Cheila Vanessa da Silva Diogo Fonseca Pereira

ASSESSMENT OF SIX PHTHALATES AND ONE ADIPATE ON BEER SAMPLES

Master in Quality Control

Water and Food

Work supervised by Prof. Doctor José de Oliveira Fernandes and Doctor Sara Cristina da Silva Cunha

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Cheila Vanessa da Silva Diogo Fonseca Pereira

DETERMINAÇÃO DE SEIS FTALATOS E UM ADIPATO EM AMOSTRAS DE CERVEJA

Mestrado em Controlo de Qualidade

Água e alimentos

Orientação feita pelo Prof. Doutor José de Oliveira Fernandes e Doutora Sara Cristina da Silva Cunha

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Abstract

The use of plastics has a greatly increase in the last decades leading to a rising exposure to the toxic effect of its components. Phthalates and adipates are added to plastic to increase its plasticity; because these compounds are not chemically bonded with the plastic leaching and possible migration to the environment are likely. These compounds have documented harmful health effects, mainly endocrine disrupting effects in humans.

Food contaminants can be of very different natures, such as biological, chemical, or physical, and although food packaging has the purpose to protect food products, in itself, can also be a source of contamination. Besides packaging, food contamination with phthalates may result from other sources such as production and storage equipment's, handling, and atmosphere. Beer is one of the most consumed beverages in the world, and due to their production process and types of packaging, can be a source of phthalate exposure in humans. The aim of this study was to optimise and validate a method based on dispersive liquid-liquid microextraction (DLLME) coupled to gas chromatography with mass spectrometry (GC-MS) analysis for the simultaneous determination of six phthalates and one adipate in commercial beer samples. The developed method had good linearity (r^2>0.96), low limits of detection (0.3-1.5 µg/L) and quantification (1-5 µg/L) and good intraday (<12%), except DMP (<20%), and interday (<13%) precision. The matrix suppression effects observed were surpassed by the use of matrix-matched calibration curves. The application of the method on commercial beer samples demonstrated the presence of five out of six phthalates, and of the only adipate studied, with levels ranging from 1.77 to 205.40 µg/L. The presence of the target analytes was correlated with the alcohol content, and varied with the type of packaging and with the production origin, with higher levels detected in alcoholic samples, packed in aluminium cans from industrial production.

Keywords: beer, phthalates, DLLME, GC-MS

Resumo

O uso de plásticos tem aumentado nas últimas décadas, levando a uma maior exposição aos efeitos tóxicos dos seus componentes. Ftalatos e adipatos são compostos adicionados ao plástico para aumentar a sua plasticidade, no entanto, por não se encontrarem ligados covalentemente ao plástico, estes compostos podem libertar-se e migrar para o ambiente. Os efeitos tóxicos destes compostos para a saúde humana já estão documentados, especialmente os efeitos de desregulação endócrina.

Os contaminantes alimentares podem ser de diferentes tipos, biológicos, químicos ou físicos, e, apesar das embalagens alimentares terem o propósito de proteger os alimentos desses mesmos contaminantes, podem, também, ser fonte de contaminação. Os ftalatos podem contaminar os alimentos por diversas vias, para além do embalamento, como o equipamento usado no processo de produção, armazenamento, manuseamento e via ambiental. A cerveja é uma das bebidas mais consumidas no mundo, e devido ao seu processamento e embalamento, podem ser uma fonte de exposição a ftalatos. O objetivo deste trabalho foi otimizar e validar um método baseado em microextração dispersiva líquido-líquido (DLLME) acoplado a uma análise por cromatografia gasosa com espectrometria de massa (GC-MS) para determinar simultaneamente seis ftalatos e um adipato em amostras de cerveja. O método apresentou boa linearidade ($r^2 > 0.96$), baixos limites de deteção (0.3-1.5 µg/L) e de quantificação (1-5 µg/L) e baixos coeficientes de variação (intraday <12%, exceto DMP (<20%); interday <13%). Os efeitos de matriz observados foram ultrapassados pelo uso de curvas de calibração em matriz. Nas amostras analisadas verificou-se a presença de cinco dos seis ftalatos e do único adipato estudados, com concentrações entre 1.77 µg/L e os 205.40 µg/L. Verificou-se que a contaminação por ftalatos/adipato em cervejas está relacionada com presença de álcool nas amostras e varia conforme o tipo de embalagem e a origem de produção, sendo que foram detetados níveis mais elevados em amostras com álcool, embaladas em latas de alumínio e de origem industrial.

Palavras-chave: cerveja, ftalatos, DLLME, GC-MS

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Abbreviation/Acronyms List

ACN – Acetonitrile

BBP – Butyl benzyl phthalate

C18 – Octadecylsilane

CF-SPME – Cooling-Fibre Solid-Phase Microextraction

CMR – mutagenic or toxic for reproduction

DAD – Diode Array Detector

DAP – Diamyl Phthalate

DBP – Di-butyl Phthalate

DCHP – Di-cyclohexyl Phthalate

DEEP – Di-ethoxyhexyl Phthalate

DEHA – Di-ethylhexyl Adipate

DEHP – Di-ethylhexyl Phthalate

DEP – Di-ethyl Phthalate

DIBP – Di-isobutyl Phthalate

DIDP – Di-isodecyl Phthalate

DINP – Di-isononyl Phthalate

DI-SPME – Direct Immersion Solid-Phase Microextraction

DLLME – Dispersive Liquid-Liquid Microextraction

DMEP – Di-methoxyethyl Phthalate

DMP – Di-methyl Phthalate

DNBP – Di-n-butyl Phthalate

DNOP – Di-n-octyl Phthalate

DPP – Di-pentyl Phthalate

d-SPE – Dispersive Solid-Phase Extraction

EC – European Commission

EDC – Endocrine Disrupting Chemical

EFSA – European Food Safety Authority

EI – Electron Impact

EtOH – Ethanol

EU – European Union

FID – Flame Ionization Detector

GC – Gas Chromatography

GMP – Good Manufacturing Practices

HF-LPME – Hollow-Fibre Liquid-Phase Microextraction

HMW PEs – High Molecular Weight Phthalates

HPG – Hypothalamic-Pituitary-Gonadal Axis

HPLC – High Performance Liquid Chromatography

HS-SPME – Head-Space Solid-Phase Microextraction

IBCEP – Butyl Cyclohexyl Phthalate

IBP – Isobutyl Phthalate

IS – Internal Standard

IT-SPME – In-tube Solid-Phase Microextraction

IV – Intravenous

LC – Liquid Chromatography

LDH – Layered Double Hydroxides

LLE – Liquid-Liquid Extraction

LMW PEs – Low Molecular Weight Phthalate

LOD – Limit of Detection

LOQ – Limit of Quantification

LPME – Liquid-Phase Microextraction

MAC – Maximum Accepted Concentration

MBP – Monobutyl Phthalate

MCIHPP - Mono(carboxy-isoheptyl) Phthalate

MCIOP - Mono(carboxy-iso-octyl Phthalate

MCMHP - Mono(2-carboxymethylhexyl) Phthalate

m-DSPE – Magnetic Dispersive Solid-Phase Microextraction

MECPP - Mono(2-ethyl-5-carboxypentyl) Phthalate

MEHHP - Mono(2-ethyl-5-hydroxyhexyl) Phthalate

MEHP – Mono(2-ethylhexyl) Phthalate

MeOH – Methanol

MEOHP - Mono(2-ethyl-5-oxohexyl) Phthalate

MEP – Monoethyl Phthalate

MHINP - Mono(hydroxy-isononyl) Phthalate

MINP - Mono-isononyl Phthalate

MIP – Molecular Imprinted Polymer

MMP – Monomethyl Phthalate

MOINP - Mono(oxo-isononyl) Phthalate

MRL – Maximum Residue Limit

MRM – Multiple Reaction Monitoring Mode

MS – Mass Spectrometry

MS/MS –Tandem Mass Spectrometry

OML – Overall Migration Limit

PEs – Phthalate

PET – Polyethylene Terephthalate

PSA – Primary Secondary Amine

PVC – Polyvinyl Chloride

Q – Single Quadrupole

QqQ – Triple Quadrupole

QuEChERS – Quick, Easy, Cheap, Effective, Rugged and Safe

SDME – Single-Drop Microextraction

SIM – Single Ion Monitoring Mode

SML – Specific Migration Limit

SPE – Solid-Phase Extraction

SPME – Solid-Phase Microextraction

TDA – Terephthalic Acid

TDI – Tolerable Daily Intake

UA-DLLME – Ultrasound Assisted Dispersive Liquid-Liquid Microextraction

UV – Ultraviolet

USVA-DLLME – Ultra-Sound Vortex-Assisted Dispersive Liquid-Liquid Microextraction

Introduction

1. Beer

Beer history dates as far back as 5000 BC; there are several reports on the production of fermented beverages all over the world in Ancient Egypt, Mesopotamia, and China. Firstly, the fermentation process was used for cereal conservation, which later gave rise to the use of this technique to produce beverages (1, 2). There have been several different techniques and recipes over the years, and with the different technological advances and improvements it was possible to obtain a standardized product (2).

Brewing is the process of fermentation of a carbohydrate by yeast metabolism in the absence of oxygen leading to the production of alcohol and carbon dioxide. The fermentation can occur at high or low temperatures, which results in different styles of beer. This process has several stages: malting, where the cereal used is germinated and then roasted; milling of the dry cereal and addition of water; mashing of the mixture, followed by boiling; addition of hops and cooling; fermentation with the specific yeast strain; maturation at low temperatures for several weeks; filtration in industrial production; carbonation; microbiological stabilization; and finally packaging, with aluminium cans or glass bottles. Several raw materials can be used, mainly barley and wheat, but also, rice, maize, and oats, together with water, hops and yeast (3, 4).

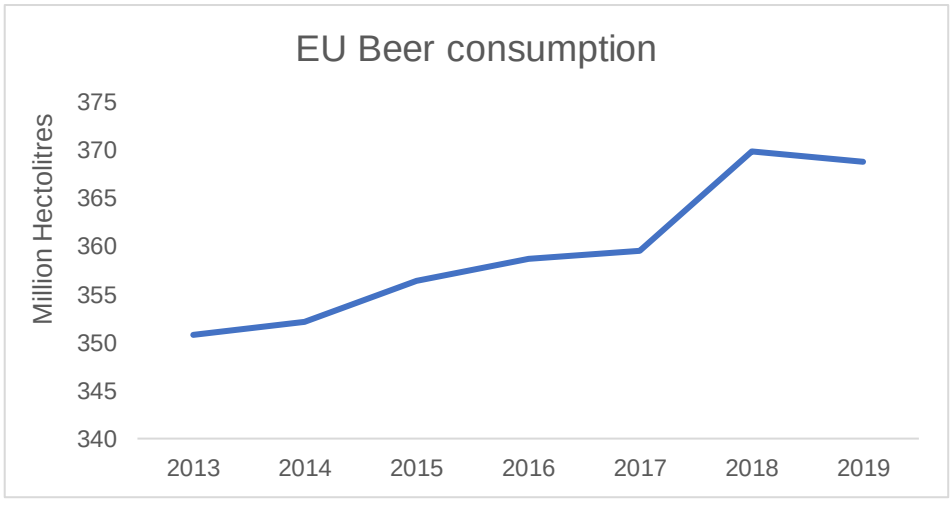

Figure 1 - Consumption of beer in Europe 2013-2019 (Million hectoliters). Adapted from Brewers of Europe

Nowadays, in Europe, there are about 80 different styles of beer, Pale Ale, Pilsner, Lager, or Stout, for example (5). Beer is one of the most consumed alcoholic beverages in the world, with a growing tendency (Figure 1), mainly due to the already established beer

markets in Western Europe and North America and also, due to the convergence of drinking patterns caused by the increased contact and influence across countries over time (6, 7). In Europe, in 2019, 402 million hectolitres of beer were produced, and 369 million hectolitres of beer were consumed (5).

The Portuguese population consumed, in 2018, 51 L of beer per capita, with an overall consumption of 5 million Hectolitres in the same year (Figure 2). Also, Portugal was the European country with the highest percentage of On-trade Consumption of beer (hospitality industry such as, breweries, bars, restaurants and hotels) (Figure 3) (5).

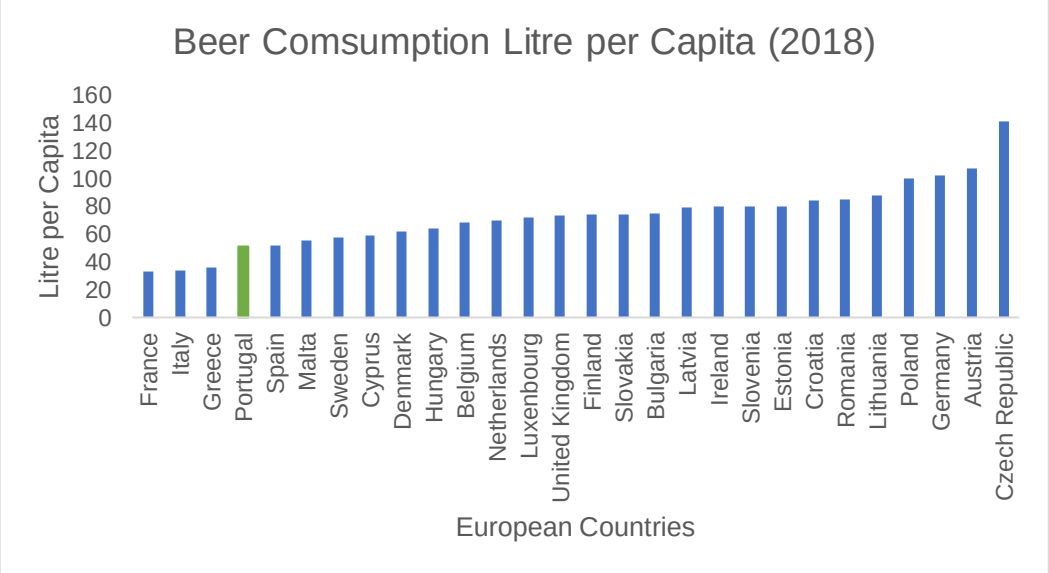

Figure 2 - Consumption of beer in the European Countries in 2018 (Litre per Capita). Adapted from Brewers of Europe

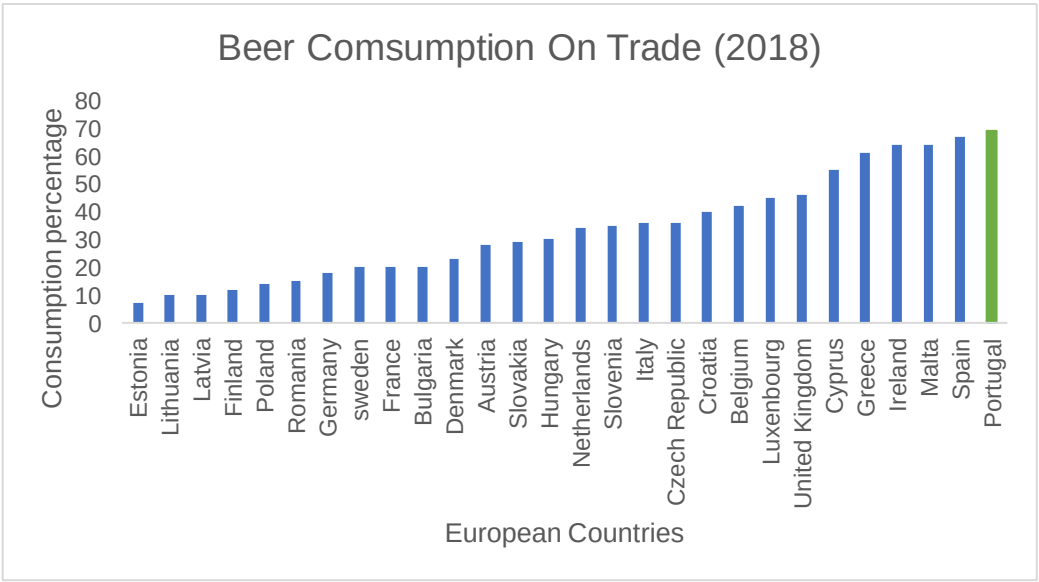

Figure 3 - Consumption of beer On-trade in European Countries in 2018 (%). Adapted from Brewers of Europe

The global beer trade is concentrated in a few multinational companies, which permitted the growth of micro-brewery or craft-brewery businesses. Due to their smaller market, these types of breweries can be more imaginative in their production process, resulting in different flavours, and increasing their competitiveness and market value (2, 6). Consequently, there has been in the last years a rise in the number of active breweries (Figure 4).

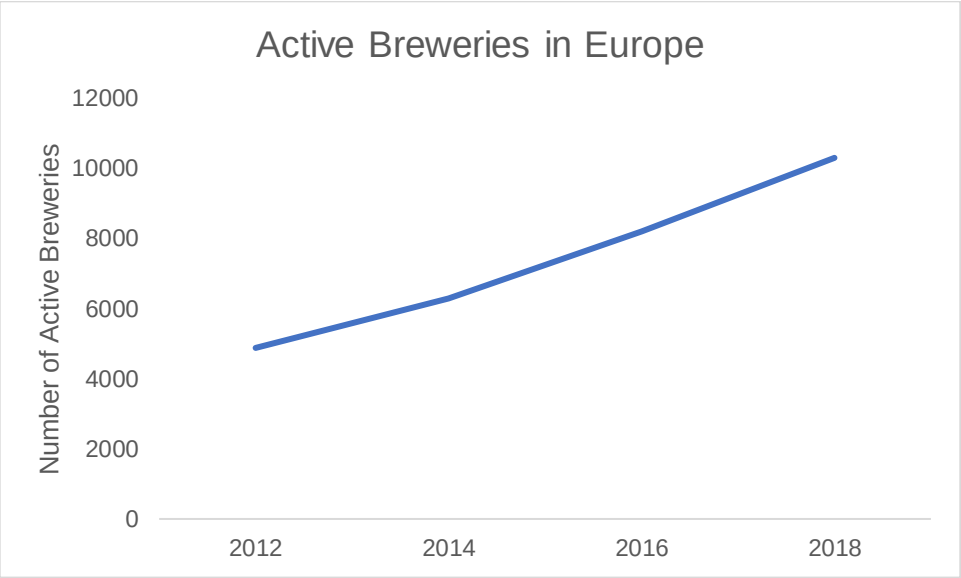

Figure 4 - Rise of the number of active breweries 2012-2018 in Europe. Adapted from Brewers of Europe

2. Food Packaging

Packaging is the enclosure of products in a bag, box, cup, tray, tube, bottle, or other container in order to contain, protect and/or preserve said products. There are several levels of packaging: the primary package is in direct contact with the product, such as metal cans, glass bottles or paper cartons, while the secondary package is usually a case or box that contains several primary packages for distribution. Similarly, a tertiary package can be made of several secondary packages (8-10).

Firstly, the product must be properly contained by the package, so as to avoid leakage, loss, and/or contamination. Secondly, the package must protect the product of outside contamination and assure quality preservation. There are several environmental sources of contamination such as water, gases, odours, microorganisms, and dust, being necessary to take into consideration physical forces such as vibrations or shocks that may alter the packaged product. Thirdly, a package must be convenient for the user, either in its usability, such as the ability to use said product outdoors or in a rush, or its portability, that can be harmed by inappropriate size and shape. Lastly, the package must be appealing to the customer, in aspect and publicity (9, 10).

Several different materials are used in food packaging, glass, metals, which includes aluminium, tinplate and steel, paper, and plastic. Food packages usually include different types of materials in order to achieve the best functionality and aspect. Glass is inert, odourless, impermeable to gases and vapours, can be sterilized, provides insulation, and can be produced in different shapes. However, due to its weight and breakage susceptibility, its use implies high transportation costs. Metal packages are very versatile, as they offer physical protection, act as a barrier, are foldable and recyclable; aluminium and steel are the most used metals in food packaging. Usually, these types of packages have a layer of protective polymer coating, this way the food products do not gain a metallic taste. Paper is commonly used in boxes, milk cartoons, bags or wrapping paper, however, plain paper can not be used to contain food products for long time periods due to poor barrier and insulation properties, consequently paper must be laminated or impregnated with other materials such as waxes or resins in order to guaranty food quality. Lastly, there's plastic packages, that can be produced by condensation polymerization or addition polymerization. The production of plastic has risen due to its low cost, versatility in size and shape production and thermosealability. Different types of plastic have been used in food packaging such as polyesters, polystyrene, polyamides, and polyvinyl chloride (PVC). The last type of plastic is heavy and stiff plastic, with high resistance to chemicals and stable electrical properties. With the addition of plasticizers, such as phthalates and adipates, PVC becomes more malleable and can be used in bottles and packaging films (11-14).

Plasticizer is a substance that is incorporated into plastic in order to enhance flexibility and workability. Phthalates (PEs) and adipates are very common plasticizers, classified as external plasticizers because they are not chemically bonded with the plastic, and can migrate to the packed product (15). Of all the plasticizers produced, 90 % are used in the industry incorporated in PVC and can be used in polymer coatings (10). Plastic is a blend of a polymer and a plasticizer, which are diesters of phthalic acid, a group of chemicals with a wide range of industrial applications for over 50 years (16, 17). The PEs fills the spaces in the polymer system providing plasticity and flexibility to the plastic, being the percentage of PEs blended, which may range up to 70%, directly proportional to plasticity (18).

Migration is the transfer of substances from the package to the food product. It can be classified by two forms: overall migration, which is the sum of all substances released per unit of area of the packaging material, and specific migration, that relates to the migration of a specific substance (10).

2.1. Phthalates

Phthalates were first produced in 1920, and rapidly allowed the growth of the PVC industry (19). The reaction of phthalic anhydride with alcohols of different chain lengths results in phthalate esters, for example, methyl esters from the reaction with methanol. At room temperature, PEs are almost colourless, odourless oily liquids, and depending on how long their chain is, are increasingly fat soluble. Characteristics such as low melting point and high boiling point make them excellent plasticizers (16, 19). PEs are classified as Low Molecular Weight PEs (LMW PEs) and High Molecular Weight PEs (HMW PEs), according to the length of R and R' side chains (Figure 5).

Because PEs are only physically bound with the polymer, changes in the environment such as temperature, pH, radiation, or contact with solvents, may cause an accelerated release of PEs to the air and other media and, consequent, migration to the environment. Contaminations may occur in food, drinks, soil, air, water, and blood (medical devices), which result in environmental and health hazards (17, 18, 20-22). Only in 1970, the migration tendency of phthalates into the environment was discovered, and since then several studies were conducted to verify human exposure (23, 24). Depending on their molecular weight, there is a higher or lesser level of bioaccumulation. e.g. DEP, with a low molecular weight is more readily bioaccumulated than DEHP, with a high molecular weight (25).

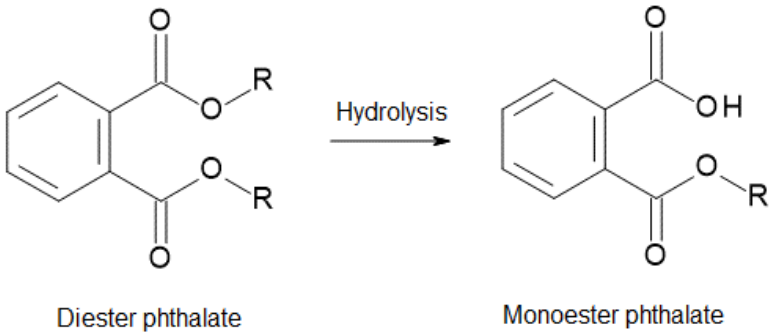

Figure 5 - Common phthalate chemical structure

2.2. Most common Phthalates

Table 1 – List of the most common phthalates. Adapted from Benjamin et al., 2017 (18) and Giuliani et al., 2020 (16)

Phthalate	Abbreviation	Formula	Molecular Weight (g/mol)
Di-methyl Phthalate	DMP	C10H10O4	194.2
Di-ethyl Phthalate	DEP	C12H14O4	222.2
Di-butyl Phthalate	DBP	C16H22O4	278.3
Di-isobutyl Phthalate	DIBP	C16H22O4	278.4
Benzyl-butyl Phthalate	BBP	C19H20O4	312.4
Di-cyclohexyl Phthalate	DCHP	C20H16O4	330.4
Di-ethylhexyl Phthalate	DEHP	C24H38O4	390.6
Di-isononyl Phthalate	DINP	C26H42O4	418.6
Di-isodecyl Phthalate	DIDP	C28H46O4	446.7

Di-ethylhexyl phthalate (DEHP)

DEHP is produced by the reaction of 2-ethylhexanol with phthalic anhydride. It is widely used as a plasticizer in PVC, with a presence up to 40 % for domestic and industrial use such as flooring, sealants and paint, toys, cables, garden hoses, gloves, and wall coverings, also, in food packaging, blood storage bags and medical devices (16, 26-28). DEHP was the first phthalate produced as a plasticizer, in order to improve the flexibility, durability, and workability of hard plastic PVC in the 1930s (22, 29). Afterward the production of PEs grew and diversified (19). After human exposure to DEHP, this compound is metabolized to mono-(2-ethylhexyl) phthalate (MEHP) by hydrolysis, which in turn is again extensively metabolized until it is eliminated via urine (30). Various metabolites of DEHP have been found in rodents and human urine, such as mono(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono(2-ethyl-5-oxohexyl) phthalate (MEOHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) and mono(2-carboxymethylhexyl) phthalate (MCMHP). Studies show that MECPP in urine and MDMHP in serum are stronger biomarkers of DEHP exposure when compared to MEHP (28).

It is considered a reproductive toxicant 1B – presumed reproductive toxicant (31, 32) and has a TDI (tolerable Daily Intake) of 0.05 mg/kg of body weight (bw)/day (d) by EFSA (27).

Butyl-benzyl phthalate (BBP)

BBP is produced by a reaction of butanol and benzyl chloride with phthalic anhydride. Over 90% of BBP use is in plasticizing PVC or other polymers for flooring, sealants and paints, it is also used in food wraps and packaging, but in less extent (33). The major exposure of

BBP through foods is by migration into fatty foods. It is rapidly metabolized into monoester metabolites, that can be excreted via urine or be conjugated with glucuronic acid and then be eliminated via urinary excretion (26)

It is considered a reproductive toxicant 1B (31) and has a TDI of 0.05 mg/kg bw/d established by EFSA (33).

Di-butyl phthalate (DBP)

DBP is produced by the reaction of n-butanol with phthalic anhydride. DBP is used mainly in as a plasticizer for polymers (76%), adhesives (14%), printing inks (7%) and other products (3%), with significant use in food wrappers and packages (34, 35). DBP is mostly excreted as a glucuronide-conjugate, monobutyl phthalate (MBP) (28)

It is considered a reproductive toxicant 1B (31) and has a TDI of 0.05 mg/kg bw/d by EFSA (34).

Di-isobutyl phthalate (DIBP)

DIBP is produced by the reaction of isobutanol with phthalic anhydride. It is used mainly in paints, lacquers, and cosmetics. The exposure routes of DIBP are oral ingestion and dermal, with a rapid metabolization into monoisobutyl (MIBP). In recent years, it has been reported an increasing exposure to this compound, due to its use as a substitute for DBP (36, 37).

It is considered a reproductive toxicant 1B (31).

Di-isononyl phthalates (DINP)

DINP is produced from octene and n-butene and is mainly used as a plasticizer in PVC (95%), and in inks, pigments, adhesives, sealants, paints, and lubricants (16, 28, 38).They are first metabolized into mono-isononyl phthalate (MINP) and then transformed into various secondary metabolites before excretion in urine, such as mono(carboxy-iso-octyl phthalate (MCIOP), mono(hydroxy-isononyl) phthalate (MHINP), mono(oxo-isononyl) phthalate (MOINP) and mono(carboxy-isoheptyl) phthalate (MCIHPP) (28).

Di-isodecylphthalate (DIDP)

DIDP is mainly used as a plasticizer in PVC. DIDP and DINP are mixtures that overlap chemically and cannot be chemical distinguished, therefore for DINP and DIDP there is a group restriction for migration, with a TDI of 0.15 mg/kg bw (39).

Di-ethyl phthalate (DEP)

DEP is produced by the reaction of ethanol and phthalic anhydride in the presence of sulfuric acid and is mostly used in personal care products, coatings, dyes, and pesticides (16, 28, 40). DEP is mostly excreted in urine in the monoester unconjugated form monoethyl phthalate (MEP) (28, 41).

Di-methyl phthalate (DMP)

DMP is produced by the reaction of methanol and phthalic anhydride and is mostly used in cosmetic products, solvents, paints, and rubbers. It is mostly excreted in urine as monomethyl phthalate (MMF) form (28).

Table 2 - List of common phthalates and their metabolites

Phthalates	Metabolites
DMP	MMP
DEP	MEP
DBP	MBP
DEHP	MEHP
	MEHHP
	MEOHP
	MECPP
	MCMHP
DINP	MINP
	MCIOP
	MHINP
	MOINP
	MCIHPP

2.3. DEHA (di-ethylhexyl) adipate

DEHA is used as a plasticizer substitute of DEHP, due to the lower reproductive toxicity and endocrine disrupting effects (42), consequently it has a broad environmental incidence. DEHA is considered as a safer alternative to DEHP, as it did not show anti-androgenic effects (43), and testicular toxicity (44) in rats. With the research in mind, a TDI of 0.3 mg/kg and an SML (specific migration limit) of 18 mg/kg were specified for DEHA (45, 46). The most important route of exposure is via contaminated food (47).

3. Routes of exposure to phthalates

PEs are ubiquitous contaminants, therefore, there are many routes of exposure (16, 48). As mentioned above, HMW PEs, such as DEHP, and DINP are used predominantly in PVC polymers and plastisol applications, for example, plastic, food packaging, food processing materials, vinyl toys, vinyl floor coverings, and building products. LMW PEs, DMP and DEP, are used in non-PVC applications, such as personal care products, paints, and adhesives (16, 29, 48).

The major source of exposure to LMW PEs are cosmetics and personal care products, perfumes, shampoos, make-up or nail-polishes, while the major source of exposure to HMW PEs is diet, with migration of PEs from food packaging materials to food products, specially fatty products stored at high temperatures (48). Pharmaceuticals and medical devices are also a source of exposure to PEs, mainly in pills with enteric coatings, intravenous storage bags, ventilator tubing, IV infusion catheters, PVC exam gloves, among others (48). Consequently, PEs have been found in several biological matrices such as urine, blood, saliva, amniotic fluid, breast milk, and cord blood (49-54).

These compounds are ubiquitous in the environment and can be found in the air (aerosols and indoor air), rivers, marine water, marine sediments, soil (sewage and wastewater treatments), and biota (16, 29). Different PEs will behave differently in the environment and food chains depending of several physicochemical properties such as water solubility, lipophilicity, abiotic degradation and biodegradation processes and others (16). In the aquatic system, the major sources of PEs contamination are leaching, drainage and atmospheric deposition, with DMP, DEP, DBP, BBP and DEHP as the most present in surface water. Biodegradation is the major mechanism for PEs degradation in aquatic and terrestrial systems, where PEs with shorter alkyl chains are more readily biodegraded and mineralized, and PEs with longer chains have to be, first, transformed into compounds with shorter chains (16). In the soil, the most detected PEs are DBP and DEHP, with higher concentrations in cultivated soils, therefore, human agricultural activities combined with the use of plastics, for example plastic greenhouses, are a major source of PEs contamination (16, 55). In the air, PEs are detected in both gas and dust phases, with DIBP and DBP more present in the first and DEHP more abundant in the last phase. It is believed that anthropogenic activities are a major source of contamination due to the high levels detected in urban areas (16, 56).

Table 3 - Most common routes of exposure and examples

Entry Route	Examples and via of entrance
Food	Wrappers, bottles, cocking aids, infant formula, and milk
Water	Ingestion, bathing, and washing waters
Inhalation	Indoor air, dust, fragrances, and perfumes
Medication	Casings of timer releasing pharmaceuticals
Medical devices	Bags, tubbing, implants, dialysis, blood transfusion, and dentures
Cosmetics	Creams, deodorants, moisturizers, shampoos, nail polish, lipstick, hair dyes
Clothing	Artificial leather, waterproof clothes footwear
Toys	Mouthing, rubbing, and playing
Construction materials	Pipes, flooring, wall covering

Infants and children are more exposed to PEs due to their hand-to-mouth behaviour, than adults. In the general population, the levels of PEs exposure are estimated in the order of tens of µg/kg bw/day (18).

4. Phthalate's metabolism

Due to their ubiquitous presence in the environment, PEs negative impact is evermore increasing. There are several exposure routes, from environmental release during production processes up to elimination processes of plastic products, and, also, migration from direct contact with food via packages, processing, transportation and/or preparation. Consequently, the major human exposure route is considered the consumption of PEs-contaminated food and water. There are other sources, such as dermal, by the use of contaminated cosmetic products and clothing, and intravenous injection (16, 48).

Due to their widespread use, the exact contribution of the different sources and routes of exposure to PEs is unknown. Ingestion has been considered a very important route of exposure, also dermal and parenteral exposure from medical devices (29, 57). The uptake of PEs is dependent on various factors, such as dose, route of exposure and molecular weight. After exposure, PEs are metabolized quickly and excreted through urine and faeces. Phase I biotransformation allows for the metabolization of polar and LMW PEs, such as DEP, into hydrolytic monoesters (hydrolyzation of one of the ester bonds by esterase or lipase). Meanwhile, the HMW PEs are firstly metabolized into hydrolytic monoesters, and secondly into hydrophilic oxidative metabolites (enzymatic oxidation of the alkyl chain). Afterwards, the monoesters and the oxidative metabolites are either excreted through urine and faeces or, they may suffer phase II biotransformation and produce glucuronide conjugates that are more water soluble, and then be excreted via urine (18, 29). The

cleavage of the ester bond occurs within 2 hours of the PEs entry into the circulatory system (18). A study by Schwedler and colleagues (58), whose results demonstrate that most Europeans excrete phthalates metabolites in their urine, is a reflection of the widespread of PEs as contaminants, and their increased use in the industry.

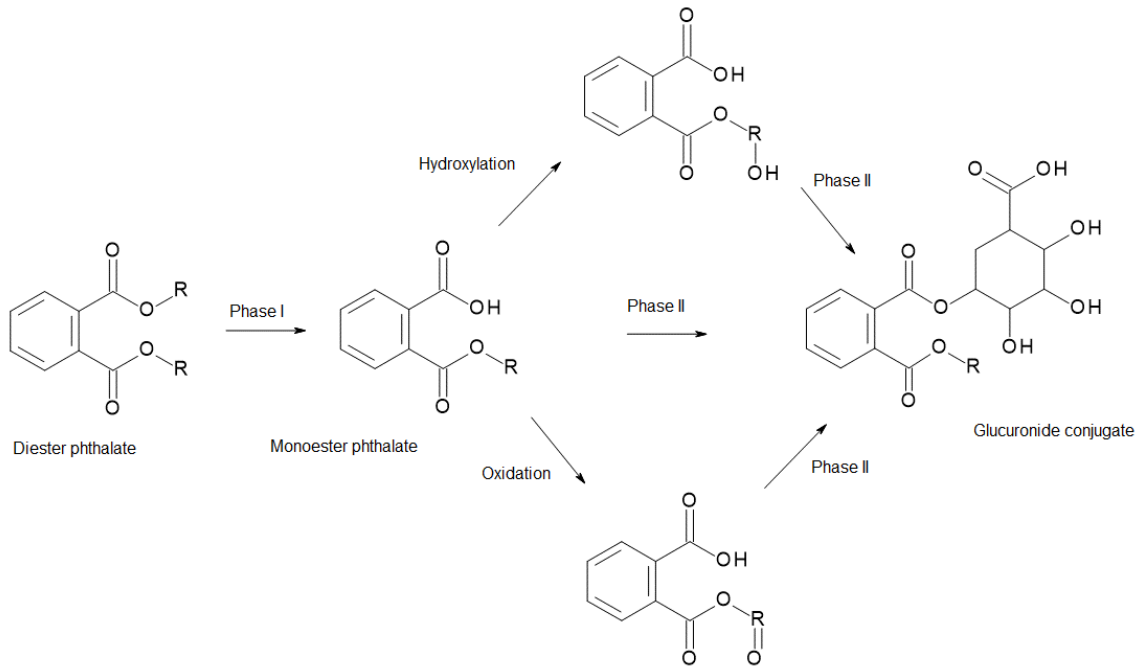

Figure 6 - Phthalate metabolism

5. Health effects

The widespread use and consequent exposure of PEs are of great concern to human health. After transformation into primary and secondary metabolites, PEs are capable of acting as endocrine disrupting chemicals (EDC) (16). PEs exposure has been linked to several health issues, such as endocrine and reproductive dysregulation, infertility, altered foetal development, cardiotoxicity, hepatotoxicity, asthma, and allergies (16, 18).

The endocrine system is composed of several glands located in several organs, brain, gastrointestinal system, kidneys, pancreas, ovaries, testes, thyroid, etc, that secrete hormones into the circulatory system and regulate several functions in the organism. PEs can act as hormone sensitizers and are able to disrupt or impair these functions in the organism (18).

The effects usually observed in male rats after exposure to long-chained PEs are cryptorchidism, decreased testosterone levels, testicular atrophy, Sertoli cell abnormalities, decreased weight of androgen-dependent organs, reduction of daily sperm production and

lower epididymal sperm counts (28). The best-known effect of PEs in male's health is the "phthalate syndrome" where these compounds interact with the hypothalamic-pituitary-gonadal axis (HPG axis) and dysregulate the signalling pathways in steroid homeostasis and biosynthesis. Also, they may cause functional impairment of Sertoli cells, with subsequent meiosis, spermiogenesis, and testosterone production by Leydig cells inhibition (16, 29, 59). PEs exposure in humans is associated with decrease in sperm quality, sperm aneuploidy, decrease of sperm count, reduced sperm head sizes and abnormal sperm tails (60-62)

PEs can interact directly with oestrogen receptors or indirectly as regulators or co-activators of transcription factors or by an independent pathway in which they modulate metabolic enzymes that are vital for oestrogen receptor metabolism, and are capable of modifying the genomic and non-genomic activity of the female reproductive system (18). General studies of biomonitoring and risk assessment in women reported detection of PEs in every fluid, such as urine, saliva, blood, cord blood, amniotic fluid, follicular fluid, and breast milk (18). Especially in pregnant women, the effects of PEs are felt not only on the women but also on their children (16, 18, 63). Animal studies demonstrated links between DEHP exposure and ovarian toxicity, including prolonged oestrus cycles, suppressed/delayed ovulation, reduced granulosa cell size, which leads to smaller preovulatory follicles, and decreased circulating oestradiol (29). PEs, such as DEHP, DEP, DBP and BBP can cross the placental barrier and affect foetal development, such as reduced gestational age and increased birth loss. PEs exposure may also lead to endometriosis, infertility and reduced yield of oocytes (16). Additionally, DEHP has been detected in maternal milk, which leads to exposure of new-borns to this contaminant (16, 51).

Some studies have related PEs with metabolic diseases. Diabetes is a metabolic disease which results in high blood glucose levels, being a common disease worldwide. Lifestyle changes due to industrialization and rapid economic development may contribute to an increase in diabetes incidence. Additionally, the substantial increase in human exposure to synthetic chemicals, such as PEs, may lead to that increase (16, 48). A study by Sun, Cornelis (64), reported that women with higher concentrations of PEs in their urine had a higher risk of diabetes diagnosis. Castro-Correia and colleagues found no significant differences between PEs metabolites concentrations in urine between recently diagnosed diabetic children, children with type 1 diabetes and healthy children, however, they had a small sample pool (17). PEs have also been associated with cardiotoxicity (26), wherein the study of Olsen et al., 2012, a correlation between coronary heart disease and rising PEs concentration (65) was found, also, another study found an association between HMW PEs and increased blood pressure in children (66), and Werner and co-workers (2015) found an

association between increased diastolic blood pressure in pregnant women and PEs (67). PEs have been associated with several human cancers such as skin, liver, prostate and breast cancer (16). A study by Rodgers et al., 2018 (68) found higher MEP concentration in women with breast cancer than in healthy ones. A study by Zhu team in 2018 (69) showed that phthalates induced proliferation in prostate cancer cells.

6. Legislative Limits for Phthalates/Adipates

The migration of compounds from food packaging materials to food has become one of the major sources of assumed food toxicity. Considering this, PEs are a worldwide threat to the environment and human health. There are several legislations in place to control the use of these compounds and to protect consumers. Firstly, there was Framework Directive 89/109/EEC which establishes the principles of “inertness” and “safety” as two basic principles for food-contact material and decrees that any material, article, or its components should be inert in a way to not cause any health hazard, intolerable change in the food composition or degradation of the quality of the food. This directive was later substituted by 1935/2004/EC which gives general rules for new topics on active food-contact materials and safety measures, such as not endangering human health, not altering the food composition in an unacceptable way, not altering taste, texture, or odour and produced according to Good Manufacturing Practices. Another directive, 2002/72/EC, is related to the basic rules and guidelines on food-contact plastics, but only on simple materials made of plastic and plastic gasket in lids. Regulation 10/11/EU, is the most recent, replacing the latter ones, and considers the use of phthalates likely to contact with food and beverages, in this regulation, there is a list of certain PEs, BBP, DBP and DEHP, that are considered toxic for reproduction, CMR category 1B in annex IV of Regulation EU No. 143/2011 EC, and states that these are banned beginning of 1st January 2015 (16).

The European Food Safety Authority (EFSA) has reviewed and defined Tolerable Daily Intakes (TDI) for several PEs, as seen in table 4 below.

Table 4 - Tolerable Daily Intakes of phthalates defined by EFSA

Phthalates	TDI µg/Kg bw
DBP	50
DEHP	50
DINP	50
DIDP	150
BBP	50

The EU has established limits, Specific Migration Limits (SMLs) which is the Maximum Accepted Concentration (MAC) of a substance released from a material into food and food simulants, in food and beverage-contact plastic materials (Table 5). Other PEs that are not as strictly regulated and do not have a SML value, have a limit of 60 mg/Kg. The Overall Migration Limit (OML) must not surpass 10 mg of all compounds in 1 dm^2 of contact surface between the food product and the package.

Table 5 - Specific Migration Limits in plastic food contact materials established by the EU

Phthalates/Adipates	SML mg/Kg
DBP	0.3
DEHP	1.5
DINP	9
DIDP	9
BBP	30
DEHA	18

DINP, DIDP, and BBP can only be used as “Plasticizer in repeated use materials and articles”; “Plasticizer in single-use materials and articles contacting non-fatty foods except for infant formulae and follow-on formulae as defined by Directive 2006/141/EC or processed cereal-based foods and baby foods for infants and young children as defined by Directive 2006/125/EC”; “Technical support agent in concentrations up to 0.1% in the final product”.

7. Phthalates in Food

Food is the main source of exposure to PEs in humans. The major sources of PEs contamination in food are PVC tubbing, food-packaging films, PVC gaskets in metallic caps for glass jars, printing inks, paper and board packaging, PVC gloves, aluminium foil-paper

laminates, coatings on cookware, and polyethylene terephthalate (PET) in PET bottles (16, 70). Consequently food products can be contaminated in the production process, transport process, storage, or handling (16, 71). Food with a higher lipid content is more commonly contaminated with PEs, due to their lipophilic nature (16, 48).

Food contact materials are meant to come into contact with food, for example, packaging and containers, kitchen equipment, cutlery, and dishes. Modern packaging materials are supposed to have multiple purposes such as protection of food products from damage and external sources of contamination, preservation, ease of transportation and of storage, and provide the consumers the information on ingredients and nutritional data (72). In the last decades, with the evolution of production of packaging materials, there is an effort to produce packaging safe for consumers, with minimal environmental impact and at a low cost. One of the biggest problems in packaging production is the migration of harmful compounds to food products.

7.1. Alcoholic beverages

Ethanol is a contributing factor for the high susceptibility of PEs contamination in alcoholic beverages (16, 71). Grinbaum and colleagues (71), analysed sources of PEs and factors that favour their diffusion into wines and verified that the most important factor to promote contamination is the alcohol content, where the risks of migration of PEs to musts are very low, contrarily to the risks of migration to wines.

There are some studies on the detection of PEs in alcoholic beverages. The team of Carrilo, in 2008, was the first to determine the presence of PEs in different samples of wine. The authors used a HS-SPME (Head-space Solid-phase Microextraction) method coupled to GC-MS (Gas chromatography with Mass spectrometry) and selected 10 wine samples from different areas and with different packages, glass bottles (n=6), cartons (n=2), and bag-inbox containers (n=2). The bottled samples were also differentiated by their stoppers: one piece cork (n=2), agglomerated cork (n=2) and synthetic (n=2). DBP was the most detected compound, present in all samples. The bottles samples had a higher diversity of PEs – DMP, DEP, DBP, DEHP and BBP without significant difference on the type of stopper (73). Del Carlo et al., 2008 (74) analysed 62 wine samples with a SPE (Solid-phase Extraction) method coupled to GC-MS. The samples were catalogued by their origin, commercial, private wine producers, and pilot plant, and by their package, polyethylene film brick, and glass bottle. The authors found that the frequency of PEs detection was dependent on the type of sample and not on the type of packaging material, where commercial wines (385

µg/L) had a higher concentration of PEs than private producers (204 µg/L), and pilot plant (138 µg/L) samples. The most detected PEs were IBP and DEHP.

Ye team, in 2009, (75) used HS-SPME-GC method to extract PEs from four beer samples collected from three different breweries, and found that DBP and DEHP were detected in three samples, DAP was detected in two and DNOP was detected in one sample. Two of the samples were from the same brewery, however with different alcohol content, and while in sample A1 (3.5% v/v ethanol) three different PEs were detected (DBP, DAP, and DEHP), in sample A2 (4.0% v/v ethanol) there was no presence of PEs contamination.

In the study of Fierens and co-workers (76), there was an analysis of several food products and packaging materials sold in Belgium for the presence of PEs. The alcoholic samples included in the study were 18 samples of beer, and out of all the food products, these samples had the lowest phthalate concentrations, with detection of DNOP, DEP, DIBP, and DNBP, with a maximum concentration of 1.2 µg/kg.

A few years later, Cinelli et al., 2014 (77), used a SPE method with Amberlite XAD-2 adsorbent coupled to GC-FID (Gas chromatography with Flame Ionisation Detector) to analyse the presence of PEs in different alcoholic beverages, such as wine samples in glass bottles and Tetrapack, and vodka melon liqueur samples. The authors detected DEHP in both samples of white and red wine in Tetrapack containers, in two out of three samples of red wine, in both samples of white wine in glass bottles, and in the vodka sample. DEHP was the most detected compound with a maximum concentration in the vodka sample at 22.4 µg/L. DBP was the second most detected phthalate, present in one sample of white wine (Tetrapack), two samples of red wine and one sample of white wine (glass bottle), and in the vodka sample (highest concentration). In the vodka sample, it was also detected BBP and IBP. Out of 8 samples tested, only one had showed no presence of phthalates. The concentrations detected were all below the SML imposed by the EU. In the same year, Russo and co-workers, from the same team, analysed several beverages with an SPE method with Amberlite XAD-2 adsorbent coupled to GC-MS. Four alcoholic beverages – three beers (Italian, Dutch, and German) and a whisky and coke light drink – were included in the samples. DEP, DBP, and DEHP were detected on the four samples, with DEHP at a higher total concentration. DIBP was detected in the three beer samples, with the highest concentration in the German beer (2.45 µg/L), BBP was detected in the Italian beer sample and the whisky and coke light drink, with a higher concentration in the beer sample (0.81 µg/L). DMP was detected only in the Dutch beer at a concentration of 1.89 µg/L and IBCEP was detected only in the sample of the Italian beer at a very low concentration (0.08 µg/L) (78). A different team, Fan et al., 2014 (79), developed a method based on IL-DLLME

coupled to HPLC-DAD to detected PEs in 30 samples of Chinese white spirit and 11 samples of red wine. The authors found that DBP was the most detected PEs, and in 63% of Chinese white spirits, the concentrations were above the SML imposed by the EU (0.3 mg/kg). DIBP and DEHP were also detected in the Chinese white spirit samples at a frequency of 97 and 93 %, respectively. BBP was found in only two samples of this beverage, at low concentrations. In red wines, only DIBP and DBP were detected at low concentrations, at a frequency of 36%. Hayasaka (80) in 2014, developed a method with HPLC-MS/MS with an extra HPLC column (hold-back) upstream the injection valve, in order to quantify PEs in wine and eliminate the influence of laboratory contaminants. The hold back column was capable of delaying the elution of any PEs sourced from the HPLC system, resulting in two distinguishable elution peaks. The samples used were red (n=5) and white (n=5) wines, and the author found that DNBP (up to 9.3 µg/L) was the most detected PE, and DIBP (up to 10.7 µg/L) was mostly detected in red wine.

March and Cerdà (81) team, developed a method based on in-vial membrane assisted-LLE (Liquid-liquid Extraction) coupled to GC-MS and analysed several samples of alcoholic beverages for PEs contamination. The samples included brandy, two red wines, one white wine, one sangria, and three beers, one of which had low alcohol content. The brandy sample had the highest total concentration of PEs (DEP and DBP), followed by the sangria (DBP and DPP), and the red wine samples (DBP and DPP). The beer samples had the lowest concentrations detected, especially the sample with low alcohol content (DBP and DEP). DBP was the most common phthalate. Cao et al., 2015 (82), detected three PEs (DBP, BBP, DEHP) and DEHA, at a low level, in alcoholic beverage samples from the 2013 Canadian Total Diet Study, with DBP at the highest concentration of 0.0142 µg/Kg. In the same year, Fasano and colleagues determined the level of PEs in wine packed in tetra pack, and detected DBP and BBP in both white and red wines, with DBP levels higher in red wines and BBP in white wines; DEHP levels were below the limits of detection (83). The screening of six samples of Chinese spirits was performed by Wang et al., 2015 (84), all samples were contaminated with PEs at different levels. The authors found DMP, DEP, DIBP, DMEP, DEEP and DPP at low levels, but also detected DBP and DEHP at 1.95 and 1.96 mg/Kg, respectively, which concentrations are above the MAC levels allowed by the EU and above the Maximum Residue Level (MRL) established by China Nation Health Agency.

Jurica et al., 2016 (85) performed a screening of 20 samples of plum spirit in glass bottles from different countries of Central and Eastern Europe and observed the migration of PEs during the different phases of the production process. DBP and DEHP were detected in the highest concentrations. The authors verified that the concentration of PEs detected

increased with the moving stages of the production process. At the pureeing stage, the concentration of DEHP was lower when compared to the distillation phase, most probably due to the acidic medium provided by the plum distillate, which promoted the extraction of PEs from the plastic and rubber equipment used in production. Pérez-Outeiral et al., 2016 (86), developed a method for the detection of PEs in liquid food and water. In wine samples, two in glass bottles and two in Tetra pack box, only DBP was detected at a low concentration in one sample not specified. Vidal, Ibañez (87) developed a method to detect endocrine disruptors (EDs) in beverages. One sample of red wine had a concentration of DBP above the level permitted by the EU, all others were below. DEP was only detected in red wine samples. Cachaça contained high levels of DEHP. From all the alcoholic samples, beer had the lowest concentration of PEs.

Carnol et al., (88), proceeded to the quantification of six PEs in samples of beer from different breweries. PEs were detected in all samples in a range of 1.01-64.56 µg/L, with DEHP the most frequent. DBP and DEP were less frequent but with higher concentrations. The authors found no statistical differences between the results of beer samples stored in cans or stored in bottles (glass or aluminium), however, there were differences between samples from different breweries, which suggests that PEs contamination may result from the production process. Montevecchi and colleagues (89), analysed a brandy series of 27 years. In samples with ageing higher than 15 years, DBP had the highest concentrations, which most probably was due to the base wines, the long ageing process and use of, now, outdated equipment. Wang et al., 2017 (90) developed a method that couples GC-MS and DLLME for the extraction of PEs in beverages. In the liquor samples, 15 PEs were detected, contrasting from the other non-alcoholic beverages. Barciela-Alonso, Otero-Lavandeira (91) tested white and rosé wine samples bottled in Tetra Brik packages for the presence of PEs, and found higher presence of PEs in white wines, with BBP, DBP, DEP and DMP, while in rosé wine, only DEP and DBP were detected.

Aghaziarati et al., 2020 (92) developed a method with on-line IT-SPME coupled to HPLC-UV for the determination of PEs in beverage samples. The samples included five different alcoholic beverages Whisky Scottish Star, Whisky Black, Whisky Mont, beer, and a traditional Iranian drink. Only three samples were contaminated with PEs, DAP was detected in Whisky Black at a concentration of 7.4 µg/L, DMP was detected at a concentration of 5.2 µg/L in Whisky Mont and, lastly, DEHP was detected in the beer sample at a concentration of 3.7 µg/L. Rodríguez-Ramos et al., 2020 (93) developed a method based on QuEChERS and GC/MS for the detection of PEs in beer, cider, and grape juice. BBP was found in most beer samples in the range of 0.14-0.19 µg/L, but not present in cider

samples. DINP was detected at a range of 0.5–2.1 µg/L in cider, but not detected in beer samples. DIDP was present both in beer and cider samples.

As the research shows, there are several examples of contamination of PEs in alcoholic beverages, and while their levels are, usually, below the limit stipulated by the EU, one must be concerned by the effects of long-term exposure with day-to-day consumption of these food products. It is, as well, of remark, the presence of several PEs which are not legislated yet, which may lead to an underestimation of true PEs exposure.

7.1.1. Beer samples

From the 16 papers found in the literature dealing with the presence of PEs in alcoholic beverage samples 9 are referred to beer samples. However, only 2 authors were mainly focused on these types of samples (75, 88). Still, both authors were developing new methods and only had a small pool sample to test the methods, consequently, there isn't a study focused on the determination of PEs in beer samples with a sample large enough to draw more certain conclusions. As referred previously, beer is one of the most consumed drinks in the world (6, 7) and with the ubiquitous nature of phthalates, this is a topic worth researching further. It is also of notice the lack of Portuguese studies on this subject in spite of beer being a highly consumed drink in the country.

Table 6 - Occurrence of phthalates in alcoholic beverages

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	
Wine	DMP	HS-SPME		GC-MS	0.61 ± 0.01	Carrilo et al., 2008 (73)
	DEP				4.22 ± 0.50	
	DBP				5.37 ± 0.05	
	DEHP				7.40 ± 0.09	
	BBP				4.29 ± 1.18	

DMP – di-methyl phthalate; **DEP** – di-ethyl phthalate; **DBP** – di-butyl phthalate; **DEHP** – di-ethylhexyl phthalate; **BBP** - butyl benzyl phthalate; **IBP** - isobutyl phthalate; **DAP** - diamyl phthalate; **DNOP** - di-n-octyl phthalate; **DDP** - di-decyl phthalate; **DIBP** - di-isobutyl phthalate; **DNBP** - di-n-butyl phthalate; **DCHP** - di-cyclohexyl phthalate; **IBCEP** - butyl cyclohexyl phthalate; **BMEP** - bis(2-methoxyethyl) phthalate; **DEEP** - di-ethoxyhexyl phthalate; **DINP** - di-isononyl phthalate; **BMPP** - bis(4-methyl-2-pentyl) phthalate; **DBEP** - bis(2-nbutoxyethyl) phthalate; **DHXP** - di-hexyl phthalate; **DMEP** - di-methoxyethyl phthalate; **DPP** - di-pentyl phthalate; **HS-SPME** - Head-Space Solid-Phase Microextraction; **SPE** - Solid-Phase Extraction; **LLE** - Liquid-Liquid Extraction; **DLLME** - Dispersive Liquid-Liquid Microextraction; **IL-DLLME** -Ionic Liquid Dispersive Liquid-Liquid Microextraction; **MA-LLME** - Microwave Assisted Liquid-Liquid Microextraction; **d-SPE** - Dispersive Solid-Phase Extraction; **QuEChERS** - Quick, Easy, Cheap, Effective, Rugged and Safe; **USVA-DLLME** - Ultra-Sound Vortex-Assisted Dispersive Liquid-Liquid Microextraction; **SPME** - Solid-Phase Microextraction; **MIP-SPE** - Molecular Imprinted Polymer assisted Solid-Phase Extraction; **IT-SPME** - In-tube Solid-Phase Microextraction; **SFOD** - Solidification of Floating Organic Drop; **GC-MS** - Gas Chromatography coupled to Mass Spectrometry; **GC-MS/MS** - Gas Chromatography Tandem Mass Spectrometry; **GC-FID** - Gas Chromatography coupled to Flame Ionization Detector; **HPLC-DAD** - High Performance Liquid Chromatography coupled to Diode Array Detector; **HPLC-MS/MS** - High Performance Liquid Chromatography Tandem Mass Spectrometry; **LC-DAD** - Liquid Chromatography coupled to Diode Array Detector; **HPLC-ESI-MS** - High Performance Liquid Chromatography coupled to Mass Spectrometry; **HPLC-UV** - High Performance Liquid Chromatography coupled to Ultraviolet Detector; **n.d.** – not detected; **TPA** - Terephthalic Acid; **LDH** - Layered Double Hydroxides.

Table 6 (continued)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	
Wine (Glass bottle)	DMP	SPE		GC-MS	n.d.	Del Carlo et al., 2008 (74)
	DEP				n.d.	
	IBP				260	
	DBP				244	
	BBP				269	
	DEHP				242	
Wine (polyethylene film)	DMP				n.d.	
	DEP				n.d.	
	IBP				173	
	DBP				240	
	BBP				252	
	DEHP				276	
Wine (producer)	DMP				n.d.	
	DEP				n.d.	
	IBP				254	
	DBP				125	
	BBP				237	
	DEHP				133	
Wine (pilot plant)	DMP				n.d.	
	DEP				n.d.	
	IBP				197	
	DBP				n.d.	
	BBP				n.d.	
	DEHP				61	

Table 6 (continued)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	
Beer	DMP	HS-SPME		GC-FID	n.d.	Ye et al., 2009 (75)
	DEP				n.d.	
	DBP				2.66 ± 0.37	
	DAP				1.28 ± 0.11	
	DEHP				5.24 ± 0.26	
	DNOP				0.77 ± 0.08	
	DINP				n.d.	
	DDP				n.d.	
Beer	DMP	LLE		GC-MS	0.1	Fierens et al., 2012 (76)
	DEP				0.1	
	DIBP				0.1	
	DNBP				0.2	
	BBP				n.d.	
	DEHP				n.d.	
	DCHP				n.d.	
	DNOP				n.d.	
Beer	DMP	SPE		GC-MS	1.89 ± 0.33	Russo et al., 2014 (78)
	DEP				0.99 ± 0.21	
	DIBP				2.45 ± 0.35	
	DBP				4.36 ± 0.44	
	IBCEP				0.88 ± 0.11	
	BBP				0.81 ± 0.16	
	DEHP				5.07 ± 0.13	

Table 6 (continued)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	
Alcoholic beverages	DMP	SPE		GC-FID	n.d.	Cinelli et al., 2014 (77)
	DEP				n.d.	
	DBP				13.6	
	BBP				6.3	
	IBP				8.2	
	DEHP				22.4	
White spirits	DIBP	IL-DLLME		HPLC-DAD	379.0	Fan et al., 2014 (79)
	DBP				336.0	
	BBP				<14.0	
	DEHP				9.0	
Red wine	DIBP				<5.0	
	DBP				<7.3	
	BBP				<7.0	
	DEHP				<6.7	
Wine	DMP	LLE		HPLC-MS/MS	1.8	Hayasaka, 2014 (80)
	DEP				1.2	
	DIBP				10.7	
	DNBP				9.3	
	BBP				6.3	
	DEHP				4.0	
	DINP				6.0	
	DIDP				1.8	

Table 6 (continued)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (μg/L)	
Alcoholic beverages	DMP DEP DBP BMEP DPP	MA-LLME		GC-MS	n.d. 5.0 ± 0.9 65.0 ± 7.0 n.d. 32.0 ± 4.0	March and Cerdà, 2015 (81)
Alcoholic beverages	DBP BBP DEHP DEHA	n-hexane	d-SPE	GC-MS	0.0142 <0.00359 <0.0140 <0.0023	Cao et al., 2015 (82)
Wine	DBP BBP DEHP DEHA	QuEChERS		GC-MS	8.72 ± 1.41 3.08 ± 0.89 <2.25 2.39 ± 0.42	Fasano et al., 2015 (83)
Chinese spirits	DMP DIBP DBP DEHP DEP DMEP DPP DEEP	n-hexane	-	Isotope dilution GC-MS/MS	0.166 ± 0.00089 0.695 ± 0.0022 1.946 ± 0.0051 1.955 ± 0.095 0.004 ± 0.0001 0.015 ± 0.00011 0.018 ± 0.00016 0.015 ± 0.00078	Wang et al., 2015 (84)

Table 6 (continued)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	
Plum spirit	DMP	dichloromethane	-	GC-MS	n.d.	Jurica et al., 2016 (85)
	DEP				16.7 ± 15.3	
	DIBP				38.3 ± 13.9	
	DBP				414.5 ± 355.9	
	BBP				78.9 ± 39.7	
	DEHP				423.8 ± 524.6	
Wine	DBP	UA-DLLME-SFOD		GC-FID	<0.78	Pérez-Outeiral et al., 2016 (86)
	BBP				n.d.	
	DCHP				n.d.	
	DEHP				n.d.	
	DNOP				n.d.	

Table 6 (continued)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	
Lager beer	DEP	SPE		LC-DAD	4.7	Vidal et al., 2016 (87)
	DBP				1.1	
	DEHP				18.2	
Stout beer	DEP				n.d.	
	DBP				74.7	
	DEHP				16.6	
Red Wine	DEP				56.0	
	DBP				334	
	DEHP				80.3	
White Wine	DEP				n.d.	
	DBP				32.4	
	DEHP				18.2	
Cachaça	DEP				25.8	
	DBP				40.5	
	DEHP				140	

Table 6 (continued)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples		Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	Range (µg/g)	
Beer	DMP DEP DBP BBP DEHP DEHA	SPME		GC-MS	<0.58 23.80 ± 7.32 37.14 ± 6.43 1.49 ± 0.58 1.74 ± 0.59 0.48 ± 0.23		Carnol et al., 2017 (88)
Brandy	DBP DEHP DINP	USVA-DLLME		GC-MS		0.03 – 0.43 0.13 – 4.18 1.68 – 6.68	Montevecchi et al., 2017 (89)

Table 6 (continue)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	
Alcoholic beverage	BMPP	DLLME		GC-MS	0.1540	Wang et al., 2017 (90)
	DEEP				0.2825	
	DEHP				1.0890	
	BBP				0.4271	
	DBEP				0.8929	
	DCHP				0.5426	
	DPHP				1.0510	
	DNOP				1.8190	
	DIBP				0.5450	
	DBP				0.0263	
	DHXP				0.3324	
	DMEP				0.2117	
	DMP				0.0915	
	DEP				0.0870	
	DPP				0.2183	

Table 6 (continued)

Matrix	Phthalate	Sample pre-treatment		Method	Positive Samples	Reference
		Extraction	Clean-up	Detection method	Concentration (µg/L)	
Wine	DBP	MIP-SPE		HPLC-ESI-MS	2.5 ± 0.01	Barciela-Alonso et al., 2017 (91)
	BBP				0.3 ± 0.02	
	DEP				1.4 ± 0.3	
	DMP				5.0 ± 0.2	
Alcoholic beverages	DMP	IT-SPME (TPA/LDH)		HPLC-UV	5.2	Aghaziarati et al., 2020 (92)
	DBP				n.d.	
	DAP				7.4	
	DEHP				3.7	
Beer	DIPP	QuEChERS		GC-MS/MS	n.d.	Rodríguez-Ramos et al., 2020 (93)
	DNPP				n.d.	
	BBP				0.19 ± 0.08	
	DBEP				0.4 ± 0.2	
	DNOP				0.9 ± 0.7	
	DINP				n.d.	
	DIDP				1.1 ± 0.8	
Cider	DIPP				0.4 ± 0.2	
	DNPP				0.3 ± 0.1	
	BBP				n.d.	
	DBEP				n.d.	
	DNOP				n.d.	
	DINP				2.1 ± 0.2	
	DIDP				2.0 ± 0.2	

8. Phthalates Analysis: Extraction and Detection

The beginning of the “Plastic Era” occurred with the invention of Celluloid from Parkesine, which revolutionized the movie and photography industries. Since then plastic has substituted other materials like glass, wood, paper and metal (94).

The analysis of PEs is difficult due to their ubiquity in an analytical laboratory, and when analysing PEs in food matrixes it’s necessary to have into account the complexity of the food, the possibility of interfering compounds and matrix effects (94).

8.1. Sample preparation and extraction

Correctly preparing the food sample is a very important step because of its complexity with the presence of lipids, proteins, carbohydrates, organic acids, and others; furthermore, PEs are usually present in low concentrations, and there is a good chance of contamination during analysis, due to the ubiquity of PEs. For this reason, the methodologies for sample preparation involve always extraction and clean-up steps (94-96).

Solvent-based extraction techniques

The most common methodologies for the extraction of PEs from food are solvent-based extraction techniques, especially with non-fatty liquid food samples, such as drinks. Different solvents of lipophilic nature can be used, for example, n-hexane, cyclohexane, and dichloromethane (85, 94, 97, 98).

Liquid-liquid extraction (LLE) is normally used without further steps, when analysing samples of drinks, such as alcoholic beverages, or with an additional step of clean-up when analysing solid or semi-solid samples (99).

The Quick, easy, cheap, effective, rugged and safe (QuEChERS) method is the most commonly used when combining extraction and clean-up, usually with solvents such as acetonitrile (ACN) or ethyl acetate, following clean-up step with sorbents such as primary secondary amine (PSA) or octadecylsilane (C18) (94, 100). This approach was first used to extract pesticides from foods, however, nowadays, can be used for the extraction of several other compounds (72, 101). First, the sample is homogenized, the analyte of interest is extracted, then there is a dehydration phase with salts, followed by clean-up and, at last, analysis of the supernatant (83, 93). This method was used to extract PEs in wine samples by the team of Fasano, in 2015, (83) and in 2020, by Rodríguez-Ramos, in beer and cider samples (93).

Nowadays, however, there is a search for Green Chemistry methods that use less volume of organic solvents, are simpler and faster. Miniaturized liquid-phase microextraction (LPME) technique uses very low volumes of solvent, that is water immiscible, and acts as the acceptor phase in the extraction of the compounds of interest from a very small volume, millilitres, of an aqueous phase, the donor phase. This technique can be divided into three: single-drop microextraction (SDME), where the extractant is a drop suspended on the tip of a syringe taken after the extraction process; hollow-fibre-LPME (HF-LPME), where the extraction occurs in a liquid membrane that is in the pores of a hydrophobic porous hollow fibre in which there is the acceptor phase; and dispersive liquid-liquid microextraction (DLLME), where the extractant is mixed with an organic solvent miscible with water, called the disperser, and is then injected in the aqueous phase to form a cloudy solution that has a wide contact surface between the sample and the extractor. Then, after extraction, with centrifugation, a drop of the water-immiscible solvent that contains the analytes of interest is collected. In this last technique, the extraction solvents must have low volatility, be water-immiscible and be dispersant-miscible, to produce adequate extraction (99, 102, 103). Pérez-Outeiral et al., 2016 (86) used ultrasound-assisted DLLME followed by solidification of floating organic drop to determine the presence of PEs in food simulants and liquid samples with acceptable results. Montevecchi et al., 2017 (89) used ultrasound-vortex-assisted DLLME coupled to GC-MS for the determination of PEs in distillates with good results.

Sorbent-based extraction techniques

Solid sorbents can also be used for the extraction of PEs in food, with low solvent use. One of the most used methods is solid-phase microextraction (SPME) because it is a versatile method for the extraction in either gas or liquid phase and low consumption of solvents (104). This technique is commonly applied in the headspace mode (HS-SPME), in which the extraction fibre does not contact directly with the sample but with the headspace above the sample, or direct-immersion mode (DI-SPME), where the extraction fibre contacts directly with the sample, this mode has greater efficiency of extraction (70, 72). The most used sorbents in SPME are polymeric sorbents and sol-gel sorbents (88, 99, 105). This method combines sampling, extraction, purification, concentration, and injection in one procedure (73, 99). Moreira et al., 2015 (106) used CF-SPME (cooling fibre, SPME) to analyse eight PEs in spices and roasted chicken meat stored in plastics. Ye et al., 2009 (75) used an SMPE-GC method to determine trace PEs in beer samples.

The conventional method solid-phase extraction (SPE) can also be used for the extraction of PEs, with the use of sorbents such as molecular imprinted polymers (MIPs), C18 or

nanomaterial-based sorbents, with C18 the most common. It is based on the partition coefficient, where the separation occurs between the solid sorbent and the mobile phase and is dependent on the composition of the sorbent material and the shape and size of the sorbent bed (70, 95). Vidal et al., 2016 (87) used this method to extract PES from several beverage samples, such as water, beer, wine, cachaça and juices, with subsequent separation and detection by LC-DAD and LC-FID.

Magnetic-dispersive SPE (m-DSPE) is characterised by speed, simplicity and low consumption of sorbents and solvents, however, due to the commercial unavailability of magnetic particles modified with the specific functional groups, it is a less used technique (94).

Table 7 - Main extraction methods and their characteristics. Adapted from Haji Harunarashid et al., 2017 (96)

<table>
<tr><th>Extraction methods</th><th>Extractants</th><th>Advantages</th><th>Disadvantages</th></tr>
<tr><td>LLE</td><td>Organic solvents</td><td>Non-fatty liquid samples: no clean-up procedure.
Low cost.
Reduced retention time.</td><td>Oil and fatty extract: clean-up with different SPE phase.
Fatty solid foods: addition of aluminium oxide and sodium chloride solution to decrease interference from proteins, fats, and other components; addition of sodium chloride/sodium sulphate to eliminate water.</td></tr>
<tr><td>DLLME</td><td rowspan="3">Chloro-containing organic extractants

Ionic liquids as green extractants.</td><td>Better efficiency, simplicity and rapidity than LLE.
Few µL organic solvent required
Fast
Inexpensive
Simple equipment
Low cost</td><td rowspan="3">Possible environmental pollution due to the chloro-containing organic solvents but only microliters are used.
Ionic liquids are: Unstable; Tendency to decompose when in contact with some metallic catalysts; The synthesis of ionic liquids requires few toxic solvents; Complex purification process; High cost; Limited wide application.
Samples are not well separated: may require further centrifugation.
Disperser solvent peaks may overlap with analyte peaks.</td></tr>
<tr><td>UA-DLLME</td><td>Reduce the volume of solvent used
Simple, inexpensive, and more reliable than DLLME.
Reduced solvent volume
Improved extraction efficiency.
Able to analyse matrices with large alcohol content.
Detect trace and ultra-trace levels.</td></tr>
<tr><td>UVA-DLLME</td></tr>
</table>

Table 7 (continued)

Extraction methods	Extractants	Advantages	Disadvantages
SPE	C18 C8 Polystyrene XAD-2 adsorbents Multiwall carbon nanotubes (MWCNTs)	Reduced solvent use. Improved extraction efficiency. Yield more purified extracts.	Often requires extensive sample handling and treatment of sample prior to analysis. High blank values. Requires clean-up using Florisil. Clogging of cartridges.
MIP-SPE	Polymer	Higher selectivity, sensitivity, and reliability than SPE	Usually require a polymer synthesis step
SPME	PDMS/DVB (polydimethylsiloxane/-divinylbenzene) fibre	In comparison to SPE: simple and efficient, low cost, solvent-free, does not require any prior sample preparation, able to reduce the risk of secondary contamination High sensitivity	Limited lifetime with the use of fibre due to the fragility and degradation. Batch- to-batch variation, artefact formation and low repeatability. Low capacity
HS-SPME		No sample manipulation is required and hence minimizing cross contamination from glassware, solvents, and samples.	Fibres have tendency to break and are relatively expensive Difficulty in quantification
DI-SPME		Simple, reduce the volume of solvents used, better linearity, repeatability and sensitivity	
QuEChERS	Organic solvents	Fast, simple, and inexpensive. Low solvent usage and waste. Minimum handling. Only requires few devices to carry out this procedure.	May require primary secondary amine to remove possible co-extracted matrix ingredients that can be mistaken as analyte and eluted at the same time.

8.2. Analytical determination

The physicochemical properties of the analyte and the sensitivity required are the bases for the selection of the instrumental technique for separation and detection. Therefore, the most used techniques are liquid chromatography (LC) and gas chromatography (GC) coupled to mass spectrometry (MS) detection system (72, 96).

Gas Chromatography

In this technique, volatile compounds are separated by their vapour tension and relative affinity for a stationary phase inside an open tubular column. PEs are volatile and thermo-stable, which results in GC as the most commonly used separation technique in food products (72). The polarity of the analytes is the most important parameter when selecting an analytical column, therefore due to their non-polar nature, non-polar fused-silica columns (5% phenyl/95% dimethylpolysiloxane) are mostly used for separation (74, 95, 107, 108). GC-MS, has been the most used approach to determine PEs due to its high sensitivity and reliability (73, 74, 76, 78, 81-85, 88-90, 93).

Liquid Chromatography

As a reliable alternative to GC, there is LC separation for the analysis of PEs, with HPLC being the most used technique. Due to the non-polar characteristic of PEs, C18 columns are the most used. A gradient elution is necessary due to differences in the physicochemical properties of PEs; therefore acetonitrile (ACN)/water is the most common choice, followed by MeOH/water, due to its lower viscosity coefficient and higher elution power. It can also be added formic or acetic acid to improve elution (94, 95). The team of Barciela (91) used HPLC as a separation and detection technique after MIP-SPE extraction of PEs in wine samples. Also, Garcia Ibarra and co-workers (109) used HPLC-MS/MS to separate and detect four PEs in cereal product samples.

Detectors

An MS detector with electron impact ionization (EI) is the most used, due to its specificity and high sensitivity, for detecting PEs at the low levels they are present in food matrice. A single quadrupole (Q) with single ion monitoring (SIM) mode or a triple quadrupole (QqQ) with multiple reaction monitoring (MRM) mode can be used. Other detectors used are FID, in the works of Ye, Cinnelli, Cirillo and Pérez-Outeiral (75, 77, 86, 110), UV, by Aghaziarati in 2020, (92) and DAD (Diode Array Detector), in the analysis of alcoholic samples by the teams of Fan and Vidal (79, 87, 94, 95). Electron Impact (EI) is the most applied ionization technique because the contents of PEs in food samples are generally at ultra-trace levels.

The enhanced selectivity, by using SIM mode, is another advantage of the MS detector, because it reduces the requirement for chromatographic separation and increases the sensitivity of detection (72).

Internal Standard

Several compounds have been used as internal standards (ISs), d_{10}-pheanthrene, deuterated-DEHP (d_4-DEHP), or deuterated-dibutyl phthalate (d_4-DBP), and non-deuterated compounds, such as BBP, anthracene, or pyrene. Usually, ISs are added at the beginning of the process, to correct any irreproducibility (94, 107).

9. Challenges in the determination of PEs

The biggest challenge in PEs analysis is their ubiquitous presence in the laboratory. In analytical laboratories, plastic materials are widely used, due to their low price, which can result in background signals of PEs presence contaminating the samples. Also, PEs contamination is not only from plastic materials of the laboratory such as plastic containers or pipette tips but also from solvents, sorbents, or from the environment air (72, 95, 96, 111). The products used to clean the laboratory and personal hygiene products used by the researcher may also be sources of contamination (107).

There are several strategies to reduce or avoid contamination, such as rising the plastic materials with an organic solvent, substitute plastic materials by Teflon, aluminium, stainless steel, or glassware, clean volumetric glassware with oxidising agents, and calcinate non-volumetric glassware at 450-550 ºC after cleaning with water and organic solvents (94, 111). Also, using high purity solvents certificated with the lowest possible contamination, purified with aluminium oxide, and using PEs free gloves and pipette tips (72, 94, 95).

The sampling should be performed in glass containers, and these should be cleaned with solvents and dried at 400 ºC. The containers must be stored shut as there is the possibility of absorption of phthalate from the laboratory atmosphere onto the glass walls of the container. The lids used to seal the containers must also be analysed and/or cleaned. In the sampling process, any contact of the sample and personnel's hands or plastic gloves must be avoided, being preferred the use of metallic instruments such as spatulas or forceps. The samples then must be stored at 4ºC, if the analysis is performed either in the day of the collection or in the next few days, otherwise in should be kept at -20 ºC due to phthalate biodegradation (107, 111). During analysis, procedural blanks during each set of

samples should be performed, where the contamination from the different materials, solvents and sorbents is tested (94).

Another source of phthalate contamination is the chromatographic system, particularly in the inlet and gas supply system, with septa, liners, and rings. In splitless injection mode there is the formation of a solvent vapour that could be able to extract phthalates from these parts of the equipment. The vial caps are also a concern with phthalates contamination, therefore certain precautions should be enforced, either the use of aluminium or tin caps, free of PEs, the use of materials such as aluminium foil as replacement of the caps in the vials, and not inject twice from the same vial (107).

Experimental procedure

1. Aim of the study

Phthalates are considered ubiquitous contaminants of the environment due to their considerable use in the plastic industry. These contaminants have come to attention in the last years due to their harmful health effects and presence in food via migration from plastic packages and/or from plastic components of production equipment's. The main objective of this work was to optimise and validate a method based on DLLME extraction and GC-MS detection for the simultaneous determination of six phthalates (DMP, DEP, DIBP, DBP, BBP and DEHP), which are commonly used plasticizers and therefore frequently food contaminants, and one adipate (DEHA), which is now mostly used as a substitute of DEHP, in commercial beer samples, obtained from several markets in the Porto region, Portugal, in order to evaluate the possible contamination of a food product that is widely consumed.

2. Materials and Methods

2.1. Standards and Reagents

Analytical standards of dimethyl phthalate (DMP), diethyl phthalate (DEP), di-isobutyl phthalate (DIBP), di-butyl phthalate (DBP), benzyl butyl phthalate (BBP), di-ethylhexyl adipate (DEHA), di-ethylhexyl phthalate (DEHP), and internal standard dioctyl phthalate-d4 (DNOP-d4), with standard purity of ≥99%, were obtained from Supelco/Sigma-Aldrich (St. Louis, MO, USA). The working solutions at 10 μgL^{-1} and 100 μgL^{-1} were prepared in ethanol (EtOH), HPLC grade, and kept refrigerated (~ 4 °C) until the analysis. Hexane was used as extraction solvent, and methanol (MeOH) HPLC grade, and purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Materials

MeOH, EtOH and hexane HPLC grade solvents were tested for the presence of phthalates and MeOH was found to have the least concentration. Therefore, it was selected as the washing solvent and blank solution. The same batch of solvent was used throughout the experiment.

Due to the ubiquitous nature of phthalates, all materials other than pipette tips were glassware. The glassware was carefully washed and previously rinsed with EtOH, and

MeOH before use, also, calcinated when possible. The plastic pipette tips were left overnight in EtOH at 70 ºC, rinsed with EtOH and dried before use. All vial caps had a layer of aluminium foil to avoid phthalate contamination.

3. Sampling

Beers (n= 66) of different brands (n= 50), composition, alcohol content (0 – 8,5 %) and packaging (aluminium can (C), glass bottle (B), pressurized (P)) were bought in several local supermarkets of Porto, Portugal (Table 8). The samples were kept refrigerated, at 4 ºC, until time of analysis.

Table 8 – Commercial beer samples acquired from various markets in Porto

Sample	Brand	Container	Alcohol %	Sample	Brand	Container	Alcohol %
S1	A	Aluminium can	5.2	**S34**	V	Glass bottle	4.7
S2	A	Glass bottle	<0.5	**S35**	W	Aluminium can	4.9
S3	A	Glass bottle	5.2	**S36**	X	Aluminium can	4.2
S4	B	Aluminium can	5	**S37**	Y	Aluminium can	5
S5	C	Aluminium can	6.8	**S38**	Z	Aluminium can	4.5
S6	D	Aluminium can	5.6	**S39**	Z	Aluminium can	0
S7	E	Aluminium can	7.8	**S40**	AA	Aluminium can	5
S8	F	Aluminium can	4.9	**S41**	AB	Aluminium can	5.1
S9	G	Aluminium can	7.9	**S42**	AB	Glass bottle	5.1
S10	H	Aluminium can	7.5	**S43**	AC	Aluminium can	5.2
S11	I	Aluminium can	5.6	**S44**	AD	Aluminium can	5
S12	J	Aluminium can	4.9	**S45**	AE	Aluminium can	4.5
S13	J	Aluminium can	<0.5	**S46**	AF	Glass bottle	5.3
S14	K	Aluminium can	5.4	**S47**	AG	Glass bottle	5.2
S15	L	Aluminium can	6	**S48**	AH	Glass bottle	8
S16	L	Glass bottle	6	**S49**	AI	Glass bottle	4.8
S17	M	Aluminium can	5.8	**S50**	AJ	Glass bottle	6
S18	M	Aluminium can	0	**S51**	AK	Glass bottle	5
S19	O	Aluminium can	5.9	**S52**	AL	Glass bottle	7.5
S20	O	Glass bottle	5.9	**S53**	AM	Aluminium can	0
S21	P	Aluminium can	5	**S54**	AN	Glass bottle	5.3
S22	P	Aluminium can	0	**S55**	AO	Glass bottle	7.9
S23	P	Glass bottle	5	**S56**	AP	Glass bottle	5.9
S24	Q	Aluminium can	4.1	**S57**	AQ	Glass bottle	6

S25	Q	Aluminium can	0	**S58**	AR	Glass bottle	5
S26	R	Aluminium can	6.2	**S59**	AS	Glass bottle	4.5
S27	S	Aluminium can	4.6	**S60**	AT	Glass bottle	4.9
S28	T	Aluminium can	5	**S61**	AU	Glass bottle	4.9
S29	T	Glass bottle	5	**S62**	AV	Glass bottle	5.2
S30	U	Aluminium can	5	**S63**	AX	Glass bottle	6.2
S31	U	Aluminium can	0	**S64**	AY	Glass bottle	8.5
S32	U	Glass bottle	5	**S65**	AZ	Glass bottle	6.6
S33	V	Aluminium can	4.7	**S66**	A	Pressurized beer	5.2

4. Extraction procedure

A DLLME extraction procedure, previously developed by Caldeirão et al., 2021 (112) in herbal based soft drinks, was adapted and used. A sample volume of 10 ml was first degasified by sonication for 15 minutes and added to a glass centrifuge tube. Next, the samples were spiked with 50 µg/L of IS (DNOP-d4), and 300 µL of n-hexane were added. The tube was capped with a layer of aluminium foil and centrifuged for 3 minutes at 1800 rpm. The resulting extract, 200 µL, was transferred to an insert, placed inside an injection vial caped with aluminium foil and a volume of 1 µL was injected into the GC-MS/MS system.

Due to the difficulty of finding a blank solution without phthalates, a MeOH (methanol) solvent was used as blank.

5. GC-MS conditions

An Agilent 7890B gas chromatograph equipped with an Agilent 7693A auto-sampler (Agilent, Little Falls, DE, USA), and electronically controlled split/splitless injection port, coupled with a 7000C triple quadrupole mass spectrometer (Agilent Technologies, Inc., Santa Clara, CA, USA) with electron ionization (EI) chamber, was used for PE and DEHA analysis.

GC separation was achieved on a Phenomenex ZB-35HT Inferno TM column (30 m x 0.25 mm x 0.25 µm film thickness (Phenomenex, USA). The oven temperature started at 90 ºC, was held for 1 minute, then increased to 300 ºC at a rate of 20 ºC min^{-1} and held for 5 minutes, The total run time was 16.5 minutes. Ultrahigh-purity helium (99.999%; Gasin, Portugal) was used as carrier gas at a rate of 1.0 mL min^{-1}. The injector was maintained at 300 °C in pulsed splitless mode (0.5 min purge-off, 35 psi), and 1.0 µL of the extract was

injected. A Merlin Microseal TM septum (Agilent) was used to prevent silicone rubber contamination on analysis due to septum degradation through repeated injections. The triple-quadrupole MS was operated in multiple reaction monitoring (MRM) mode, detecting three transitions per analyte (table 9). The electron energy was 70 eV and the temperatures of the transfer line, ion source, and quadrupole were 300, 230, and 150 °C, respectively. Helium was used as quenching gas (2.25 mL min^{-1}) and nitrogen as collision gas (1.5 mL min^{-1}). System control and data acquisition were performed in MassHunter® software.

Table 9 – GC-MS/MS conditions

Analyte	Precursor ion (m/z)	Product ion (m/z)	Collision energy (kV)	Run time (min)	Time window
Dimethyl phthalate	164	78	20	6.985	1
	163	135	10		
	133	105	5		
Diethyl phthalate	177	149	5	7.726	2
	176	149	5		
	150	122	10		
	149	121	10		
Di-isobutyl phthalate	223	149	5	9.058	3
	167	149	5		
	149	121	15		
Di-butyl phthalate	223	149	5	9.618	4
	205	149	5		
	149	121	15		
Di-ethylhexyl) adipate	**129**	111	60	11.126	5
	129	101	5		
Benzyl butyl phthalate	206	149	5	11.803	6
	206	105	25		
	149.1	121	15		
Di-ethylhexyl phthalate	279	148.9	15	11.975	6
	167	149	5		
	149	121	15		
Dioctyl phthalate-d4 (IS)	283	153	10	13.09	7
	153	153	5		
	153	125	10		

Quantification ions in bold

Results and Discussion

1. Sample Optimization

The method chosen for the simultaneous extraction of 6 phthalates and 1 adipate from beer samples was based on a DLLME method previously developed by the research team in which I was integrated. DLLME is an eco-friendly method with the use of small volumes of solvent (few µL) that produces sensible and rapid results (112). Pre-sample treatment was not used, as is usual, with liquid samples.

2. Method Performance

Limits of Detection and Limits of Quantification

The limits of quantification (LOQ) were determined as the lowest linear concentration in the calibration curve and the limits of detection (LOD) were determined at signal-to-noise ratio of 3 (Table 11). DIBP, DBP and BBP had lower LOD and LOQ (0.3 µg/L and 1 µg/L, respectively) than DEHA and DEHP (0,6 µg/L and 2 µg/L, respectively) and DMP and DEP (1.5 µg/L and 5 µg/L, respectively). These results are similar to the study of Pérez-Outeiral and colleagues (86) which used an ultrasound-assisted DLLME/GC-FID, with LOD and LOQ of 0.7 – 2.82 µg/L and 1.93 – 8.47 µg/L, respectively. However, Wang team in 2017 (90) had lower LOD and LOQ (0.003 – 0.570 µg/L and 0.01 – 1.86 µg/L, respectively) with a DLLME/GC-MS based method using methanol and carbon tetrachloride as dispersive and extractive solvents.

Linearity

Linearity was determined by matrix-matched calibration by analysis of beer sample spiked at ten concentration levels (1, 2, 5, 10, 20, 50, 75, 125, 175 and 200 µg/L). The standard solutions were prepared by spiking 10 mL of a sample (free of analytes of interest) with the appropriated concentration of phthalate and adipate solutions, prepared in MeOH, and the extraction process was performed as described above, with the addition of the IS (d4-DNOP, 50 µg/L) before DLLME extraction to account for possible losses during the extractive process.

The calibration curves were constructed by plotting the compound/IS ratio against the concentrations of the analytes. The results demonstrated good linearity within the tested concentrations, with correlation coefficients (r^2) above 0.96 for all analytes (Table 11).

Precision

Intra-day and inter-day precision were determined at 5 µg/L, 35 µg/L, and 85 µg/L, where six spiked samples were extracted and analysed in two different days for a period of two weeks. Intra-day %RSD (relative standard deviations) were below 20.5% for all compounds at the three concentration levels, and inter-day %RSD, at concentration of 35 µg/L, were below 13.5% for all compounds. The presence of the IS was crucial to the improvement of the method repeatability.

Matrix Effect

To evaluate the matrix effect, the slopes of calibration curves obtained from solvent (EtOH) and from the matrix (standard added to beer samples commercially acquired) were compared.

Table 10 - Results of slopes obtained from the calibration curves in solvent (EtOH) and in matrix (beer)

Phthalate	**Solvent (EtOH)**		**Matrix**	
	CC slope	r^2	CC slope	r^2
DMP	0.0013	0.979	0.0008	0.983
DEP	0.0027	0.997	0.0019	0.991
DIBP	0.0034	0.989	0.0013	0.986
DBP	0.0032	0.981	0.0014	0.982
DEHA	0.0018	0.999	0.0001	0.964
BBP	0.0007	0.996	0.0004	0.975
DEHP	0.0021	0.996	0.0002	0.976

When analysing food samples there are usually high matrix effects observed, that negatively affect the quantification of the target compounds. The percentage of matrix effects was calculated for each compound tested, by the ratio of the slopes of the calibration curves in the matrix (beer sample) and in solvent (EtOH) multiplied by 100, in order to obtain the percentage of suppression or enhancement (Eq. 1).

Equation 1:

$$Matrix\ effect\ (\%) = \frac{m(CC\ matrix)}{m(CC\ EtOH)} x\ 100$$

A percentage of 100 indicates that there are no significant matrix effects, while values above indicate enhancement and values below show suppression (113). All compounds show matrix suppression effects (Figure 7), most with values ranging from 44% and 70%, with the exception of DEHA with a value of 6% and DEHP of 10%. Fierens et al., 2012, analysed the presence of phthalates in several food groups, with a method based on LLE for water based samples and verified that beer samples were especially affected by matrix interferences (76).

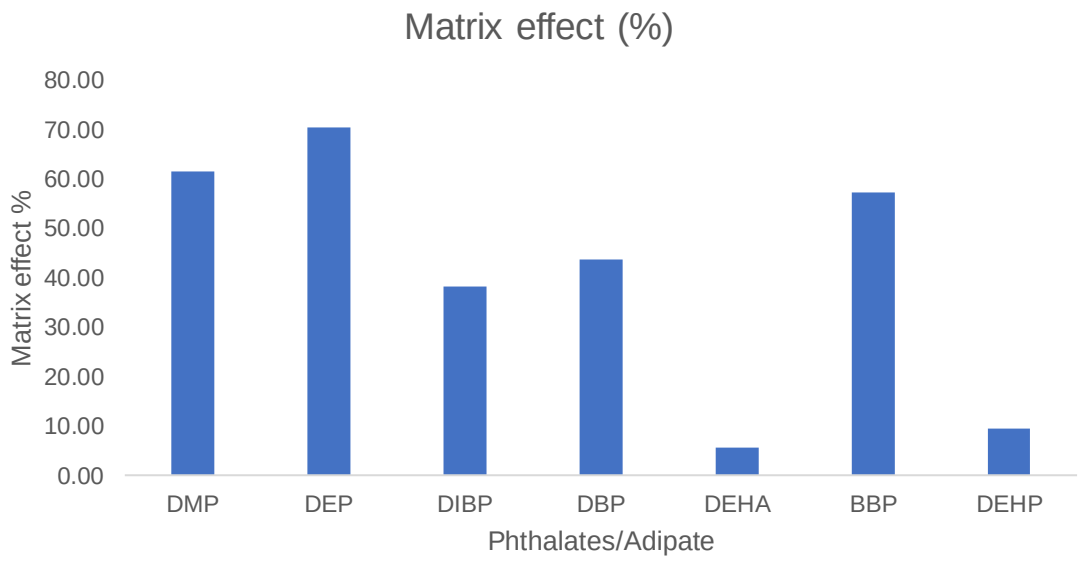

Figure 7 - Observed matrix effect (%) in the studied compounds

Table 11 – Performance of the analytical protocol for the studied analytes in spiked beer samples, with DLLME followed by GC-MS

Phthalate	Linearity		LOD	LOQ	Intra-day precision % RSD			Inter-day precision % RSD
	CC slope	r^2	(µg/L)	(µg/L)	5 (µg/L)	35 (µg/L)	85 (µg/L)	35 µg/L
DMP	0.0008	0.983	1.5	5	10.43	17.41	20.4	11.48
DEP	0.0019	0.991	1.5	5	11.69	12.28	1.94	13.34
DIBP	0.0013	0.986	0.3	1	3.10	4.81	1.36	7.23
DBP	0.0014	0,982	0.3	1	2.37	5.61	1.77	2.54
DEHA	0.0001	0.964	0.6	2	1.72	2.79	2.49	3.28
BBP	0.0004	0.975	0.3	1	3.95	4.97	4.30	13.36
DEHP	0.0002	0,976	0.6	2	1.98	3.55	7.19	6.98

3. Occurrence of phthalates/adipates in beer samples

The optimized method was applied to extract and quantify six phthalates and one adipate from 66 beer samples obtained from various local supermarkets in the region of Porto, Portugal. The results are presented in table 11.

In all the analysed samples (n=66), thirty-two presented positive results above the LOQ of the method. In twelve of the samples, only one PEs was detected – samples S7, S15, S27, and S66 were positive for the presence of DEHP (2.82, 16.25, 5.11, and 4.20 µg/L, respectively); in samples S13, S45, S52, S62, S64, and S65, only DEHA was detected (22.27, 5.24, 12.22, 10,74, 49.87, and 8.49 µg/L, respectively); BBP was the only phthalate detected in S51 (1.33 µg/L); and in sample S50 only DBP was detected (1.77 µg/L) (Figure 9).

Seven samples were positive for two analytes, in the case of S1 and S8, DEHA and DEHP were both detected (3.10 and 2.93 µg/L; 4.76, and 7.23 µg/L, respectively), in samples S3 and S5 were detected DBP and DEHP (1.77 and 7.94 µg/L; 2.34, and 11.44 µg/L, respectively), in sample S14 were detected DIBP and DEHA (3.39 and 3.06 µg/L, respectively), and in samples S21 and S25 both DEHA and BBP were detected (31.75 and 5.12 µg/L, respectively) (Figure 9).

DBP, DEHA and DEHP were detected in samples S4 (Figure 8) and S6 at concentrations of 7.59/10.85, 12.50/4.65, and 29.50/13.57 µg/L, respectively. DIBP, DBP and DEHA were detected in sample S17 (4.29, 8.86, and 17.85 µg/L, respectively). And DIBP, DEHA and BBP were detected in sample S44 (2.12, 205.40, and 9.11 µg/L, respectively). The concentration of DEHA in S44 was the highest concentration detected of all compounds (Figure 9).

Lastly, sample S29 had the highest number of compounds detected (n=5), DEP, DIBP, DBP, DEHA, and BBP were detected at concentrations 26.78, 56.43, 2.29, 4.24, and 14.68 µg/L, respectively (Figure 9).

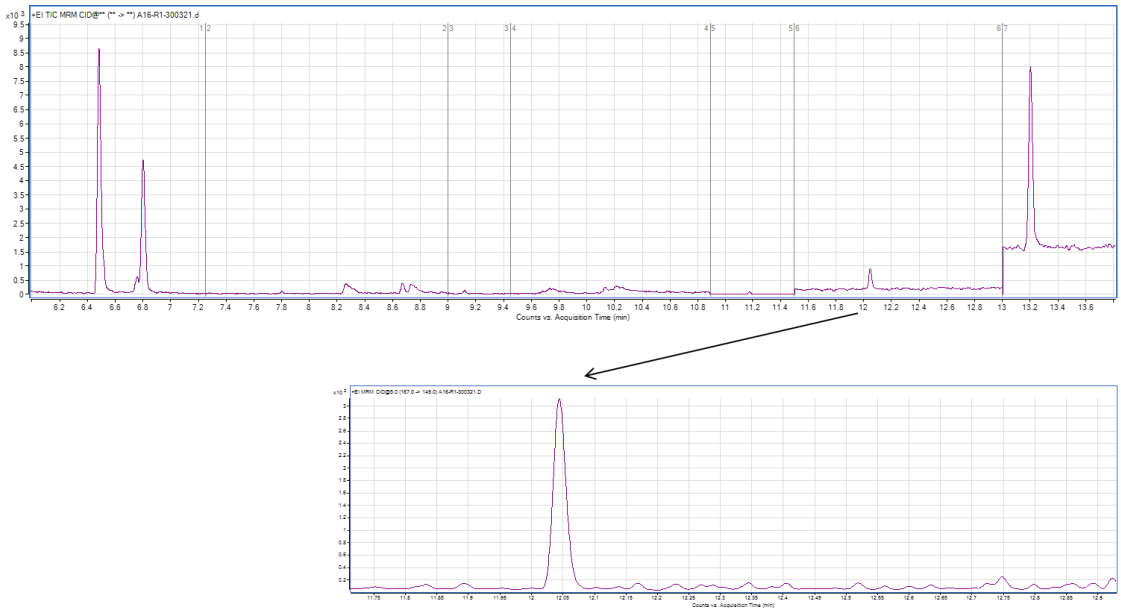

Figure 8 - Chromatogram of sample S4 with detection of DBP, DEHA and DEHP. Arrow points to the peak of DEHP.

Some PEs have SML to food products dictated by the EU, as is the case for DEHP, 1.5 mg/Kg and DEHA, 18 mg/Kg, and in our samples, the detected concentrations were below those limits. Other PEs are not as strictly regulated, have a limit of 60 mg/Kg, therefore the concentration of DEP, DIBP and BBP detected are also below the legislated limit.

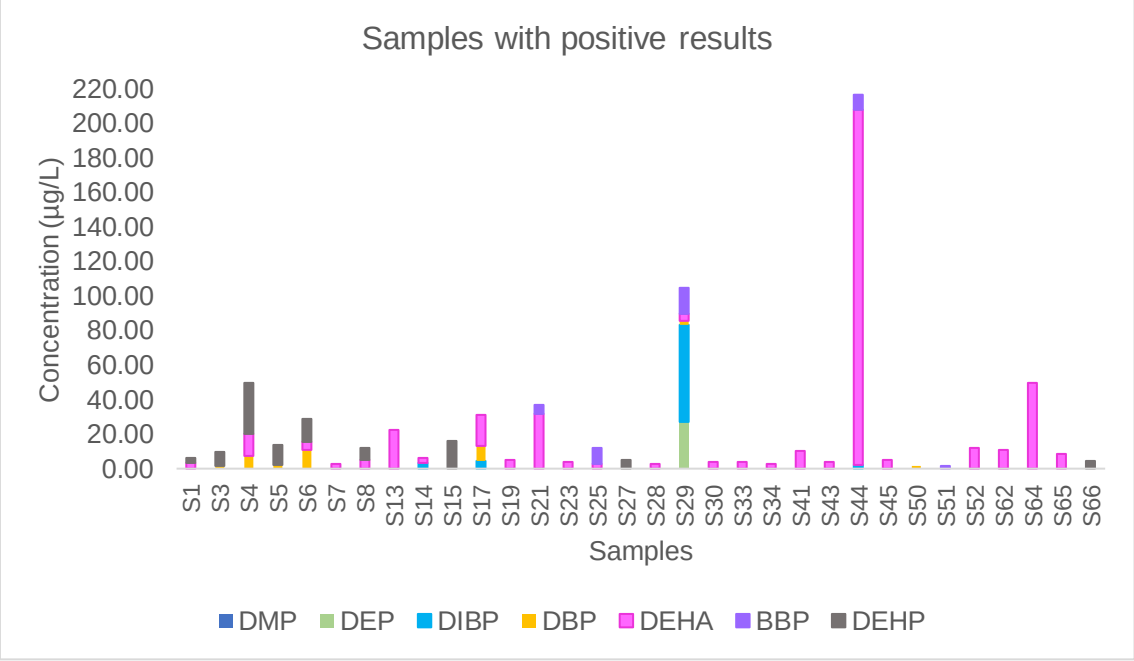

Figure 9 – Samples with detected phthalates concentrations above LOQ

DEHA was the most detected compound and the one detected at the highest concentration, followed by DEHP and DIBP. DMP, although a very common phthalate, was not detected in any sample (Figure 10).

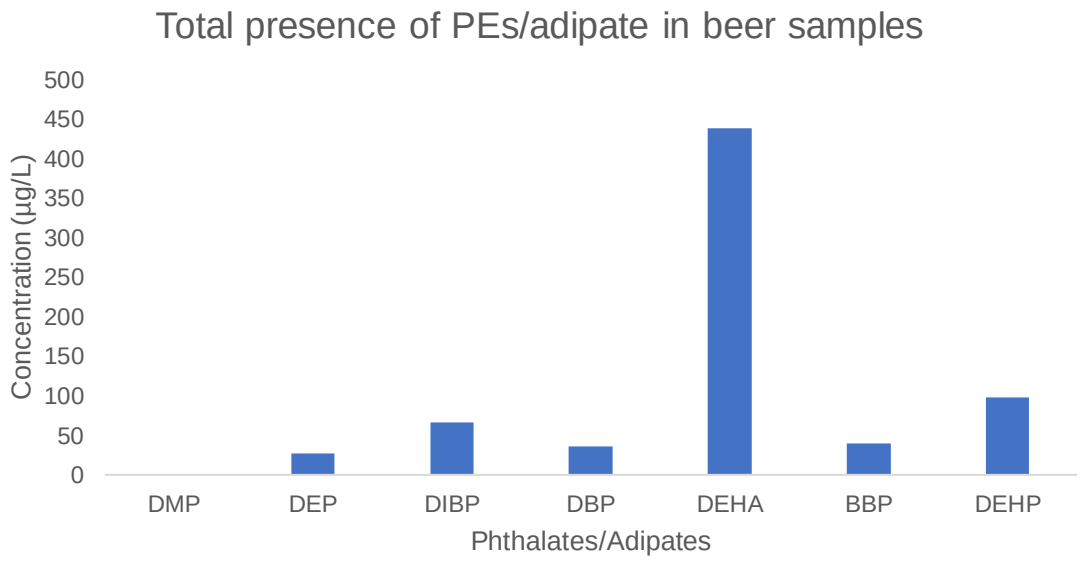

Figure 10 - Total presence of phthalates/adipate in 66 commercial beer samples

Table 12 – Phthalates and adipate levels (µg/L) measured in 66 commercial beer samples from markets of the region of Porto, Portugal

Sample	Phthalates/Adipate						
	DMP	DEP	DIBP	DBP	DEHA	BBP	DEHP
S1	n.d.	n.d.	n.d.	n.d.	**3.10**	n.d.	**2.93**
S2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S3	n.d.	n.d.	n.d.	**1.77**	<LOQ	n.d.	**7.94**
S4	n.d.	n.d.	n.d.	**7.59**	**12.5**	n.d.	**29.50**
S5	n.d.	n.d.	n.d.	**2.34**	<LOQ	n.d.	**11.44**
S6	n.d.	n.d.	n.d.	**10.85**	**4.65**	n.d.	**13.57**
S7	n.d.	n.d.	n.d.	n.d.	**2.82**	n.d.	n.d.
S8	n.d.	n.d.	n.d.	n.d.	**4.76**	n.d.	**7.23**
S9	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
S10	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
S11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S13	n.d.	<LOD	n.d.	n.d.	**22.27**	n.d.	n.d.
S14	n.d.	n.d.	**3.39**	n.d.	**3.06**	n.d.	n.d.
S15	n.d.	n.d.	n.d.	n.d.	<LOD	n.d.	**16.25**
S16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S17	n.d.	n.d.	**4.29**	**8.86**	**17.85**	n.d.	n.d.
S18	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
S19	n.d.	n.d.	n.d.	n.d.	**5.08**	n.d.	n.d.
S20	n.d.	n.d.	n.d.	n.d.	<LOD	<LOQ	n.d.
S21	n.d.	<LOD	<LOD	<LOQ	**31.75**	**5.12**	n.d.
S22	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
S23	n.d.	n.d.	n.d.	n.d.	**3.74**	n.d.	n.d.
S24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. – non detected; DMP and DEP – LOD: 1.5 µg/L; LOQ: 5; DIBP, DBP and BBP – LOD: 0.3 µg/L; LOQ: 1 µg/L; and DEHA and DEHP – LOD: 0.6 µg/L; LOQ: 2 µg/L

Table 12 (continuation)

Sample	Phthalates/Adipate						
	DMP	DEP	DIBP	DBP	DEHA	BBP	DEHP
S25	n.d.	n.d.	n.d.	<LOD	**2.80**	**9.37**	n.d.
S26	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
S27	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	**5.11**
S28	n.d.	n.d.	<LOQ	n.d.	**2.94**	n.d.	n.d.
S29	n.d.	**26.78**	**56.43**	**2.29**	**4.24**	**14.68**	n.d.
S30	n.d.	n.d.	n.d.	n.d.	**3.96**	n.d.	n.d.
S31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S33	n.d.	n.d.	n.d.	n.d.	**3.80**	n.d.	n.d.
S34	n.d.	n.d.	n.d.	n.d.	**2.84**	n.d.	n.d.
S35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S36	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S37	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S38	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S39	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S40	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S41	n.d.	n.d.	n.d.	n.d.	**9.99**	n.d.	<LOD
S42	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S43	n.d.	n.d.	n.d.	n.d.	**3.93**	n.d.	n.d.
S44	n.d.	n.d.	**2.12**	<LOQ	**205.40**	**9.11**	n.d.
S45	n.d.	n.d.	n.d.	n.d.	**5.24**	n.d.	n.d.
S46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S47	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S48	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S49	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.

n.d. – non detected; DMP and DEP – LOD: 1.5 µg/L; LOQ: 5; DIBP, DBP and BBP – LOD: 0.3 µg/L; LOQ: 1 µg/L; and DEHA and DEHP – LOD: 0.6 µg/L; LOQ: 2 µg/L

Table 12 (continuation)

Sample	Phthalates/Adipate						
	DMP	DEP	DIBP	DBP	DEHA	BBP	DEHP
S50	n.d.	n.d.	n.d.	**1.77**	n.d.	n.d.	n.d.
S51	n.d.	n.d.	n.d.	n.d.	<LOD	**1.33**	n.d.
S52	n.d.	n.d.	n.d.	n.d.	**12.22**	n.d.	n.d.
S53	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S54	n.d.	n.d.	n.d.	n.d.	<LOD	n.d.	n.d.
S55	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S56	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S57	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
S58	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S59	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
S60	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
S61	n.d.	n.d.	n.d.	n.d.	<LOD	n.d.	n.d.
S62	n.d.	n.d.	n.d.	n.d.	**10.74**	n.d.	n.d.
S63	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	n.d.
S64	n.d.	n.d.	n.d.	n.d.	**49.87**	n.d.	n.d.
S65	n.d.	n.d.	n.d.	n.d.	**8.49**	n.d.	n.d.
S66	n.d.	n.d.	n.d.	n.d.	<LOQ	n.d.	**4.20**

n.d. – non detected; DMP and DEP – LOD: 1.5 µg/L; LOQ: 5; DIBP, DBP and BBP – LOD: 0.3 µg/L; LOQ: 1 µg/L; and DEHA and DEHP – LOD: 0.6 µg/L; LOQ: 2 µg/L

In the literature, only nine works report on the presence of phthalates in beer samples. The most reported PEs were DBP, DEP, DEHP, DNOP, DIBP and BBP, which is in accordance with our results. However, in this work, the most detected EDs was the adipate DEHA, which is very commonly used instead of DEHP, and there wasn't any other reference to a screening of this compound in beer samples.

The concentrations detected seem to be in accordance with the other studies: usually, the contamination of PEs is low, below the SML imposed by the EU, and in the studies with a screening of various food groups, such as Fierens et al., 2012 (76), that includes beer samples, and Cao et al., 2015 (82), that includes wine as beverage samples, the alcoholic samples have a lower concentration of phthalates, when compared to other food groups such as fish and fish products, condiments or oils and fats, which may be explained by the low fat content of beer samples and the lipophilic nature of phthalates (114). Also, when a comparison is made between different alcoholic beverages, usually the beverages with a higher alcohol content have a higher concentration of PEs, as is demonstrated in the study of Fan et al., 2014 (79), where the authors analysed Chinese spirits and red wine samples, the spirits have a much higher alcohol content and a higher detection frequency of PEs; also in the work of Vidal et al., 2016 (87), where the pooled sample included several alcoholic beverages and between Cachaça, red and white wine and beer, the beer samples had the lowest concentration of PEs; and, in the work of Aghaziarati et al., 2020 (92), where the whisky samples had a higher PEs concentration than the beer sample. As referenced, the alcohol content is a major factor in the migration of phthalates from package materials, tubbing and other equipment in the production process due to their high solubility in ethanol (71, 85).

4. Types of Packaging

Three different types of packaging were studied, aluminium cans (n=37), glass bottles (n=28) and pressurized beer (n=1). Twenty-two positive samples were packed in aluminium cans (S1, S4, S5, S6, S7, S8, S13, S14, S15, S17, S19, S21, S23, S25, S27, S28, S30, S33, S41, S43, S44 and S45), nine samples in glass bottles (S3, S29, S34, S50, S51, S52, S62, S64 and S65), and one sample in the form of pressurized beer (S66). As it is shown in figure 11, in the aluminium can packed samples, five compounds were detected, DIBP, DBP, DEHA, BBP and DEHP, the DEHA at the highest average concentration of 9.45 µg/L. In the samples packed in glass bottles, there is a higher diversity of detected compounds, DEP, DIBP, DBP, DEHA, BBP and DEHP, and again, DEHA is the highest detected analyte,

however with a lower average concentration of 3.16 µg/L. In the pressurized beer sample, only DEHP was detected at a low concentration of 4.20 µg/L.

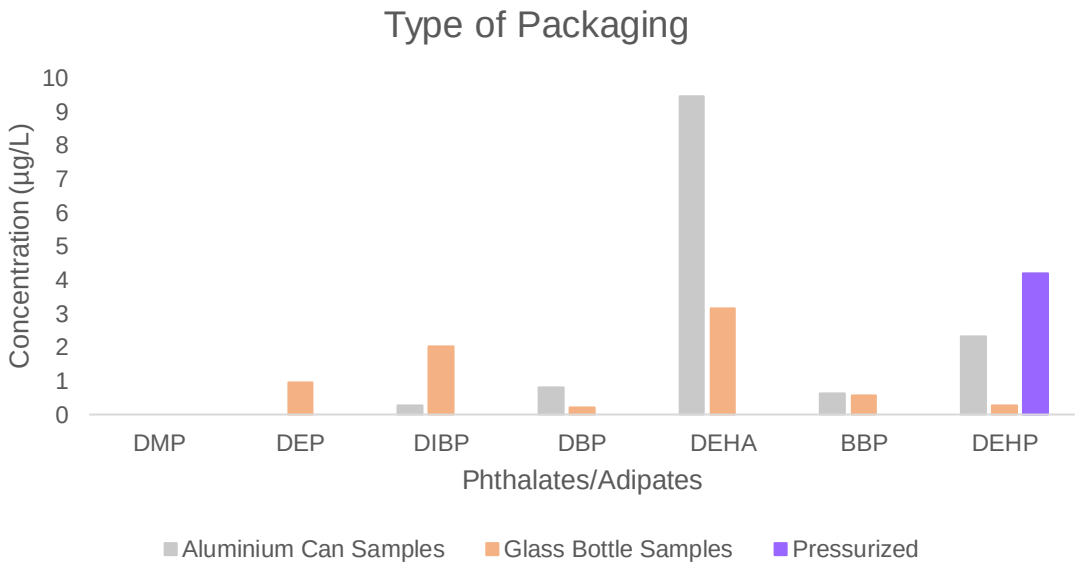

Figure 11 – Detection of phthalates/adipate in different types of packaging from commercial beer samples

The beer samples in aluminium cans have a higher average concentration of phthalates/adipates (13.48 µg/L) but less diversity with five different compounds, while the beer samples in glass bottles have a lower average concentration of phthalates/adipates (7.19 µg/L), but a higher number of different compounds was detected (n=6).

The compounds present in both aluminium cans and glass bottles are DIBP, DBP, DEHA, BBP and DEHP. DEHP is the only analyte present in all three types of packaging, aluminium cans, glass bottles and pressurized beer, reasonably explained by the widespread use of this specific phthalate in the plastic industry.

In the studies on PEs contamination in beer, only recently it is shown a differentiation and characterization of the type of packaging of the samples tested, Carnol et al., 2017 (88), tested the presence of different PEs and one adipate in 15 samples of Luxembourgish beer, 3 samples packed in cans, 10 samples packed in glass bottles and 2 samples packed in aluminium bottles, and found that, there was no statistical difference between the different packages, however, they had a small pool sample. Rodríguez-Ramos et al., 2020 (93) tested the presence of PEs in alcoholic and non-alcoholic beverages, including 10 samples of beer, 5 in glass containers, 2 in plastic containers and 3 in aluminium containers. The authors detected four different PEs (BBP, DBEP, DNOP and DIDP) in the samples in plastic containers, and consequently, these samples had a higher average concentration of PEs.

In the glass containers samples only BBP was detected at a low concentration and there was no detection in the samples stored in aluminium. It is difficult to analyse the effect the type of package container has on beer samples, due to the few studies available with an accurate description of the containers, because usually the studies are focused on a variety of different samples and not different packages.

Studies on wine, such as Carrillo et al., 2008 (73) and Del Carlo et al., 2008 (74), where the wine samples were also selected and characterised by their package, research its influence on phthalate content. In the first study, the packages are glass bottles, cartons and bag-inbox containers, and the authors found that samples packed in plastic had lower average concentrations of PEs than those packed in glass bottles or cartons, with the highest average concentration in the bottled samples. In the second study, the samples were packed in polyethylene coupled film brick and glass bottles from commercial, local production and pilot plant origins. The authors did not find influence of the type of packaging in phthalate content and considered environmental and production contamination origins.

The sample of pressurized beer is only contaminated with DEHP, and the low concentration may indicate that the contamination is due to the tubbing used in the beer dispenser equipment, and not the beer storage recipient.

5. Alcoholic vs. Non-alcoholic samples

The effect of alcohol in the migration of phthalates/adipates in the samples analysed was also studied. There were 59 samples of alcoholic beer, out of which 31 were contaminated and 7 samples of non-alcoholic beer, with only one positive sample.

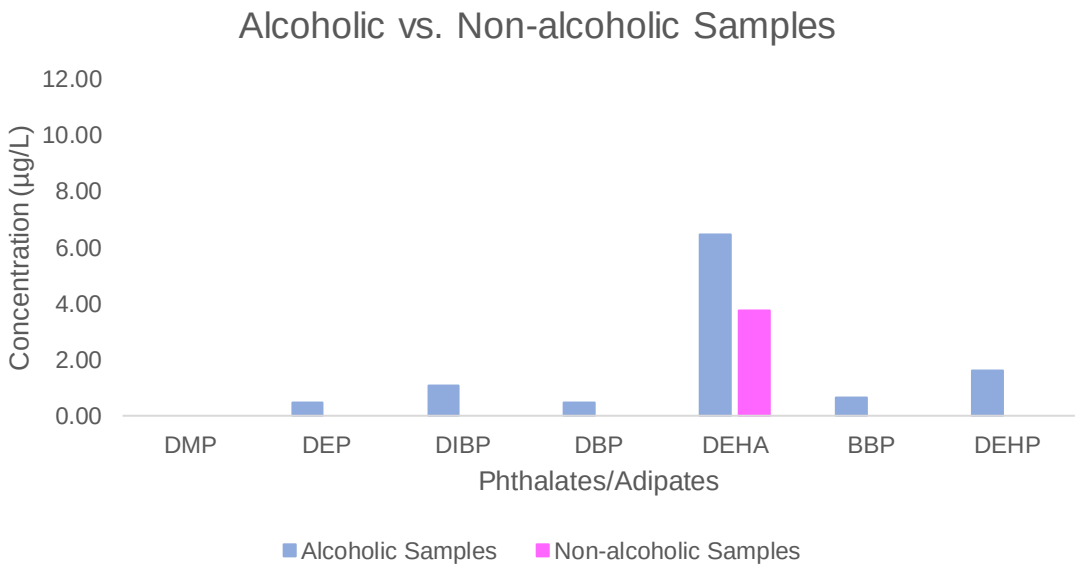

Figure 12 - Detection of phthalates/adipate in alcoholic and non-alcoholic commercial beer samples

The average concentration of phthalates/adipate in alcoholic samples is 10.73 µg/L, while there was only one detection of the analytes in the non-alcoholic samples at a low concentration (3.74 µg/L). Several studies have demonstrated that the presence of alcohol favours the migration of phthalates from packaging to food products, wherein the same tendency was observed, all the positive samples had alcohol (16, 79, 85).

Assuming that in both alcoholic beers and non-alcoholic beers are exposed to a similar concentration of PEs from plastic equipment and tubbing during production and packaging during storage. The presence of alcohol in beer samples may be an influencing factor in PEs migration during production and from the packages to the final product. Despite no studies on the presence of PEs in non-alcoholic beers have been reported.

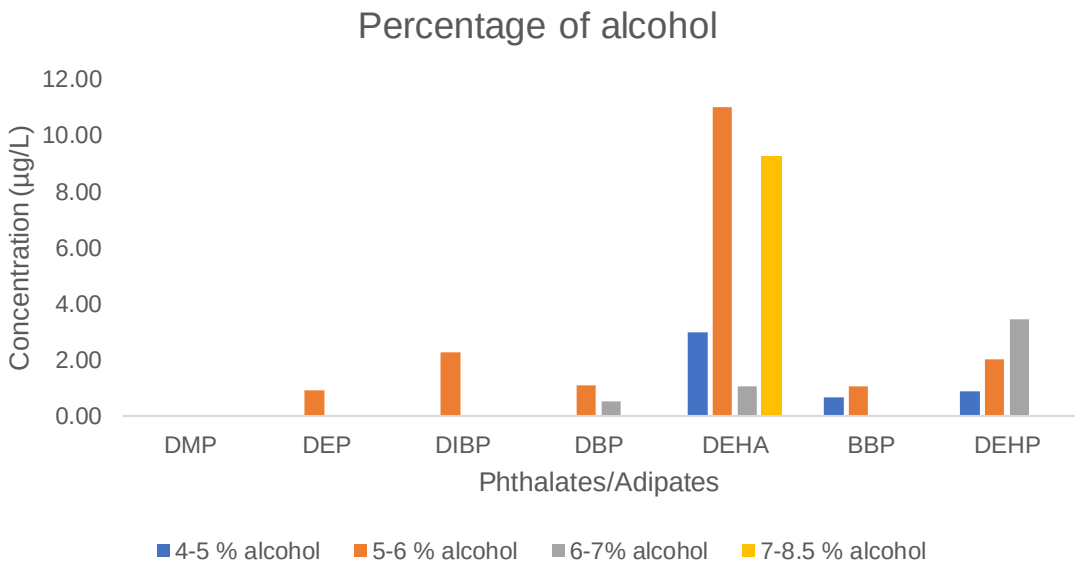

Figure 13 - Detection of phthalate/adipate in alcoholic samples with different alcohol percentage

In order to assess if different concentrations of alcohol could lead to different concentrations of PEs in the alcoholic samples, these were grouped and analysed in four different categories: (1) samples with a percentage of alcohol between 4 and 5 (n = 14); (2) samples with a percentage of alcohol between 5 and 6 (n = 29); (3) samples with a percentage of alcohol between 6 and 7 (n = 8); and (4) samples with a percentage of alcohol between 7 and 8.5 (n = 7).

The results demonstrate that there is a higher average and diversity of PEs in the samples with a lower alcohol percentage (5-6 % alcohol), contrary to what could be expected, which could indicate that other factors, such as the manufacturing environment of the samples or the type of container material, could have a bigger influence on the contamination of these types of samples.

6. Origin of the samples

With a rising of the market and demand of craft beers, these types of samples were also included in our selection. Craft beer was considered any sample labelled as “craft” or “artisanal”, 53 beer samples were of industrial origin and 13 were craft beers.

Four samples of craft origin were positive for DEHA (S7, S8, S62 and S64) and one sample was also positive for DEHP (S8), with an average phthalate/adipate concentration of 5.8 µg/L. In 28 samples of industrial origin DEP, DIBP, DBP, DEHA, BBP and DEHP were detected (average concentration of 11.87 µg/L) (Figure 13).

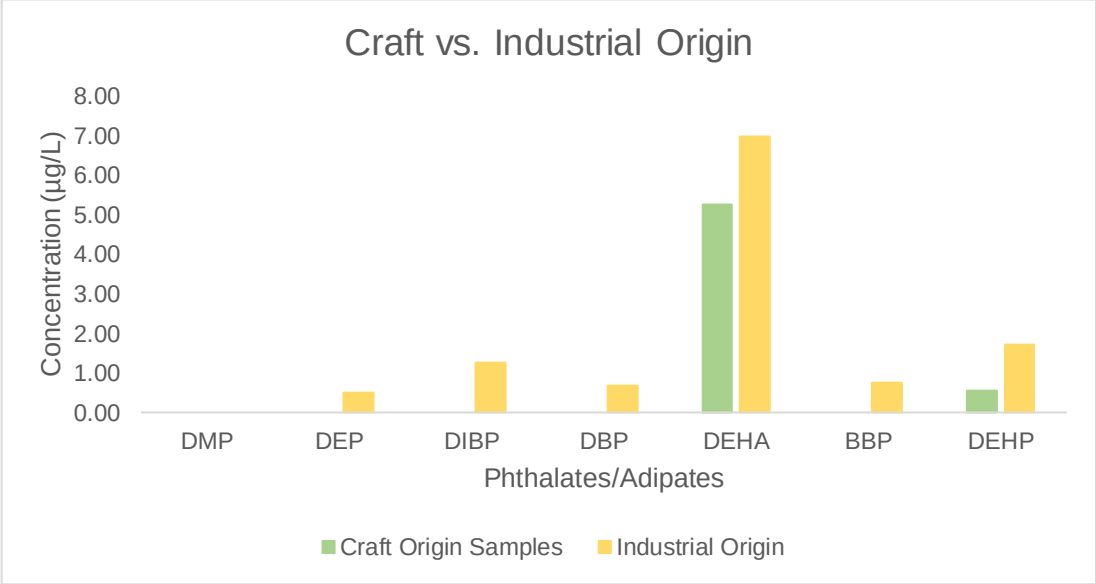

Figure 14 – Detection of phthalates/adipates in commercial beer samples of craft and industrial origin

The samples from industrial origin had a higher concentration of plasticizers contamination than the craft beer samples, probably due to the equipment used during processing. In an industrial environment, modern and sturdy equipment is necessary to maintain the necessary production quota of high volume for a long period of time, consequently plastic with its versatility, durability and low cost of production is a very common material. On the other hand, in craft beer production the materials used may have a lower plastic component. There are no studies on the presence of PEs in craft beers, therefore a comparison with other studies is not possible.

In other type of samples, such as wines, the study of Del Carlo et al., 2008 (74) is the only that considered the origin of the samples as a possible determinant factor in contamination.

The authors had commercial samples, private wine producer samples, and experimental pilot plant samples, and found that the frequency of PEs detection was dependent on the type of sample. While some PEs were detected in all samples, and seem to have an environmental contamination origin, other PEs, such as DBP and BBP, had a higher frequency of detection in commercial samples compared to the pilot plant samples, which may be due to the production process, as the authors recognized that only stainless-steel tanks and tubbing were used in the pilot plant.

Nowadays, there is a higher demand for craft products, which manufacture process uses more traditional techniques and equipment with less plastic. The industrialization of food processing, while leading to great advances in production times and yield, and diminished costs also uses a more automatic process with plastic tubbing and other components, which may result in higher plasticizers contamination rates.

7. Differences between samples of the same brand

Whenever possible, different types of beer, such as alcoholic, non-alcoholic and of different packaging's, of the same brand were purchased.

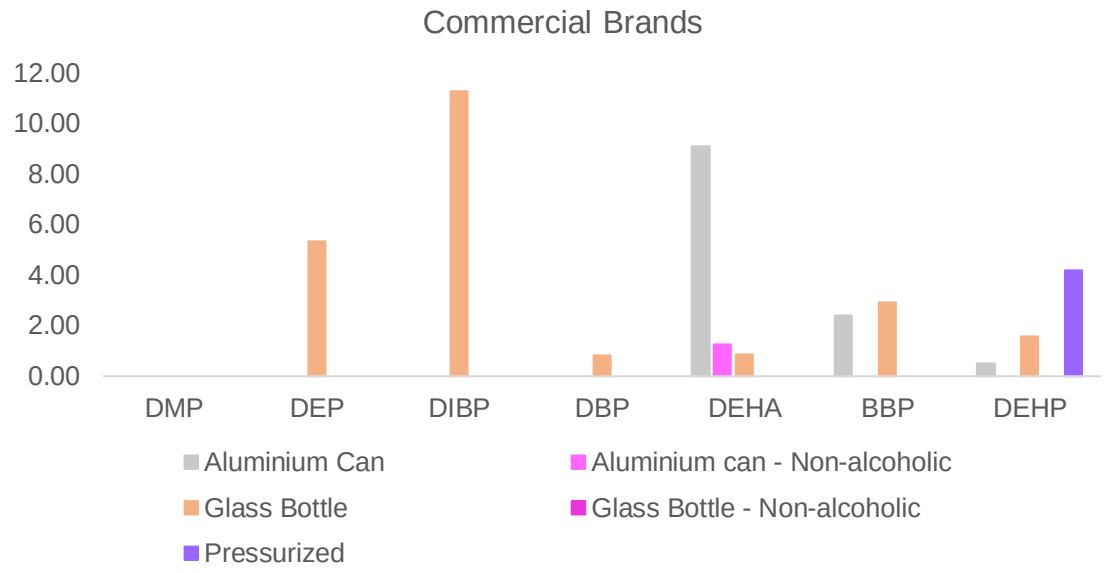

Figure 15 - Detection of phthalates/adipates in commercial brands beer samples

Commercial brand beer samples are considered as international and national well-known mass-produced brands, and include brand A (S1, S2, S3 and S66), brand P (S21, S22 and S23), brand Q (S25 and S25), brand T (S28 and S29), brand U (S30, S31 and S32) and brand AB (S41 and S43).

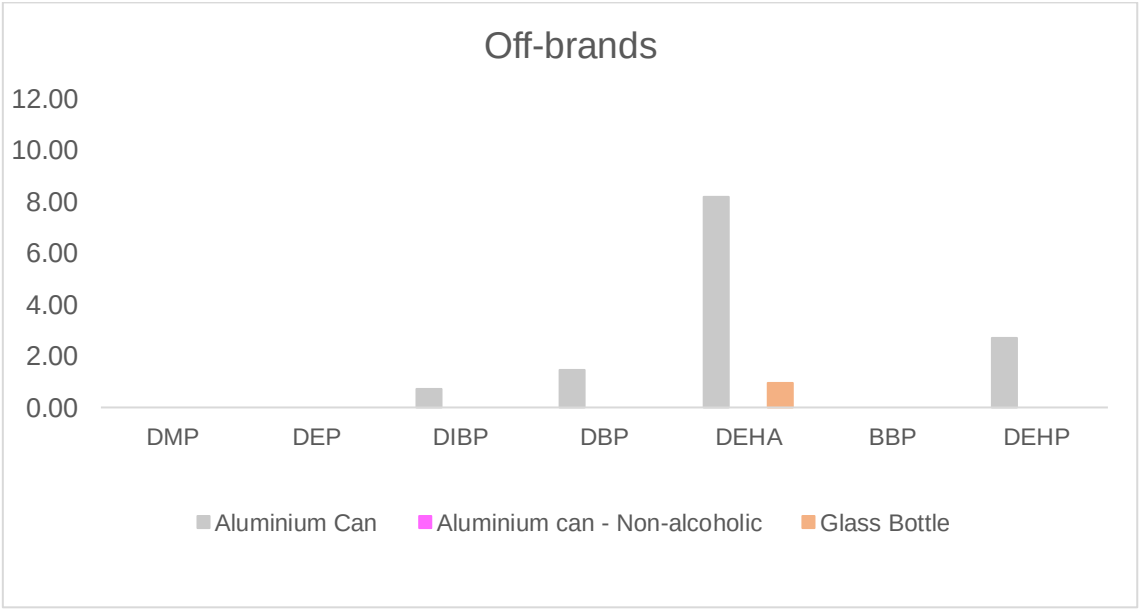

Figure 16 - Detection of phthalates/adipates in off-brand beer samples

Off-brand beer samples are considered as less known brands produced at a smaller scale, and include brand J (S12 and S13), brand L (S15 and S16), brand M (S17 and S18), brand O (S19, and S20), brand V (S33 and S34) and brand Z (S38 and S 39).

When we compared different samples, with different alcohol contents and different packages of the same brand, which supposedly have similar production conditions and quality control standards, differences were found. In commercial brand samples, a higher diversity of compounds were detected – DEP, DIBP, DBP, DEHA, BBP and DEHP, in a wide variety of types of packaging – aluminium can, glass bottle and pressurized beer. As it is possible to see in figure 14, there is a higher presence of the analytes in glass bottles, contrarily to what occurs in off-brand samples where there is a higher analytes content in aluminium cans. This may be explained by the production environment, where the contamination can be environmental or due to the processing equipment. Commercial brand samples are produced at a much larger scale than off-brand samples, which means a more industrialized environment prone to several sources of phthalate/adipate contamination, such as air, tubbing and/or storage recipients; also the contamination may be sourced to the bottles in their production, transportation and handling, before contact with the sample. It is of notice that, the only non-alcoholic beer sample with a positive result for the presence of phthalate/adipate is from a commercial brand, once again pointing to an environmental contamination, since there is no alcohol content to aid in the compounds migration to the sample. Non-withstanding, alcohol content is a major factor in phthalate contamination, as all positive sample but one, are alcoholic.

On the other hand, in off-brand samples, where the production is smaller, the contamination seems to be mainly caused by the package, as the lower diversity of analytes detected (DIBP, DBP, DEHA and DEHP) are concentrated mainly in samples packaged in aluminium cans. Alcohol content is also a factor, since the non-alcoholic samples were all negative in these off-brand samples.

Still, it must be taken into consideration that these samples were from different batches with different production and expiration dates, which may also explain the different results. Nevertheless, the same pattern observed in the evaluation of all the samples is observed within samples, with a major influence of alcohol content on the migration of phthalates, since most positive samples are alcoholic.

For a more thorough analysis of the possible sources of contamination it would be necessary to collect several samples from all the different stages of beer production, both in an industrial scale (larger and smaller) and at a craft/artisanal level. Also, it would be important to known the composition of all plastic materials used in the production process,

both of the equipment – tubbings, tanks, and other pieces, and any protective gear used by the workers that may contact with the product. The packages should also be analysed because different brands may used packages produced by different companies that may have a different percentage of plasticizers integrated in their plastic recipients or coverings. All of these factors may have a different contribution to phthalate/adipate contamination in the same type of food products, resulting in different contamination levels, and therefore should be evaluated.

Final remarks

The present work reports on an adaptation and application of a DLLME-GC-MS based method on commercial beer samples for the simultaneous detection and quantification of six phthalates (dimethyl phthalate, diethyl phthalate, di-isobutyl phthalate, di-butyl phthalate, benzyl-butyl phthalate and di-ethylhexyl phthalate) and one adipate (di-ethylhexyl adipate). The DLLME extraction procedure presents good linearity and precision, with low LOD and LOQ, and the matrix suppression effects observed were surpassed by the use of matrix-matched calibration curves. An adjustment to the method such as addition of a sonication step may allow for an improvement of the extraction procedure. Nevertheless, the method allows the detection of the target analytes at low concentrations in the order of few µg/L. This method was applied to sixty-six samples of commercial beer samples from several markets in Porto, with different types of packages (aluminium can, glass bottle and pressurized beer), different alcohol contents (alcoholic and non-alcoholic) and different manufacture origins (craft and industrial origin). The detected concentrations were all below the legislated SML. The most occurring compound was DEHA followed by DEHP, while DMP was not found in any sample. The occurrence of the compounds was dependent on the alcohol content, as all but one positive sample were alcoholic. Samples packaged in aluminium cans had a higher average concentration than samples packaged in glass bottles, probably due to the inner plastic film that protects the beer from metal of the can. The origin of the sample, craft or industrial, seems to be another influencing factor on phthalate contamination, as there was a much higher average concentration and diversity of phthalates/adipate in samples from industrial production. Likewise, when a comparison is made between samples of commercial brands and off brands, with different packaging and alcohol content, the same trend in results follows with higher DEHA concentration and higher average concentration in alcoholic samples, however commercial brand samples had higher phthalate detection in glass bottles, while off-brand samples had higher detection in aluminium samples. The difference may be the result of environmental contamination in the commercial samples. This study revealed that beer is a probable food exposure source to these contaminants, however, there is very few research on the subject with these types of food matrices. Also, this work demonstrated the presence of several different phthalates in the samples, even those that are not as controlled by food quality measures and have a higher legislated SML, indicating a necessity of a review of those limits. While their health effects may not be as severe at low concentration, the ubiquitous nature of these compounds means that we are susceptible to exposure from various sources leading to a cumulative exposure.

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