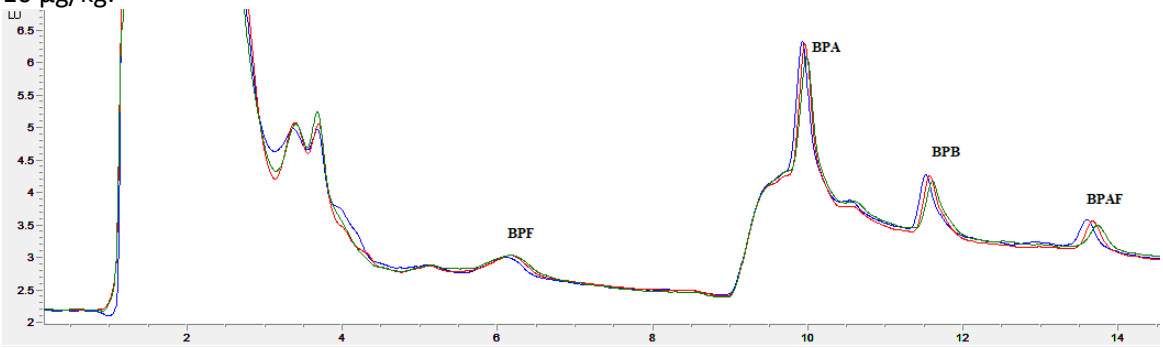


INTB: interfering compound for bisphenol B.

4.3.3.3 Repeatability

Independent test results have been obtained under repeatability conditions, that is with the same method on identical test items in the same laboratory by the same operator using the same equipment. Repeated chromatograms were obtained for all the spiked foodstuff samples, and in figure 19 chromatograms referred to three replicates of extracted spiked dried tomatoes at 10 µg kg^{-1} are given as an example.

Figure 19. Three replicates of chromatograms obtained for extracted dried tomatoes spiked at 10 µg/kg.



BPF: bisphenol F, BPA: bisphenol A, BPB: bisphenol B; BPAF: bisphenol AF.

Repeatability results have been provided as values of the coefficient of variation (CV) (see table 12).

Table 12. Coefficients of variation (CV) referred to foodstuffs spiked with bisphenols at 10 µg kg and processed with the developed extraction method.

	CV_{BPF} (%)	CV_{BPA} (%)	CV_{BPB} (%)	CV_{BPAF} (%)
Dried blueberries	6.74	4.56	6.26	1.91
Dried tomatoes	2.09	8.25	7.90	11.56
Raw ham	-	8.18	6.82	8.30
Tuna in oil	14.70	2.59	1.77	7.94

The coefficients of variation have been estimated by performing three replicates for each food sample.
BPF: bisphenol F, BPA: bisphenol A, BPB: bisphenol B; BPAF: bisphenol AF.

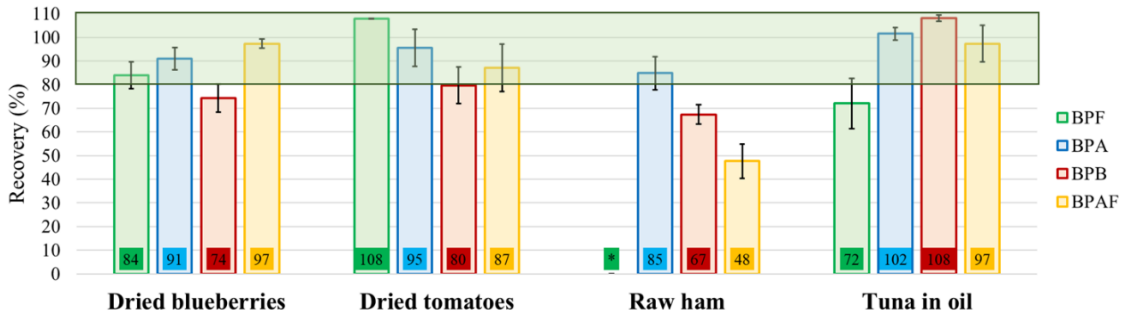
Samples have been spiked at 10 µg/kg, thus the reference value of CV for reproducibility is 22% (Côté et al. 2012). However, under repeatability conditions the reference value might be equal at most to two thirds of the above value of 22% (Commission Decision 2002/657/EC). Therefore, 15% has been considered the reference value for repeatability. As it can be seen in table 12, all the CVs are below the reference value of 15%.

4.3.3.4 Recovery

Recovery means the percentage of the true concentration of a substance recovered during the analytical procedure. According to Commission Decision 657 (Commission Decision 2002/657/EC), recovery values should be in the range 80-110% for mass fraction ≥ 10 µg

kg^{-1}. Recovery values are shown in figure 20. BPA recoveries resulted in the abovementioned range for all the food samples . BPF, BPB, BPAF recovery values have been found to be included in the 80-110% range, or slightly below for all the food samples except for raw ham samples. This might be due to the complexity of the raw ham samples and their high fat content.

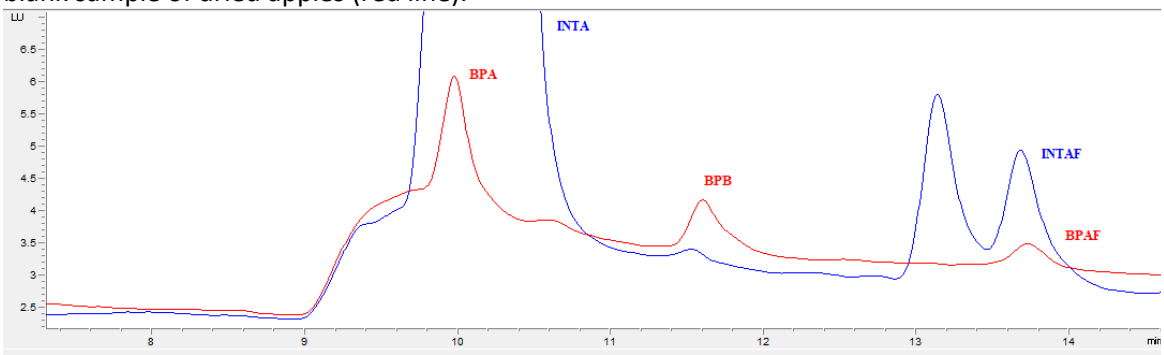
Figure 20. Recovery values for extracts of food samples spiked at 10µg/kg.



Recovery values have been estimated by performing three replicates for each food sample. The error lines correspond to the standard deviation of these three replicates. *: BPF cannot be detected in raw ham due to the presence of interfering compounds. BPF: bisphenol F; BPA: bisphenol A; BPB: bisphenol B; BPAF: bisphenol AF.

The performances on dried apple samples have not been reported because the samples did not seem to be free from BPs as shown in figure 21. Indeed, the samples may be contaminated with BPA and BPAF during the manufacturing.

Figure 21. Chromatograms of a standard mix of BPs at 10 µg L^{-1} in NADES1 (blu line) and of a blank sample of dried apples (red line).



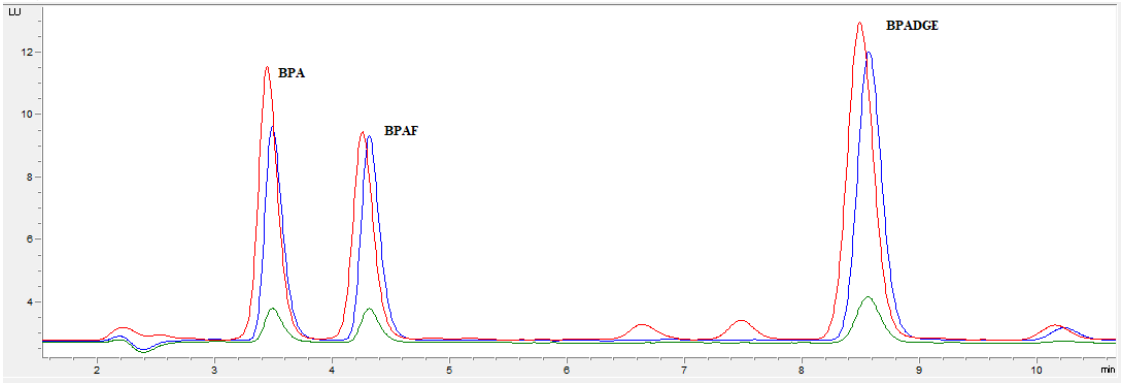
BPF: bisphenol F; BPA: bisphenol A; BPB: bisphenol B; BPAF: bisphenol AF; INTA: compound that interfere with BPA; INTAF: compound that interfere with BPAF.

4.4 Microextraction by packed sorbent (MEPS) for the clean-up of BPs NADES extracts

In view of using mass spectrometry for the detection of some FECs it might be essential to clean-up NADESs extracts of food samples . A microextraction by packed sorbent (MEPS) based protocol has been developed to clean-up NADESs extracts of food samples containing bisphenols with the main target of removing the NADESs components that can be deleterious for the mass spectrometry instrumentations, such as ion sources and analyzers.

The protocol has been developed by considering several practical, technical and theoretical issues. Prior to use MEPS for sample containing BPS the device has been cleaned with methanol and then conditioned with water. The first step has been the withdrawal of the sample solution. Several factors might influence the effectiveness of analytes retention in the MEPS cartridge, such as volume of sample solution, analytes concentration, times of syringe suction and speed of the withdrawing. Several tests have been performed on standard solution in acetonitrile, methanol and glucose-choline chloride NADES in order to assess a good combination of these variables. The barrel speed in withdrawing may be chosen in consideration of several factors, for example, NADESs viscosity, residence time of the sample solution in the silica-C18 cartridge and need for reducing time. Subsequently, in view of using the protocol for the clean-up of food samples in NADESs, a washing step made with water have been performed in order to eliminate all the NADESs residues and the sample polar interfering compounds. Pure water has been selected as washing solvent in consideration of both the apolar characteristic of the cartridge and the analyte, and the polar peculiarity of glucose-choline chloride based NADES. Several tests have been performed to assess an effective number of time and speed of the withdrawal. Subsequently, the analytes retained in the cartridge have to be released. This might be done by flushing an organic solvent. Pure acetonitrile, pure methanol, acetonitrile with 0.01% of formic acid and methanol with 0.01% of formic acid have been used as eluting solvents. The type of solvents and its volume might influence method performance. The elution solvent typology might influence the effectiveness of the release with the same times and velocity of the withdrawal. The elution solvent volume might be essential in view of carry out an increase of BPs final concentration, thus ensuring an effective instrumental detection. In order to test the effectiveness of the elution step in term of recovery, a second step with pure methanol has been performed. For example, in figure 22 are shown chromatograms of standard solution of BPS in water prior to MEPS extraction and after the first and the second elution in pure acetonitrile. The first step of elution resulted to be effective. The mean of the BPs recovered after the first step of the elution has been of 89% respect to the total recovered BPSs at the end of the second elution.

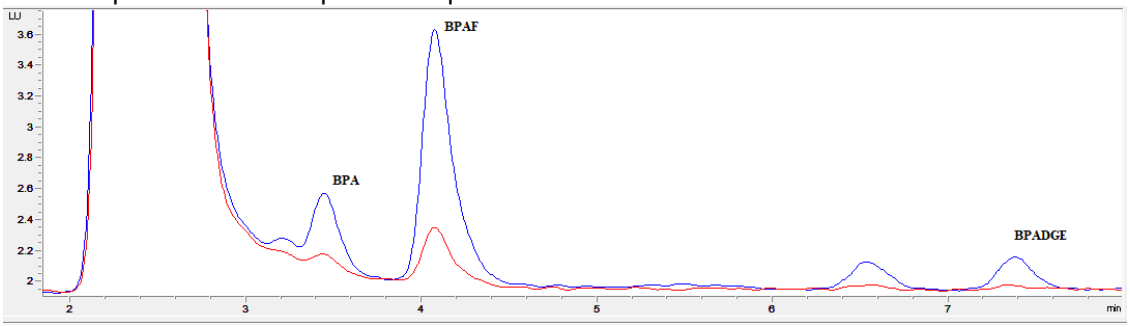
Figure 22. Chromatograms of BPSs standard solution at 1 µM (blu line) in water, BPs in acetonitrile after the first elution (red line) and BPs in acetonitrile after the second elution (green line).



The sample volume was of 500µL. The elution have been made with 250µL of pure ACN. MEPS protocol has been: standard solution withdrawal 60 times, washing with water 5 times, first elution 30 times, second elution 30 times. Mobile phase: 60/40 ACN/Acetate buffer at pH 6.00 ± 0.02. Flow rate: 1 mL/min. Excitation wavelength: 230 nm. Emission wavelength: 312 nm. BPA: bisphenol A, BPAF: bisphenol AF, BPADGE: bisphenol A diglycidyl ether.

Depending on different conditions, the first elution with methanol has provided recovery values ranging among about 50% to 93%. In consideration of all the experiments a MEPS protocol has been developed as described in "3.8 MEPS protocol for clean-up of NADES extracts". The developed method have been tested for the clean-up of BPSs food extracts in glucose-choline chloride based NADES (figure 23). The MEPS clean-up resulted to be quite effective. No interfering peaks have been detected near the retention time of the target analytes. The first elution allows the recovery of almost all the analytes respect to the total recovered also considering the second elution.

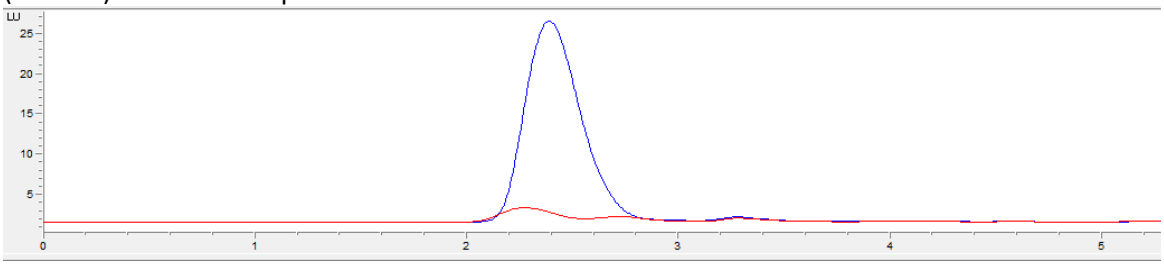
Figure 23. Chromatograms of turkey breast extracts in glucose-choline chloride based NADES cleaned-up with the developed MEPS protocol.



First (blue line) and second (red line) elution in pure methanol. Turkey breast has been spiked at 1µM with a mix of three bisphenols. Mobile phase: 60/40 ACN/Acetate buffer at pH 6.00 ± 0.02. Flow rate: 1 mL/min. Excitation wavelength: 230 nm. Emission wavelength: 312 nm. BPA: bisphenol A, BPAF: bisphenol AF, BPADGE: bisphenol A diglycidyl ether.

The developed protocol has been found to be successful also in the removal of the NADES1 components, as shown in figure 24, where are compared chromatograms of a NADES1 water solution injected before and after the MEPS clean-up.

Figure 24. Chromatograms of NADES1 diluted with water (1:1 V/V) before (blue line) and after (red line) MEPS clean-up.



Mobile phase: 60/40 ACN/Acetate buffer at pH 6.00 ± 0.02. Flow rate: 1 mL/min. Excitation wavelength: 230 nm. Emission wavelength: 312 nm.

5. CONCLUSIONS

1 Electrochemical studies

Part of the research work has been focused on the electrochemical characterization of bisphenols, alkylphenols and neonicotinoids. These experiments have been performed by using different combination of electrode materials, electrolyte solutions and applied potentials. Several observations might be done by considering the performed electrochemical studies. It has been found that the acetamiprid and the thiametoxam cannot be detected by applying an electrochemical detection method. This behaviour has been the same regardless of the working electrode used, namely gold, platinum and glassy carbon and of the applied potential range. Conversely, bisphenol A, bisphenols AF, bisphenol S and bisphenol A diglycidyl ether have been effectively detected by performing cyclic voltammetry (CV) experiments. On the basis of the CV features a multiple pulsed amperometry detection (MPAD) waveform has been developed and applied to bisphenol A detection. The developed MPAD waveform allowed a significant improvement of the response sensitivity, reproducibility and stability, with respect to the bisphenol A detection at constant potential. Furthermore, the experiments have been performed successfully by using electrolyte solutions (made by mixtures of water and organic solvents) that are typical mobile phases used in LC separation of bisphenols, alkylphenols and neonicotinoids. This gives the opportunity to couple with LC the electrochemical detection based on the MPAD waveform to perform the multianalyte determination in real samples of bisphenols.

The developed technique shown several advantages compared to the most used electrochemical detection techniques presented in the introduction paragraph. Specifically, it does not require time-consuming electrode pre-treatments, the use of biological elements and could be used to detect on-line more than one BPs at time.

5.2 Natural deep eutectic solvents (NADESs) preparation

Several tests have been carried out to study how it might be possible to prepare and eutectic mixture by starting from natural compounds. It has been observed that the formation of the NADES might depend on several factors, such as the whole mass of the starting components, the amount of added water, the presence of a preliminary grounding and mixing step, the mass ratio among the NADES components, and the mechanical continuous mixing during the preparation. In addition it has been observed that these factors may have different effects depending on the NADES type.

Moreover, it has been observed also that some NADES even if formed resulted unstable after about 12 hours from the preparation, and NADES prepared by mixing polar and apolar compounds never formed. In conclusion, it has been observed that NADES formation might be complex and depending on the nature of the components used for the preparation. To the best of our knowledge, very few works focus on the study of the experimental variables that may influence the NADESs formation and their stability.

5.3 NADES1 based extraction method

NADESs were already applied for the extraction of BPs from samples including the food ones. However, except for the research made by Noori and Ghanemi in 2019 (Noori and Ghanemi 2019), whose drawbacks have been highlighted in the introduction paragraph, NADESs have been applied only to liquid food samples. In the present work, an easy, quick and reproducible protocol was successfully applied to five different solid food matrices, namely dried tomatoes, dried blueberries, dried apples, raw ham and tuna in oil to extract BPs. Several theoretical, technical and practical issues were carefully investigated. Each step was studied in order to obtain both an effective extraction of bisphenols from solid food samples and a whole compatibility of the extract with the direct injection in HPLC-UV or HPLC-FD. For instance, it was observed that might be crucial the choice of the centrifuge tube material, the volume of NADES to be added to the samples, the type and the volume of the diluting solvent and the material of filters. The correct selection of such variables allowed the obtainment of very good performance parameters for the extraction of bisphenols. The limits of detection and quantification were all below 5.5 µg L^{-1}. In particular, the values determined for BPA resulted well below the established specific migration limits of 50µg·kg^{-1} (European Commission 2018, 213), also taking into account the whole extraction/clean-up dilution factor of 5. The extraction process showed also a good repeatability and recovery, with recovery values for bisphenol A in all the food samples included in range 80-110%. For the other bisphenols, the recoveries were also in the 80-110% range or slightly below except for raw ham samples.

Finally the advantages of the proposed extraction protocol based on NADESs can be summarized as follows:

- the protocol is effective in extracting BPs from solid food samples, requiring a sample amount very low compared to the one needed in applying traditional extraction techniques;

- the protocol represents a green alternative to the traditional extraction/clean-up methods that require large volume of organic solvents;
- is the protocol is flexible, since by properly changing some factors, such as the NADES type, it can be applied to extract other food emerging contaminants (e.g. NNs) from solid food samples.

5.4 MEPS protocol for the clean-up of NADES extracts

The extracts obtained by applying the developed NADES based extraction protocol have been demonstrated to be compatible with HPLC-UV and HPLC-FD systems. However, the presence of NADES1 components in the extracts could make them incompatible with the MS detection. Thus, a MEPS protocol was developed to overcome this problem. The proposed MEPS protocol was effective in purifying the bisphenols from interfering compounds contained in food samples and in particular from the NADES1 components that can affect the detection in MS. It was observed that several factors, such as the volume of sample solution, analyte concentration, times of syringe suction, speed of the withdrawing and the eluting solvent are crucial to develop an effective MEPS protocol. The proposed MEPS protocol might be a good starting point in order to develop and optimized method aimed for example to reduce the total time needed to perform the process.

5.6 Future prospects

The information gained by both performing the electrochemical characterization of some FECs and by applying the developed MPAD waveform, might be crucial in order to develop an effective MPAD to detect BPs after LC separation.

The studies on both NADESs preparation and application of NADESs to extract BPs from solid food samples is the starting point to develop new green and validated NADESs based extraction methods for other FCSs from different solid food matrices.

Moreover, it would be very interesting to test in MS experiments the extracts obtained by applying the developed NADES1 based protocol followed by the MEPS purification, in order to evaluate if MEPS is effective in removing NADES components from the extracts, thus ensuring a full compatibility with the MS detection, and then to extend the applicability of the NADEs based extraction protocol developed in the present PhD work.

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