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Analytical Methods

Measurement of phthalates diesters in food using gas chromatography–tandem mass spectrometry

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ABSTRACT

An analytical strategy dedicated to 4 major phthalate diesters (DiBP, DnBP, BBzP and DEHP) monitoring in food items has been developed and validated according to normalized guidelines. The method has been applied to a wide range of foodstuffs ($n = 54$) to generate first-ever occurrence data at the French level. This method involves separation and detection using gas chromatography coupled to tandem mass spectrometry, in electron ionisation with highly specific selected reaction monitoring, quantification being performed according to the isotope dilution principle. A particular attention has been paid to background contamination management at any stage of the analytical process, from the sampling to the expression of the results. Limits of reporting, defined as statistically different from background contamination, were found to be 2.7, 0.53, 0.18 and 3.4 μg kg^{-1}, and relative combined uncertainties were finally found to be 7.6%, 12.2%, 12.0% and 14.1%, for DiBP, DnBP, BBzP and DEHP, respectively.

1. Introduction

Alkyl aryl diesters of 1,2-benzenedicarboxylic acid, hereafter referred to as "phthalate diesters", are a class of anthropogenic organic substances widely used for decades in a large range of industrial and consumer applications (Sioen et al., 2012). They have been primarily used as plasticisers to increase the flexibility of plastics such as polyvinyl chloride to which the historical production has been tightly linked. Although the most known representative for this application is the bis(2-ethylhexyl) phthalate (DEHP), two other higher-molecular-weight isomer mixtures, namely diisononyl (DINP) and diisodecyl (DIDP) phthalates have become the predominant sales since the beginning of the twentieth century, reaching 83% of the total phthalate sales in Europe in 2010 (ECHA, 2013). Lower-molecular-weight phthalate diesters, such as diethyl phthalate (DEP), di-*n*-butyl phthalate (DnBP), and butyl benzyl phthalate (BBzP), are commonly used as solvents to hold colour and scent in various consumer and personal care products (Cao, 2010). Phthalate diesters are not covalently bound to the polymer they are included in and are continuously released into the air or leach from the products. As a consequence, they can be found in indoor environments and human food, and constitute therefore part of the ubiquitous environmental contaminants (Wormuth, Scheringer, Vollenweider, & Hungerbuhler, 2006).

Phthalate diesters are known to induce toxic effects on liver, kidney and the reproductive system (Swan et al., 2005; Swan, 2008; Hauser & Calafat, 2005) and act partly as endocrine disrupting agents (Chauvigné et al., 2009; Eveillard et al., 2009), raising concern of the scientific community, consumers, producers and policy makers. To date in the European Union (EU), ten phthalate diesters have been classified by the European Chemical Agency (ECHA) as carcinogenic, mutagenic or toxic for reproduction (CMR) substances, with the mention "Reprotoxic 1B". This

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classification includes categories 4 down to 1, from the least to the most hazardous. In particular, di-2-methylpropyl phthalate (DiBP, CAS 84-69-5), di-*n*-butyl phthalate (DnBP, CAS 84-74-2), *n*-butylbenzyl phthalate (BBzP, CAS 85-68-7) and di-2-ethylhexyl phthalate (DEHP, CAS 117-81-7) are listed among reprotoxic 1B CMR substances.

Such classification consequently led to a series of regulatory restrictions in the EU for four activity sectors: toys and childcare articles, medical devices, personal care products and food contact materials (FCM). Regarding FCM, the European authorities set up slight restrictions and specifications for those compounds (repeated and single-use materials, non-fatty food, infant and follow-on formulae) along with specific migration limits (SML) in the Regulations related to plastic FCM (Regulation EC, 2004; EU, 2011). Additional specific dispositions have been described, aiming at reducing exposure of children and newborns.

Phthalate diesters have been detected in the environment, in food, and in various human matrices (Cao, 2010). Direct migration from packaging films appears to be one of the main sources in food. Phthalate diesters have been detected in solid food (Schecter et al., 2013), mainly composite samples (Cao, 2010; Fromme et al., 2007) and more specifically gasket sealed fatty food (Ezerskis, Morkunas, Suman, & Simoneau, 2007), but also liquid food products (Schecter et al., 2013) such as milk (Feng, Zhu, & Sensenstein, 2005; Sorensen, 2006), wine/beer (Carrillo, Martinez, & Tena, 2008; Del Carlo et al., 2008) or olive oil (Cavaliere, Macchione, Sindona, & Tagarelli, 2008), at levels reaching regularly the mg/kg (ppm) range. Such high levels might be of concern for at least some particular sub-populations for which daily intake may reach tolerated daily intake (TDI) for DEHP (Guo et al., 2012), although extensive total diet studies are missing for better risk assessment. Other sources of human exposure have been described, such as dust with median levels close to 500 mg/kg (Abb, Heinrich, Sorkau, & Lorenz, 2009; Fromme et al., 2004). Although exposure is believed to be primarily through ingestion and inhalation, dermal exposure may also be important for some particular phthalate diesters (*e.g.* DEP). Special populations, such as neonates in intensive care units, may be highly exposed to plasticizers through the use of medical devices (Sathyanarayana, 2008; Swan et al., 2005).

Phthalate diesters exhibit short biological half-lives, metabolise quickly into monoesters, further oxidation products and glucuronide conjugates, do not accumulate and are primarily excreted in urine (Swan et al., 2005), thus qualifying urine as the most studied human fluid for phthalate exposure assessment due to additional sampling conveniences. Reported half-lives are in the range of a few hours but constant exposure makes phthalate diesters metabolites almost always detectable in Human urine. Although the main phthalate diesters (DEP, DiBP, DnBP, BBzP, DEHP, DINP) have been reported both in the environment and in foodstuffs, as well as their metabolites in Human fluids, attesting from Human exposure, more extended and integrated data are required for better exposure assessment, especially on French territory for which data remain scarce.

Most analytical methods dedicated to phthalate diesters in foodstuffs are based on gas chromatography coupled to simple quadrupole mass spectrometry (GC–MS), with electron ionisation (Wenzl & Technical Reports, 2009). However, these methods may reach specificity and sensitivity limits when dealing with trace-levels far below the SMLs. Indeed, signals may suffer the presence of coeluted isomers or matrix interferences, as reported by Fierens et al. in beer for example (Fierens et al., 2012). Tandem mass spectrometry (MS/MS) has flourished significantly in recent years, offering higher potentialities for targeted analyses, in terms of specificity and sensitivity. GC–MS/MS has been reported for phthalate diesters analysis in bottled water (Guart et al., 2013), vegetable oil and oily food (Cavaliere et al., 2008; Liu, Wang, & Wang, 2013; Sannino, 2010) and in environmental matrices (only abstracts available and in English: Li, Tian, Ren, & Wang, 2011; Luo, Guo, & Sun, 2010).

Even more important when reaching low levels, ubiquitous presence of phthalate diesters in laboratory environment generates significant procedural contaminations which engender a huge challenge to minimize and control. Sources of contamination have been identified from injector parts of the GC instrument injector (syringe, septum, insert), plastic laboratory consumables, dust, adsorption to glassware and solvents (Fankhauser-Noti & Grob, 2007; Guo & Kannan, 2012; Guo et al., 2012; Marega, Grob, Moret, & Conte, 2013), which make it a very complex problem to manage. Identifying a source represents a meticulous and long-term work not always successful. Indeed, contamination is not necessarily homogenous in a series of samples and is different from one compound to another. Moreover, dealing with systematic procedural contamination, even though it is controlled, raises methodological questions regarding reporting of the results which can upstage usual problematic of pure limits of detection or quantification. Rare are the authors who mention how they minimize and control blank levels, what levels they obtain and how they take it into account when reporting the results (Fierens et al., 2012; Sannino, 2010). For example, Sannino (2010) reported mean method blank levels ($n = 10$) to be 30 μg kg^{-1} for DiBP, 15 μg kg^{-1} for DnBP and 10 μg kg^{-1}for DEHP in their study for 10 g sample size of oily food. Fierens et al. (2012) reported a strategy taking into account procedural contamination when reporting the results.

In the present work, a method dedicated to 4 major phthalate diesters (DiBP, DnBP, BBzP and DEHP), has been developed and validated according to official documents, such as Decision 2002/657/EC and/or both LAB GTA 26 (2010) and SANCO/10684 (2009) documents. It involves gas chromatography coupled to tandem mass spectrometry for enhanced specificity and insists on procedural contamination control and minimisation at any stage of the procedure, including short and simple sample preparation as a key to minimise potential sources. Then, this method was applied to a range of foodstuffs ($n = 54$), including liquid and solid samples, food from animal and plant origin with various compositions, to generate preliminary occurrence data at the French level. This method involves separation and detection using GC-EI(+)-MS/MS with highly specific selected monitored transitions, quantification being performed according to the isotope dilution principle. A particular attention has been paid to background contamination management at any stage of the analytical process, from the sampling to the expression of the results, through consumables and solvents, sample preparation environment and instrumental conditions.

2. Materials and method

2.1. Reagents and chemicals

Toluene, ethyl acetate and *n*-hexane were of Picograde® quality and provided by LGC Promochem (Wesel, Germany). HPLC grade acetonitrile was provided by Carlo Erba Réactifs (Rodano, Italy). Ultrapure water was purified using a Milli-Q-osmosis system from Millipore (Milford, MA, USA). Native di-2-methylpropyl phthalate (DiBP, CAS 84-69-5), di-*n*-butyl phthalate (DnBP, CAS 84-74-2), *n*-butylbenzyl phthalate (BBzP, CAS 85-68-7) and di-2-ethylhexyl phthalate (DEHP, CAS 117-81-7) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), with determined purities above 98.5%. All d_4-labelled compounds (ring labelling) were purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada), including d_4-DiBP, d_4-DnBP, d_4-BBzP, d_4-DEHP, di-*n*-octyl phthalate-d_4 (d_4-DnOP) and di-*n*-propyl phthalate-d_4 (d_4-DnPrP). d_4-DnPrP and d_4-DnOP were used as external standards. Stock

standard solutions as well as dilution series and working mixes were prepared in HPLC-grade acetonitrile and stored at 4 °C in glass flasks, in the dark.

2.2. Samples selection

The analytical strategy was developed for the determination of phthalate diesters in various categories of foodstuffs entering in a typical French diet: different kinds of liquid such as water, milk, oil, wine, soft drink, coffee, tea, cream, together with various solid matrices ready to eat such as vegetables, fruits, fish, crustacean, meat, ready-cooked dishes, biscuits, chocolate, cheese, desserts have been included in the method development.

Table 1
Samples description and results (ng/g) obtained for DiBP, DnBP, BBzP and DEHP analysis. All packaging materials are plastic based (Except S10, S17).

Code	Sample description	DiBP	DnBP	BBzP	DEHP
S01	Minced beef 5% fat	<LoR	0.7	0.5	3.3
S02	Minced beef 15% fat	<LoR	0.7	0.3	6.8
S03	Minced veal	<LoR	1.9	0.3	104
S04	Pickled pork rib	2.6	0.7	<LoR	<LoR
S05	Cooked ham	4.1	0.7	<LoR	4.6
S06	Pork chipolata	<LoR	1.6	<LoR	3.9
S07	Sliced bacon	7.1	1.1	<LoR	6.2
S08	Strasbourg sausages	4.5	28.8	0.5	39.1
S09	White hake	10.4	3.1	0.6	44.8
S10	Breaded fish	6.3	5.1	1.6	34.7
S11	Sliced salmon	6.1	0.7	<LoR	7.3
S12	Smoked salmon	5.5	1.5	<LoR	8.8
S13	Fish stick	2.7	0.7	<LoR	3.3
S14	Cooked small prawn	<LoR	0.5	<LoR	5.6
S15	Fresh egg	6.2	0.8	<LoR	5.4
S16	Whole milk	<LoR	0.5	<LoR	21.8
S17	Concentrated milk	2.9	0.4	0.7	25.5
S18	Drinking yogurt	<LoR	0.3	<LoR	<LoR
S19	Pressed cooked paste cheese	5.1	10.6	0.6	720
S20	Grated Emmental	8.9	28.2	1.1	186
S21	Melted cheddar	9.4	12.1	0.7	155
S22	Pre-cooked rice	13.6	3.6	0.6	53.1
S23	Buckwheat pancake	3.9	1.0	<LoR	9.8
S24	White bread	9.9	1.9	0.6	12.0
S25	Croissant	20.9	41.0	1.9	536
S26	Marble cake	24.7	5.4	0.8	23.4
S27	Potato crisps	12.0	9.9	0.7	9.5
S28	Popped corn, bacon flavour	40.3	54.2	0.7	114
S29	Extruded chocolate flavoured cereals	8.7	3.0	1.2	57.9
S30	Pine nuts	250	74.2	6.8	2376
S31	Apples	7.4	6.0	1.0	69.7
S32	Artichoke puree	3.1	0.6	1.1	5.5
S33	Leek	3.5	0.5	<LoR	<LoR
S34	Cooked beetroot	3.6	1.5	<LoR	9.6
S35	Salad	5.8	0.7	0.3	3.1
S36	Macaroni chicken mixed meal for infant	5.0	1.8	10.9	49.8
S37	Rice fish mixed meal for infant	3.8	1.4	0.5	5.8
S38	Green vegetables lamb mixed meal	4.2	2.0	1.2	31.4
S39	Cheese burger	9.2	3.0	0.8	64.8
S40	Beef shepherd's pie	7.6	2.5	1.0	31.3
S41	Provencal pizza	23.2	4.2	1.6	42.0
S42	Rice pudding	3.2	1.2	0.8	52.4
S43	Salted butter	14.1	1.8	0.7	219
S44	Light margarine	5.4	0.9	1.5	40.4
S45	Pizza oil	7.9	2.4	0.6	67.1
S46	Sweets	18.6	4.1	1.5	106
S47	Apple-banana compote	<LoR	1.3	0.5	12.8
S48	Sweet tomato sauce	<LoR	1.8	0.3	6.1
S49	Red wine	<LoR	72.8	7.1	<LoR
S50	Beer	<LoR	<LoR	<LoR	<LoR
S51	Cola	<LoR	<LoR	<LoR	<LoR
S52	Coconut, whey and kiwi based drink	<LoR	0.3	<LoR	<LoR
S53	Orange juice	<LoR	0.3	<LoR	<LoR
S54	Lemon juice	19.0	2.2	0.5	<LoR

<LoR: lower than limit of reporting.

Individual real samples (n = 54, Table 1) were collected on 5th October 2010, at a local retailer (Nantes, France). Their selection was oriented in order to cover a large panel of compositions with various cooking and storage characteristics and to plastic packaged items exclusively. For examples, samples were packaged under vacuum, modified atmosphere, non hermetic packages, cellophane or glass jars with gasket lids. Each sample was stored as recommended (temperature) and prepared for analysis before use-by date.

Reproducibility of the method was evaluated using ten food item samples selected in order to represent all food matrix types from typical French diet. Briefly, each sample was prepared from 16 sub-samples from the same type and cooked as consumed by the French population. Selected samples were roast pork, chicken egg, salmon, cheese, grain bread, vegetables pool, full-cream milk, sweet fermented milk, fruit puree and unsalted butter. Reproducibility was further assessed using an unsweetened condensed milk, fortified by 50 $\mu g\,kg^{-1}$ of each target substance and considered as in-house quality control (QC).

2.3. Sample preparation

The sample preparation strategy was thought as short and simple as possible in order to minimise potential sources of procedural contamination. Sample size was fitted at 10 g fresh weight. Depending on the matrix type, one of the three following extraction strategy was used. (i) Solid samples were freeze-dried and grinded, allowing reaching adequate homogeneity. Dried sample was weighted in a glass flask and spiked with 10 ng of each deuterated internal standard in 50 μL of acetonitrile. Acetonitrile (15 mL) was added and sonication in a warm bath was applied during one hour. Then, sample was placed at −20 °C during at least 12 h in order to freeze fat, before centrifugation (10 min, 6000 m/s^2) and equilibration at −20 °C again. An aliquot of cold liquid phase (700 μL) was transferred in a glass vial and concentrated to ~100 μL at 40 °C under a gentle stream of nitrogen. Vial was caped and placed at −20 °C during at least 12 h. The cold liquid phase was transferred in another vial, evaporated to dryness at 40 °C under a gentle stream of nitrogen and reconstituted in 200 μL of toluene containing 10 ng of each external standard. (ii) Liquid samples and samples inappropriate for freeze-drying (*e.g.* fruit compote) were directly weighted in a glass flask and spiked similarly with internal standards. Ethyl acetate (15 mL) was added and mechanical agitation was applied during one hour. Then, sample was centrifuged (10 min, 6000 m/s^2) and placed at −20 °C during at least 12 h. An aliquot of cold liquid organic layer (1400 μL) was transferred in a glass vial. After evaporation to dryness at 40 °C under a gentle stream of nitrogen, the dry extract was reconstituted in 600 μL of acetonitrile and vortexed at 40 °C. Vial was caped and placed at −20 °C during at least 12 h. About 200 μL of cold liquid phase was transferred in another vial, evaporated to dryness at 40 °C under a gentle stream of nitrogen and suspended in 200 μL of toluene containing 10 ng of each external standard. (iii) Fatty samples (*e.g.* butter, margarine, mayonnaise) were placed in an oven at 80 °C. When fat separation was not efficient enough, 15 mL of water were added. Hot sample was centrifuged (10 min, 6000 m/s^2, 40 °C) and the upper fat phase was transferred to another glass flask. Then, a liquid/liquid partitioning was carried out with 15 mL of acetonitrile. The sample was placed at −20 °C during at least 12 h in order to freeze fat, before centrifugation (10 min, 6000 m/s^2). An aliquot of cold acetonitrile phase (700 μL) was transferred in a glass vial and concentrated to ~100 μL at 40 °C under a gentle stream of nitrogen. Vial was caped and placed at −20 °C during at least 12 h. The cold liquid phase was transferred in another vial, evaporated to dryness at 40 °C under a

gentle stream of nitrogen and suspended in 200 μL of toluene containing 10 ng of each external standard.

2.4. Instrumentation

Measurement of phthalate diesters was carried out by GC–MS/MS. GC separation was achieved using a 7890A gas chromatograph (Agilent Technologies, San José, USA) equipped with a capillary column (30 m long × 0.25 mm internal diameter, film thickness 0.25 μm) coated with low bleeding diphenyl (5%)-dimethylpolysiloxane (95%) copolymer (ZB-5HT Inferno, Phenomenex, Le Pecq, France). Injector and transfer line temperatures were set at 280 °C and 320 °C, respectively. The temperature gradient started from 100 °C (2 min) and increased to 320 °C (6.3 min). Injected volume was 1 μL (splitless mode). Helium was used as carrier gas at 1 mL/min. Detection was performed on a 7000 GC triple quadrupole device (Agilent Technologies, San José, USA). Electron ionisation (EI) was operated at 70 eV. The mass spectrometer was operated in the selected reaction monitoring (SRM) acquisition mode. Monitored transitions were selected for their sensitivity, as well as their specificity (Table 2). Thus, the *m*/*z* 149 ion was not selected as precursor ion for DEHP, in order to reach better specificity, particularly to discriminate from coeluted di-*n*-heptyl phthalate and di-cyclohexyl phthalate. Argon was used as the collision gas.

2.5. Validation design

The validation procedure was performed according to an in-house validation procedure based on the Commission Decision (2002)657/EC requirements completed with both the French LAB GTA 26 (2010) and SANCO/10684 (2009) specifications. The general validation parameters, *i.e.* specificity, precision (repeatability component), robustness and stability were assessed, as well as linearity, recovery determination and quantification limits.

Specificity was determined in different categories of foodstuffs by checking the absence of interfering compounds in the range of the expected retention time of the target analyte.

Repeatability was assessed using ten samples covering a wide range of compositions and representative for foodstuff. For each one, six replicates at two fortification levels were analysed (40 and 500 μg kg^{-1}). Samples were analysed by 3 different operators during six weeks using various consumable lots.

The robustness was also evaluated by performing minor and major changes such as matrices, levels of supplementation, operators, different instruments, and different batches of standards, solvents and materials.

Linearity was determined by building a calibration curve using 7 levels of concentration in the range [0–3000 μg kg^{-1}], according to the isotopic dilution method. The recovery of the analyses was systematically calculated for all the samples. No certified reference material being commercially available, accuracy was assessed based on differences between levels quantified in previously described fortified samples and their corresponding unfortified aliquots ($n = 3$, each), as well as through proficiency tests in sunflower oil (T1242A and T1245, FAPAS, York, UK). Relative combined uncertainty was estimated based on precision, accuracy, standards purity and homogeneity, according to Eppe and De Pauw (2002), EURACHEM/CITAC guide 2000 (Ellison & Williams, 2012) and VAM project (Barwick & Ellison, 2000). Standards purities were higher than 98.5%. Homogeneity uncertainty was stated to be similar to the value determined for certified reference material CRM 607, at 2.4%.

Table 2
Diagnostic SRM transition parameters for GC–MS/MS analysis of phthalate diesters.

Compound	Retention time (min)	Transitions	Collision voltage (V)
DiBP and DnBP	11.61 and 12.24	223.1 > 149.0	10
		149.0 > 121.1	20
BBzP	14.65	206.1 > 149.0	10
		238.1 > 132.0	5
DEHP	15.63	279.2 > 149.0	10
		279.2 > 167.1	5
d4-DiBP and d4-DnBP	11.61 and 12.24	227.1 > 153.0	10
d4-BBzP	14.65	210.1 > 153.0	10
d4-DEHP	15.63	283.2 > 153.0	10
d4-DnPrP	10.95	213.1 > 153.0	10
d4-DnOP	16.56	283.2 > 153.0	10

2.6. Procedural contamination management

Due to their ubiquitous presence in laboratory environment, phthalate diesters are systematically detected in procedural blanks. Then, assessing, minimizing and controlling their occurrence is a major concern for the analyst. Strict precautions were respected to achieve acceptable procedural blank levels: standards and sample preparations were performed in an overpressurised room in order to minimize dust deposition; plastic consumables were avoided; glassware was rinsed with organic solvents prior to use or, whenever possible, was heated in a muffle oven at 400 °C during 4 h just prior to use; internal part of flask caps were rinsed with organic solvents or protected with aluminium foil; solvent batches were carefully chosen and checked before use. Concerning instrumental conditions, injection syringe and insert were manually washed with organic solvents before injection sequences. Washing solvents were frequently changed. Several toluene injections were performed when preparing an uninterrupted sequence, in order to purge the system. Finally, the remaining procedural contamination was demonstrated to come mainly from solvents, particularly acetonitrile. The global procedural contamination was monitored through procedural blank samples included in each analytical series. Whenever a quantification result beyond determined drastic threshold level was obtained for a blank sample, the whole analytical series was discarded for the considered phthalate. These threshold levels were set according to reasonably achievable values while maintaining requirements above which intra- and inter-series homogeneity becomes out of control. Thus, laboratory requirements regarding procedural contamination threshold levels were set at 6.0, 1.0, 0.4 and 6.0 μg kg^{-1} for DiBP, DnBP, BBzP and DEHP, respectively, based on a virtual sample size of 10 g. Procedural contamination control charts were implemented for early detection of any drift and to adjust requirements if appropriate and define hereafter mentioned limits of reporting.

2.7. Reporting of the results

Limits of detection (LOD) were assessed by calculating the corresponding signal to noise ratio of 3 (S/N = 3) using the internal standard signal, but were below the permanent procedural contamination. Therefore, due to this systematic procedural contamination, the traditional LOD and LOQ were not applicable. It was indeed preferred to report quantitative results statistically higher than the procedural contamination level. Following this strategy, the procedural contamination was characterised based on the successive series and control charts. Assuming that the distribution of procedural contamination corresponded to normal distributions, the mean blank level was deduced from raw quantification levels in each sample. Then, the limit of reporting (LoR) applicable to all series was defined as three times the standard deviation of

procedural contamination as defined in Section 2.6, the mean value of procedural contamination being deduced.

3. Results and discussion

3.1. Method performances

3.1.1. Specificity

The main objective of this work consisted in developing an efficient analytical workflow in terms of detection limits, identification and method efficiency for the analysis of four phthalate diesters in foodstuffs. The MS/MS method was developed in order to provide both specificity of the signals and enough identification points to fit identification's criteria as laid down in EU Commission Decision (2002)657/EC concerning the performance of analytical methods and the interpretation of results. As a pre-requisite of the identification step (2002/657/EC), relative retention times of the native compounds to their internal standard ones shall correspond to that obtained in the calibration solution with a tolerance of ±0.5% in the case of GC separation. Relative retention times of each phthalate were calculated in the 10 representative foodstuff samples extracts and were all below ±0.05%, which fits expectations and shows high stability of this parameter. According to the same guidelines, when operating GC–MS/MS analysis, two diagnostic transitions with correct signal ratios are required to meet expected number of identification points. Signal ratios observed in the 10 representative foodstuff samples extracts were within the tolerated range based on signal ratios obtained in the calibration curves. Regarding the specificity, the ion with m/z 149.0, corresponding to $[M]^{+\cdot}$ of the anhydride phthalic acid, appears as the base peak for all the phthalate diesters in electron ionisation, excepted for the dimethyl phthalate which shows a different fragmentation pattern, starting with the observation of $[M]^{+\cdot}$ and the loss of $[OCH_3]$ as the base peak. As a consequence, ion with m/z 149.0 cannot be considered as specific of a given phthalate diester isomer. Despite expected lower sensitivity, more specific precursor ions were privileged and selected, in order to improve the specificity of the signals. Indeed, precursor ions corresponding to the loss of one side chain and specific to the other chain length were selected whenever possible. The ion exhibiting m/z 279.2, selected as precursor for DEHP (Table 2), is specific to di-octyl phthalates and allows discriminating from the two co-eluted di-cyclohexyl and di-*n*-heptyl phthalates. Although the transition 149 > 121 was selected as secondary transition for DiBP and DnBP, no co-eluted phthalate diesters could be detected. Fig. 1 shows an example of typical foodstuff ion chromatograms on a macaroni chicken mixed meal for infant (S36 sample). It appeared that although main transitions selected for quantification are less sensitive than transitions based on m/z 149 as precursor ion, their signal-to-noise ratios (S/N) are better, due to lower matrix noise at higher m/z. Therefore, they provide higher specificity as well as better detection limit. Intense, well resolved but unidentified interfering compounds also appeared on m/z 149 > 121 transition at 14.0 and 16.0 min, illustrating the lower specificity of this transition compared to transitions with more specific precursor ions. Indeed, the corresponding substances were not observed on any of the other monitored transitions. At last, labelled standards signals did not show any interference.

3.1.2. Precision (repeatability component)

Results obtained for the variability assessment are detailed in the Table 3. From a global point of view, the mean repeatability is 1.8%, 2.5%, 1.8% and 3.7%, for DiBP, DnBP, BBzP and DEHP, respectively. None of the selected matrices showed repeatability higher than 10% except sweet fermented milk and full-cream milk for DEHP, for which they were determined at 14% and 20%, respectively. These values remain however acceptable but may highlight a potential interaction between DEHP and milk components, leading to less reproducible extraction efficiency. Precision uncertainty, estimated from replicates for all 10 samples at 2 different fortification levels, were then 2.2%, 3.2%, 2.2% and 6.0%, for DiBP, DnBP, BBzP and DEHP, respectively.

3.1.3. Accuracy

The accuracy of the method was also considered as a critical point in the validation process. The internal standards were selected according to their mimetic properties with the targeted compounds. Thus, the use of commercially available d_4-labelled phthalate diesters was the optimal solution allowing the quantification of target compounds by isotopic dilution. It allowed leaving aside the potential fluctuations of the signals due to matrix effect. Due to unavailability of a certified reference material, accuracy has been assessed through two complementary approaches.

On the one hand, quantification results in fortified replicates have been compared to corresponding unfortified samples ($n = 3$ each, except for roast pork $n = 1$). Original contamination of samples was lower than 10 μg kg^{-1} for DiBP, DnBP and BBzP. However, for DEHP, original contamination levels were found up to 772 μg kg^{-1} in unsalted butter. Table 4 reports levels measured in fortified and their corresponding unfortified samples. When original level becomes non negligible compared to the fortification level, the accuracy evaluation derived from the difference between the two mean measurements can suffer a double and significant variability. Indeed, it appeared that the trueness deviation for DEHP in unsalted butter for the lower fortification level was extreme (+121%) and such result must be interpreted carefully. Finally, mean trueness deviations obtained for fortified samples were −10%, −9%, −15% and +8% for DiBP, DnBP, BBzP and DEHP, respectively.

On the other hand, accuracy was also evaluated through two sunflower oil samples (referenced T1242 and 1245) from FAPAS® proficiency tests (FERA, Sand Hutton, York, UK), even if contamination levels were relatively high (0.38–5.80 mg kg^{-1}) compared to levels usually found in food samples. Individual accuracy deviations to the assigned value were lower than ±16%, with means of absolute values at 6.9%, 11.5%, 11.5% and 12.5% for DiBP, DnBP, BBzP and DEHP, respectively. Additionally, participation to the second proficiency test resulted in acceptable Z-scores, between 0.7 and 1.2. Based on results from these two samples, accuracy uncertainty was estimated to be 6.9%, 11.5%, 11.5% and 12.5%, for DiBP, DnBP, BBzP and DEHP, respectively, which was considered as satisfying.

3.1.4. Combined uncertainty and limits of reporting (LoR)

Additionally to precision and accuracy uncertainties, standards purities and samples homogeneity were considered for combined uncertainty, as mentioned in Section 2.5. Relative combined uncertainties were finally found to be 7.6%, 12.2%, 12.0% and 14.1%, for DiBP, DnBP, BBzP and DEHP, respectively.

During the validation process, mean procedural contamination levels were found to be 1.5, 0.32, 0.12 and 1.5 μg kg^{-1}, with maximum levels at 3.3, 0.68, 0.24 and 3.3 μg kg^{-1} for DiBP, DnBP, BBzP and DEHP, respectively, based on a virtual sample size of 10 g. This way, LoR as previously defined, were 2.7, 0.53, 0.18 and 3.4 μg kg^{-1}, for DiBP, DnBP, BBzP and DEHP, respectively (see Fig. 2).

In terms of uncertainty, it should be pointed out that the estimated combined uncertainties are applicable to raw results, *i.e.* before deduction of procedural blank contribution. Theoretically, when the procedural contamination is deduced, the contribution of its uncertainty to the relative combined uncertainty should be

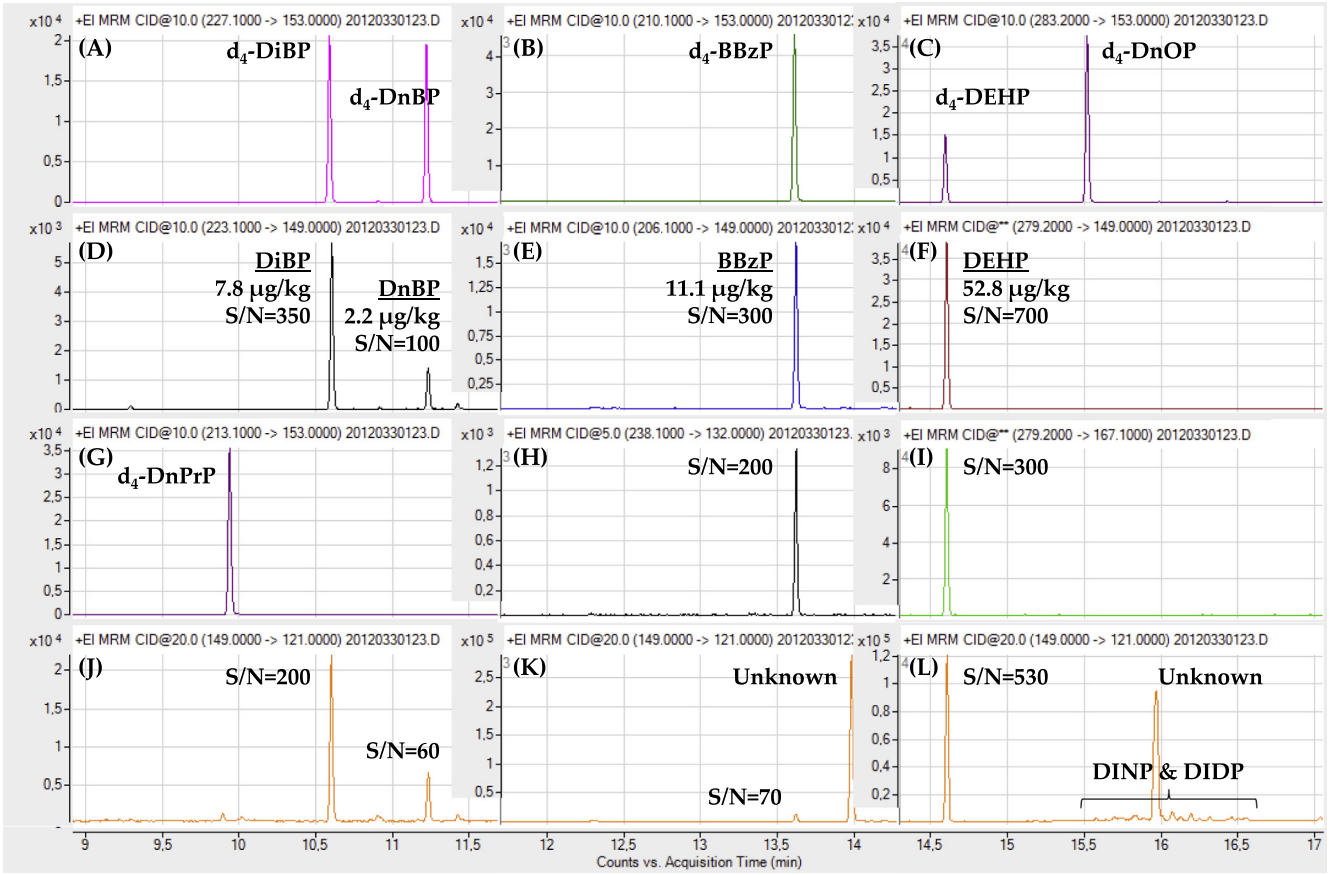

Fig. 1. Ion chromatograms obtained for S36 sample (Macaroni chicken mixed meal for infant). Columns: 3 successive acquisition windows, from 9 to 17 min of retention time; (A–C): internal standards; (D–F): quantitative transition for target compounds; (G and C): external standards; (J, H, I): secondary transitions; (J–L): generic transition for native phthalate diesters; unknown: unidentified compounds at RT = 14.0 and 16.0; DINP and DIDP: diisononyl and diisodecyl phthalate mixtures retention time zone.

Table 3
Quantification and repeatability results, in µg kg^{-1}, obtained for the 10 selected matrices, fortified at 2 different levels (40 and 500 µg kg^{-1}, $n = 6$ each).

Matrix type	DiBP	DnBP	BBzP	DEHP
Fortified at 40 µg kg^{-1}				
Fruit puree	40 (±3%)	35 (±1%)	38 (±1%)	59 (±2%)
Sweet fermented milk	38 (±1%)	35 (±1%)	40 (±1%)	49 (±14%)
Vegetables pool	40 (±2%)	35 (±2%)	39 (±2%)	67 (±5%)
Grain bread	54 (±1%)	37 (±1%)	40 (±2%)	217 (±1%)
Roast pork	37 (±1%)	33 (±1%)	38 (±1%)	190 (±2%)
Salmon	39 (±3%)	34 (±2%)	39 (±2%)	512 (±3%)
Chicken egg	39 (±2%)	34 (±2%)	38 (±1%)	35 (±2%)
Full-cream milk	36 (±6%)	32 (±8%)	37 (±7%)	57 (±20%)
Cheeses pool	42 (±2%)	38 (±8%)	39 (±2%)	329 (±1%)
Unsalted butter	42 (±3%)	37 (±2%)	44 (±2%)	851 (±1%)
Fortified at 500 µg kg^{-1}				
Fruit puree	465 (±1%)	426 (±2%)	502 (±1%)	609 (±1%)
Sweet fermented milk	489 (±1%)	451 (±2%)	507 (±2%)	470 (±3%)
Vegetables pool	462 (±1%)	424 (±1%)	492 (±1%)	557 (±1%)
Grain bread	484 (±1%)	433 (±0%)	501 (±1%)	711 (±2%)
Roast pork	458 (±1%)	423 (±1%)	490 (±1%)	585 (±6%)
Salmon	478 (±3%)	433 (±4%)	501 (±2%)	935 (±3%)
Chicken egg	482 (±1%)	445 (±1%)	496 (±1%)	418 (±1%)
Full-cream milk	473 (±2%)	440 (±2%)	496 (±2%)	448 (±2%)
Cheeses pool	462 (±1%)	421 (±6%)	470 (±1%)	701 (±2%)
Unsalted butter	468 (±2%)	430 (±2%)	481 (±2%)	1334 (±2%)

considered. Interestingly, this contribution is highly dependent on the intrinsic contamination level. The lower the intrinsic contamination level, the higher the contribution of procedural contamination uncertainty to the relative combined uncertainty. Then, in order to take into account the procedural contamination uncertainty, a more complex approach should be developed to model the relative combined uncertainty as a function of the sample contamination level.

3.2. Application to various foodstuffs samples

3.2.1. Limits of reporting (LoR)

Packaged retail food samples were obtained from a local market and analysed according to the validated method. The 54 samples were dispatched in 4 series, according to the matrix type. Obtained procedural contamination was consistent with the validation results, and even slightly better, due to gained experience in the preparation and in the injection of the extracts. To our knowledge, only Fierens et al. (2012) reported a similar approach, using six times the standard deviation of procedural contamination. None of the procedural blanks included in the present study was higher than two times the standard deviation above the mean value, highlighting a clear distinction between samples above the defined LoR and blank levels, as illustrated in Fig. 2. While the LoR calculated according to Section 2.7 were 1.9, 0.18, 0.05 and 1.8 µg kg^{-1}, displayed LoR values regarding these four series were 2.5, 0.25, 0.25 and 2.5 for DiBP, DnBP, BBzP and DEHP, respectively, in order to simplify and homogenise.

3.2.2. Selectivity

Either with validation samples or those from the local retailer, ion chromatograms usually did not show disturbing interferences in the region corresponding to retention times of target compounds. Despite a purification strategy restricted to fat

Table 4
Measured relative differences between fortified and unfortified mean levels obtained for 8 out the 10 selected matrices.

	DiBP			DnBP			BBzP			DEHP		
	UL	LFL (%)	HFL (%)	UL	LFL (%)	HFL (%)	UL	LFL (%)	HFL (%)	UL	LFL (%)	HFL (%)
Fruit puree	3.9	−8	−10	1.5	−10	−9	0.5	−15	−12	14	27	29
Sweet fermented milk	1.0	−7	−5	n.d.	−6	−4	n.d.	−11	−11	10	8	−1
Vegetables pool	5.0	−11	−11	2.3	−10	−10	1.1	−15	−14	27	10	15
Roast pork	4.8	−18	−12	1.8	−14	−10	1.2	−17	−14	162	−23	−8
Chicken egg	2.8	−9	−7	0.8	−8	−5	n.d.	−15	−13	2.2	−10	−10
Full-cream milk	1.2	−13	−8	0.4	−12	−6	n.d.	−17	−13	30	−24	−9
Cheeses pool	7.3	−13	−11	6.6	−13	−12	2.9	−19	−18	296	−8	−12
Unsalted butter	5.7	−9	−10	3.7	−9	−9	6.6	−17	−17	772	121	22

UL: mean measurement in unfortified sample (μg kg^{-1}, n = 3 except for roast pork n = 1); LFL: low fortification level (40 μg kg^{-1}); HFL: high fortification level (500 μg kg^{-1}); n.d. non detected.

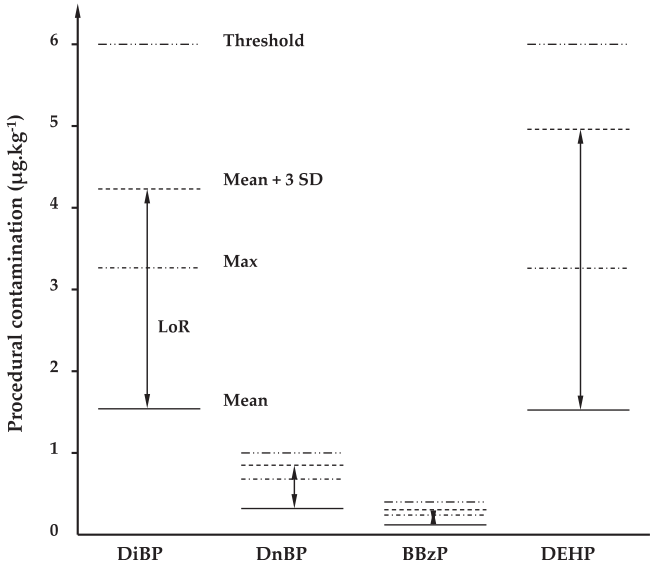

Fig. 2. Schematic representation of remarkable procedural blank values (μg kg^{-1}) obtained during the validation process for each target compound, based on a virtual sample size of 10 g. Max: maximum procedural blank value obtained; Mean + 3 SD: mean value of procedural blanks plus 3 times standard deviation; Threshold: value of procedural blank above which a batch is cancelled; LoR: limit of reporting.

precipitation at −20 °C, several decisive strategic choices led to extracts pure enough to be injected onto the GC system with no more than usual preventive maintenance required. Among those choices, the most important ones are the selectivity of extraction solvents and the triple quadrupole tandem mass filter associated to very specific transitions and relatively low collision energies. In some cases, fatty matrices from milk origin like butter or ripened cheese showed interferences for DEHP. Such matrices were always significantly contaminated with DEHP and a second analysis starting from a lower sample size enabled overcoming the trouble.

3.2.3. Contamination levels

Results including blank deduction are presented in Table 1. Although this sample set did not intend to be representative of the French population consumption habits, attention was paid to cover a large set of available plastic packaged items. The first observation is that the detection frequencies above the LoR are relatively high: 74%, 96%, 67% and 83% for DiBP, DnBP, BBzP and DEHP, respectively. Thus, the occurrence of these migrants is not limited to specific food categories as they are found in most of them. Some other macroscopic information lies in the median values observed: 5.1, 1.7, 0.55 and 12 μg kg^{-1} for DiBP, DnBP, BBzP and DEHP, respectively. Given the sampling approach, discussing the median values is not highly relevant. However, they support the assumption that DEHP would be the predominant compound in food items in terms of concentration and frequency, followed by DiBP, DnBP and finally BBzP, which is in line with observations reported by Fierens et al. (2012) on similar samples obtained in 2009–2010 in Belgian shops.

DiBP concentrations were found in the range [<LoR–250 μg kg^{-1}]. Excepted pine nuts (250 μg kg^{-1}), the most contaminated sample was extruded chocolate flavoured cereals at level of 40 μg kg^{-1}. Then, five samples showed concentrations above 14 μg kg^{-1}, including croissants, marble cake, Provencal pizza, sweets and lemon juice, all of them showing levels between 18 and 25 μg kg^{-1}. No particular factor explaining the presence of DiBP could be identified among matrix type, fat content, contact type with packaging or packaging type. Interestingly, DiBP is not authorised in the EU for FCM applications, giving rise to the question of its source of contamination (migration from non compliant FCM, ambient contamination or any other possibility). Given the unambiguous identification criteria set in the present method, analytical pitfall can be excluded.

DnBP concentrations were found in the range [<LoR–74 μg kg^{-1}], the highest concentration being found in pine nuts again. Box packaged red wine presented a level close to the maximum observed, at 73 μg kg^{-1}. Other most contaminated samples were found as follows: popped corn, croissants, Strasbourg sausages and grated Emmental cheese at 54, 41, 29 and 28 μg kg^{-1}, respectively. Again, no particular factor explaining the presence of DnBP could be identified. Regarding the regulatory limits established for FCM, even if the maximum value was below the specific migration limit (SML, 300 μg kg^{-1}), the primary packaging which is in contact with food item should not contain any DnBP because they are intended for single-use, unless DnBP was used as technical support agent in polyolefins at a concentration lower than 0.05% (Decision EU 10/2011). Therefore, the question of the source of contamination merits to be further investigated, in order to understand whether it comes from the final primary packaging and/or a previous FCM.

BBzP concentrations were found in the range [<LoR–11 μg kg^{-1}], with maximum level found in a mixed meal for infant. Noticeable values close to 7 μg kg^{-1} can be highlighted as well in the pine nuts and in the box red wine. These two samples show relatively high concentrations for the other targeted phthalate diesters. However, even if the same questions than for DnBP might be investigated, all these values were at least 2750 times lower than the authorised SML for BBzP (30 mg kg^{-1}, Decision EU 10/2011), clearly indicating that the migration is far below the established limit in simulants.

DEHP concentrations were found in the range [<LoR–2376 μg kg^{-1}], with maximum level found in pine nuts once more. Other food items containing relatively high levels of DEHP are pressed cooked paste cheese (720 μg kg^{-1}), croissants

(536 μg kg^{-1}), salted butter (219 μg kg^{-1}) and grated Emmental (186 μg kg^{-1}). A common feature to these food items is that they are among the fattiest in the sampling set, which is in line with affinity of DEHP for fat fraction. In the case of pressed cooked paste cheese, it would be interesting to investigate the concentration as a function of the distance to the surface of the item. No gradient would indicate a contamination prior to cheese ripening, while a gradient would indicate migration from a FCM to the food matrix. As regard to the SML (1500 μg kg^{-1}, Decision EU 10/2011), only the FCM used for the pine nut, if confirmed as the unique source, might be above. Other important concentration values found in the set of samples would also deserve to be interpreted in the light of restrictions set in Decision EU 10/2011, which exclusively authorises DEHP in repeated use materials and articles contacting non-fatty foods or as technical support agent in concentrations up to 0.1 % in the final product or as technical support agent in polyolefins at a concentration lower than 0.1%.

Except in the particular case of pine nut sample, no correlation could be observed between the four phthalate compounds. This pine nut sample, together with the croissants in a lesser extent, shows relatively high contents for the four compounds (first 10th percentiles). From a technological point of view, a common source is difficult to imagine, and therefore, the question of multiple origin of contaminations is raised.

4. Conclusion

The accurate determination of phthalate diesters in food constitutes a pre-requisite to the assessment of human exposure to (low) doses. While detection strategies based on GC–MS have been reported previously in the literature (Wenzl, 2009), few studies reported the use of GC–MS/MS approach and only for specific matrices. Together with a lack of specificity when using GC–MS, the main drawback of such analysis was shown to relate to background contamination. In the present work, an efficient method combining a simple sample preparation limiting background contamination with a selective and accurate GC–MS/MS detection has been developed and validated for the quantification of DiBP, DnBP, BBzP and DEHP in liquid or solid foodstuffs according to isotopic dilution principles. The use of the SRM acquisition mode allowed a very good specificity and a high sensitivity could be observed even if the sample preparation is limited to a liquid extraction followed by a delipidation step. The challenging robust quantification in complex foodstuffs was achieved with detection and quantification limits below LoR defined as 2.5, 0.25, 0.25 and 2.5 μg kg^{-1} for DiBP, DnBP, BBzP and DEHP, respectively, due to background contamination. A full validation protocol was carried out for the determination of the parameters of interest: LOD and LOQ, specificity, linearity, precision, accuracy and the relative combined uncertainty parameter which was evaluated to 7.6%, 12.2%, 12.0% and 14.1%, for DiBP, DnBP, BBzP and DEHP, respectively. The main critical parameters have been highlighted, *i.e.* background contamination, extraction pitfall and accuracy of the results, and appropriate strategies have been implemented to overcome the corresponding issues. This protocol has been successfully applied to a selection of 54 food samples selected at a local retailer, including a wide range of origins and compositions. Additionally, this protocol has been recently accredited against ISO17025 requirements and further selected and implemented for the determination of more than one thousand foodstuffs samples analysed in the frame of the second French Total Diet Study. Results from this study have previously been published concerning several chemical hazards families (*e.g.* for Polycyclic Aromatic Hydrocarbons: Veyrand et al., 2013). Results regarding phthalate diesters will be soon available.

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