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Determination of phthalate diesters and monoesters in human milk and infant formula by fat extraction, size-exclusion chromatography clean-up and gas chromatography-mass spectrometry detection

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ABSTRACT

A sensitive and reliable analytical method was developed for the simultaneous determination of five phthalate diesters and corresponding monoesters in human milk samples and infant formulas. The method involved a liquid–liquid extraction with a CH_2Cl_2/CH_3OH/NaCl 30% 2/1/0.5 (*v/v/v*) mixture, the clean-up of the extract by size-exclusion chromatography (swelling and elution solvent: cyclohexane/ethyl acetate 9/1 *v/v*), the derivatization of monoesters by trimethylsilyl-diazomethane and instrumental analysis by gas chromatography coupled with mass spectrometry. Recovery was in the range of 83–115% and precision was found between 9% and 21%. For phthalate diesters, method detection limits (MDLs) ranged from hundreds of ng/kg to 4.2 μg/kg on a fresh weight milk (f.w.) basis, depending on blank contribution evaluated in matrix. Lower MDLs (0.03-0.8 μg/kg f.w.) were achieved for corresponding monoesters. The proposed method was applied to the determination of target compounds in nine human milk samples and four infant formulas, confirming their presence in all samples. However, a generally higher contamination was assessed in artificial milk than in breast milk samples.

1. Introduction

Diesters of 1,2-phthalic acid, commonly known as phthalates, are a group of industrial chemicals mainly employed as plasticizers in the production of polyvinylchloride (PVC) and, to a minor extent, in the synthesis of other polymers [1]. Phthalates are also employed in the manufacture of countless and various materials [2], including personal care products and medical devices [3]. Over recent years, phthalates have been one of the most widely manufactured organic compound classes in the world, since their annual production is about 5 million tons [4]. As a consequence, such compounds have been found in atmospheric, terrestrial, and aquatic environments of anthropized regions [5], as well as repeatedly detected in various compartments of remote areas [6,7]

Because of the diffused presence of phthalates in the environment and their frequent use in the above-mentioned products, humans are potentially exposed through inhalation, ingestion and dermal contact throughout their life. Phthalate contamination in humans has also been found as a consequence of drug administration [8]. When phthalates enter the organism they are hydrolysed into the corresponding monoesters and then further oxidized through complex pathways [9]. Even though it is not clear which molecules, among parent compounds and the various metabolites, are more toxic, several studies have highlighted endocrine disruption properties of phthalates in humans, pointing out an association between phthalate exposure and detrimental effects on sexual characters [10,11].

The endocrine disrupting properties of phthalates, together with their ubiquitous presence in the environment, make the determination of both parent compounds and metabolites of paramount importance in human milk and infant formula, as they represent the unique nourishment for newborns in a crucial developmental period of their life. The determination of phthalate diesters and corresponding metabolites in food intended for infants should comply with toxicological evaluations that assess, for instance, tolerable daily intakes in adults of 10 μg/kg b.w. for di-*n*-butyl phthalate [12].

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In this regard, the omnipresence of phthalate diesters may give rise to blank contributions that strongly affect the actual sensitivity of the analytical method, making difficult the quantification of these analytes in human milk at ppb and especially sub-ppb levels.

In spite of their toxicological importance, phthalates and their metabolites have been investigated in human milk only to a limited extent. More in detail, two researches, which considered breast milk samples collected from Canadian [13] and German [14] women, monitored only phthalate diesters. Conversely, most of the published studies, performed in various regions of the planet (i.e. North-America, North and South Europe, East Asia), focused on the determination of phthalate monoesters [15–19], sometimes including also other polar metabolites [16,17]. In this respect, it should be noted that metabolites originating from phthalate monoesters were sporadically detected in human milk samples and found in any case at concentrations much lower than their precursors [16,17]. Surprisingly, the monitoring of a wide group of both phthalate diesters and monoesters was performed only in two studies, focusing on Swedish [20] and German [21] breast milk samples, whereas a recent research carried out in Italy was restricted to di-2-ethylhexyl phthalate and its corresponding monoester [22].

As far as infant formula is concerned, the monitoring of phthalate contamination focused mainly on the phthalate diesters [21,23–26], even though monoesters have been sporadically analysed, as well [19,21].

Human milk is a very complex matrix, due to the high content of lipids, mostly represented by esters of fatty acids, which exhibit physicochemical properties similar to target analytes. Accordingly, the analysis of phthalates in milk represents a great analytical challenge. A number of different analytical approaches have been adopted for the analysis of phthalates diesters and/or monoesters in milk samples. In this regard, it is worth mentioning that, when both phthalate diesters and monoesters were analysed, two completely different analytical approaches have been proposed.

These protocols mainly include liquid–liquid extraction (LLE) [14,18–26] and less frequently headspace solid-phase microextraction (HS-SPME) [27], QuEChERS extraction [28], solid-phase extraction (SPE) [15] and automated on-line SPE [16,17].

LLE followed by purification of the organic extract by using various clean-up approaches has been the most widely adopted extraction technique and, even recently, often applied to the analysis of phthalates in human milk and infant formula. In some cases [22,23,26], several manual analytical steps were necessary for analyte extraction and extract purification (e.g. evaporation to dryness and successive reconstitution in a proper solvent or back-extraction processes). With these analytical procedures, blank values at ppb levels or higher were found for some phthalate diesters (e.g. di-*n*-butyl phthalate and di-2-ethylhexyl phthalate), thus limiting the method sensitivity. Easier LLE procedures were achieved by adopting more straightforward extraction protocols and using automated or automatable SPE clean-up strategies [14,18,19,21,24,25]. However, background contaminations by phthalate diesters in procedural blanks were found in the ppb-level, as well [14,25]. Size-exclusion chromatography (SEC) was also employed as clean-up strategy, after LLE of milk samples [20] and infant formula [29]. In this regard, it should be noted that SEC has been suggested as one of the elective purification strategies of fatty matrix, such as human milk, in a quite recent technical report published by the European Community [30]. With this analytical approach blank contributions between a few ppb and 110 ppb were reported, depending on the study and the compound investigated.

The applicability of the HS-SPME technique to the analysis of phthalate diesters in cow milk was investigated by Feng and coworkers [27], who reported the need of long extraction times (at least 60 min at 90 °C) and detection limits varying from sub-ppb to ppb levels depending on the fat content of milk samples. In this regard, it should be underlined that lipids followed the same fate of phthalates during the enrichment process of the SPME fibre, thus giving rise to the presence of a great number of interfering peaks in the gas chromatogram.

The application of the QuEChERS extraction and clean-up method on the determination of phthalate diesters in bovine milk seemed to achieve lower background contaminations, since detection limits in the sub-ppb levels were reported [28].

Contrary to what generally reported for phthalate diesters, no significant background contamination was observed for the analysis of phthalate monoesters in both human milk and infant formula, irrespective of the overall analytical strategy employed for their determination [15–17,19]. For the analysis of monoesters in human milk, detection limits in the sub-ppb range were achieved for most phthalate metabolites using SPE on a N-vinylpyrrolidone-divinylbenzene co-polymeric sorbent as extraction and clean-up strategy, and LC–MS/MS for the instrumental quantification [15]. On-line SPE-LC–MS/MS was also applied to the analysis of phthalate metabolites in human milk, obtaining sensitivities similar to those achieved by the off-line approach; however, it should be noted that the method adopted was developed for urine and no validation was performed on milk samples [16,17].

Data concerning the levels of phthalate diesters and their metabolites in milk are necessary for assessing their potential impact on nursing mothers and their children. Accordingly, the main purpose of this study was to develop and validate an extraction and clean-up protocol for the simultaneous determination of both phthalate diesters and monoesters in human milk. The proposed method involved LLE of total fats and phthalates and their separation by SEC. In this regard, it should be remarked that phthalate diesters are hydrophobic compounds strongly partitioned in the lipid phase of milk [24] and therefore the extraction of total fats from milk samples is a recommendable procedure for their reliable analysis. Using this method, we performed for the first time the monitoring of target analytes in various milk samples collected from Tuscan donors of the Human Milk Bank of the Florence Children's Hospital. Furthermore, some infant formula widely commercialized in Italy were analysed, in order to compare the exposure to phthalates due to artificial milk consumption with that associated to breastfeeding.

2. Experimental

2.1. Standards, solvents and materials

Analytical standards, dimethyl phthalate (DMP), diethyl phthalate (DEP), di-*n*-propyl phthalate (DPP), di-isopropyl phthalate (DiPP), di-*n*-butyl phthalate (DBP), di-isobutyl phthalate (DiBP), di-*n*-pentyl phthalate (DPeP), di-*n*-hexyl phthalate (DHP), di-*n*-heptyl phthalate (DHepP), benzyl-butyl phthalate (BzBP), di-*n*-octyl phthalate (DOP), di-2-ethyl-hexyl phthalate (DEHP), di-isononyl phthalate (DiNP), di-*n*-nonyl phthalate (DNP), di-*n*-decyl phthalate (DDP), di-*n*-undecyl phthalate (DUP) and di-*n*-dodecyl phthalate (DDoP) were supplied by Sigma Aldrich (Milwaukee, IW, U.S.A.). Mono-ethyl phthalate (MEP), mono-*n*-butyl phthalate (MBP), mono-isobutyl phthalate (MiBP), mono-2-ethylhexyl phthalate (MEHP), mono-benzyl phthalate (MBzP) and mono-isononyl phthalate (MiNP) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, U.S.A.).

Labelled phthalate diesters and monoesters were obtained as following specified. Diethyl phthalate (ring-1,2,3,4-d_4) (DEP-d4), di-*n*-butyl phthalate (ring-1,2,3,4-d_4) (DBP-d4), benzyl butyl phthalate (ring-1,2,3,4-d_4) (BzBP-d4) and di-2-ethyl-hexyl phthalate (ring-1,2,3,4-d_4) (DEHP-d4) were purchased from Cambridge Isotope Laboratories, Inc., whereas di-isobutyl phthalate