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RESIDUES AND CONTAMINANTS IN BABY FOODS: DEVELOPMENT AND VALIDATION OF ANALYTICAL METHODS

RESÍDUOS E CONTAMINANTES EM ALIMENTOS INFANTIS: DESENVOLVIMENTO E VALIDAÇÃO DE MÉTODOS ANALÍTICOS

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Orientadora: Profa. Dra. Helena Teixeira Godoy

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RESUMO

O objetivo deste trabalho foi o desenvolvimento, validação e aplicação de métodos analíticos para a determinação de resíduos de agrotóxicos, contaminantes ambientais (hidrocarbonetos policíclicos aromáticos – HPAs e ftalatos), contaminantes de processamento térmico (acrilamida, 5-hidroximetilfurfural e 4-hidroxi-2,5-dimetil-3(2H)-furanona) e as poliaminas espermina e espermidina em alimentos infantis. Técnicas de preparo de amostra associadas à cromatografia a gás (CG), cromatografia a líquido de alta eficiência (CLAE) e espectrometria de massas (EM) foram desenvolvidas e otimizadas visando o consumo mínimo de reagentes e solventes orgânicos, e menor geração de resíduos químicos. QuEChERS (*quick, easy, cheap, effective, rugged and safe*) combinado com a microextração líquido-líquido dispersiva (DLLME – *dispersive liquid-liquid microextraction*) apresentou adequadas características de desempenho e sensibilidade analítica para o monitoramento de resíduos de 24 agrotóxicos no limite máximo de 10 μg kg^{-1} para alimentos infantis, usando CG-EM. Como uma alternativa à CG, um novo método empregando CLAE-EM foi desenvolvido para a análise simultânea de piretrinas naturais e resíduos de piretróides. Para isto, um simples e rápido preparo de amostra envolvendo extração líquido-líquido assistida por *salting out* (SALLE – *salting-out assisted liquid-liquid extraction*) e outro método baseado em DLLME assistida por ultrassom (UA-DLLME – *ultrasound-assisted dispersive liquid-liquid microextraction*) foram validados e comparados quanto às suas características de desempenho. Os HPAs foram analisados usando CG-EM; assim, diferentes técnicas miniaturizadas de extração foram combinadas resultando em um novo seletivo e sensível método de preparo de amostra, o qual envolve extração dos analitos com QuEChERS, limpeza do extrato por extração em fase sólida dispersiva (d-SPE – *dispersive solid-phase extraction*) com os sorventes amina primária e secundária (PSA) e C_{18}, e concentração dos HPAs no extrato final usando DLLME combinada com ultra-baixa temperatura (–80°C). Essa etapa de limpeza contribui para uma redução de 80% dos co-extrativos provenientes da matriz. Acrilamida e 4-hidroxi-2,5-dimetil-3(2H)-furanona foram simultaneamente analisadas usando CLAE-EM, cuja extração em fase sólida em cartuchos promoveu a remoção de um potencial interferente analítico, com mesma transição *m/z* 72 > 55 monitorada para acrilamida, sem afetar a acurácia e a sensibilidade analítica do método final. Outro composto formado durante o processamento térmico, 5-hidroximetilfurfural, foi investigado nos alimentos infantis usando CLAE com detecção por arranjo de diodos, após rápida extração com acetonitrila e subsequente diluição do extrato com água. Extração com acetonitrila foi também utilizada para a determinação de 7 ftalatos em alimentos infantis usando CG-EM, seguida da limpeza do extrato a baixa temperatura (–18°C), resultando em uma redução de 50% no teor total de co-extrativos da matriz. Os primeiros dados sobre poliaminas em alimentos infantis foram disponibilizados; para isto, extração por par iônico empregando bis(2-etilhexil)fosfato seguida da reação de derivatização com anidrido heptafluorobutírico assistida por micro-ondas foi otimizada usando planejamento fatorial associado à análise de superfície de resposta. A aplicação dos métodos analíticos desenvolvidos em amostras comerciais de alimentos infantis a base de frutas revelou a presença de acrilamida (35μg kg^{-1}), 4-hidroxi-2,5-dimetil-3(2H)-furanona (25–262μg kg^{-1}), 5-hidroximetilfurfural (2,3–195,4μg kg^{-1}), dietil ftalato (4,4 μg kg^{-1}), benzilbutil ftalato (3,3μg kg^{-1}), di-n-octil ftalato (1,4–2,5μg kg^{-1}), bis(2-etilhexil) ftalato (48,5–90,3μg kg^{-1}), e resíduos dos agrotóxicos procimidona (68,8μg kg^{-1}), cialotrina (0,7μg kg^{-1}) e etofenproxi (0,6 μg kg^{-1}).

ABSTRACT

The aim of this work was to develop, validate and apply analytical methods for the determination of pesticide residues, environmental contaminants (polycyclic aromatic hydrocarbons - PAHs and phthalates), processing contaminants (acrylamide, 5-hydroxymethylfurfural and 4-hydroxy-2,5-dimethyl-3(2H)-furanone) and the polyamines spermidine and spermine in baby foods. Sample preparation techniques coupled to gas chromatography (GC), high performance liquid chromatography (HPLC) and mass spectrometry (MS) were developed and optimized focusing minimal consumption of reagents and organic solvents, and lower generation of chemical residues. QuEChERS (*quick, easy, cheap, effective, rugged and safe*) combined with dispersive liquid-liquid microextraction (DLLME) presented adequate performance characteristics and analytical sensibility for the monitoring of residues of 24 pesticides at maximum limit of 10 μg kg^{-1} for baby foods, using GC-MS. As an alternative to GC, a novel HPLC-MS method was developed for simultaneous analysis of natural pyrethrins and pyrethroids residues. For this, a simple and fast sample preparation method based in salting out-assisted liquid-liquid extraction (SALLE), and other method including ultrasound-assisted DLLME were evaluated and compared regarding its performance characteristics. PAHs were analyzed using GC-MS; therefore, different miniaturized extraction techniques were combined resulting in a novel selective and sensitive sample preparation, which involves analytes extraction using QuEChERS, cleanup by dispersive-solid phase extraction (d-SPE) with primary and secondary amine (PSA) and C_{18} sorbents, and PAHs enrichment in the final extract using DLLME combined with ultra-low temperature (–80°C). This cleanup contributed to a removal of 80% of matrix co-extractives. Acrylamide and 4-hydroxy-2,5-dimethyl-3(2H)-furanone were simultaneous analyzed using HPLC-MS, whose solid-phase extraction in cartridges resulted in the removal of potential analytical interference, with same transition *m/z 72 > 55* monitored for acrylamide, without affecting accuracy and analytical sensibility of the final method. Other compound formed during heat processing, 5-hydroxymethylfurfural, was investigated in baby foods using HPLC with diode array detection, after fast extraction with acetonitrile and subsequent dilution of extracts with water. Acetonitrile-based extraction was also used for the determination of 7 phthalates in baby foods employing GC-MS, followed by low-temperature cleanup (–18°C), resulting in a reduction of 50% in the total matrix co-extractives content. The first data about polyamines in baby foods were reported; for this, ion-pair extraction with bis(2-ethylhexyl)phosphate followed by microwave-assisted derivatization with heptafluorobutyric anhydride was optimized using factorial design associated to reponse surface analysis. The application of the developed analytical methods to commercial fruit-based baby food samples revealed the presence of acrylamide (35 μg kg^{-1}), 4-hydroxy-2,5-dimethyl-3(2H)-furanone (25–262 μg kg^{-1}), 5-hydroxymethylfurfural (2.3–195.4 μg kg^{-1}), diethyl phthalate (4.4 μg kg^{-1}), benzylbutyl phthalate (3.3 μg kg^{-1}), di-n-octyl phthalate (1.4–2.5 μg kg^{-1}), bis(2-ethylhexyl) phthalate (48.5–90.3 μg kg^{-1}), and residues of the pesticides procymidone (68.8 μg kg^{-1}), cyhalothrin (0.7 μg kg^{-1}) and etofenprox (0.6 μg kg^{-1}).

SUMÁRIO

INTRODUÇÃO GERAL

Diferentes processamentos de alimentos, principalmente os que empregam altas temperaturas, são amplamente utilizados com a finalidade de reduzir a contaminação microbiológica, aumentar a vida de prateleira e conferir as características sensoriais desejadas aos alimentos. Consequentemente, estes processos térmicos podem resultar na formação de compostos potencialmente tóxicos aos humanos, a partir de reações químicas entre substâncias naturalmente presentes e/ou adicionadas nos alimentos (CAPUANO, FOGLIANO, 2011; TRITSCHER, 2004). Acrilamida, os derivados de furano 5-hidroximetilfurfural (HMF) e 4-hidroxi-2,5-dimetil-3(2H)-furanona (HDMF), e os hidrocarbonetos policíclicos aromáticos (HPAs) são alguns dos compostos tóxicos formados durante o processamento térmico dos alimentos. Adicionalmente, a ocorrência de contaminantes ambientais, incluindo os HPAs e os ftalatos, e os resíduos de agrotóxicos têm também recebido especial atenção de grupos de pesquisas e autoridades regulatórias em todo o mundo, em razão da toxicidade, dos níveis detectados e da ampla distribuição destes compostos em diversas categorias de alimentos. Desta forma, adiciona-se a preocupação da ocorrência destes compostos em produtos destinados ao público infantil.

No cenário nacional tem-se observado uma ampla variedade de produtos alimentícios destinados ao público infantil, tanto no mercado de orgânicos quanto no convencional. Muitos deles apresentam teor reduzido de açúcar e sal, ausência de conservadores na composição, e são fontes de determinadas vitaminas e minerais, fatores estes que associados à praticidade de consumo contribuem para a utilização dos mesmos. Ademais, muitos dos alimentos infantis são elaborados a partir de frutas, farinha de cereais, amido, leite e outros derivados lácteos, e durante o processamento podem ser expostos a altas temperaturas, como na etapa de esterilização, o que contribui para uma possível ocorrência de contaminantes ambientais ou compostos potencialmente tóxicos formados durante o processo térmico.

Lactentes (crianças de zero a doze meses de idade incompletos) e crianças de primeira infância (doze meses a três anos de idade) representam um vulnerável grupo à toxicidade dos contaminates presentes nos alimentos, uma vez que as suas vias metabólicas ainda são imaturas, especialmente nos primeiros meses de vida, resultando em um ineficiente mecanismo de detoxificação; além disso, a taxa de consumo alimentar por peso corpóreo é maior quando comparada aos adultos (HULIN et al., 2014; LABORD et al., 2015; LANDRIGAN, GARG, DROLLER, 2003; LANDRIGAN et al., 2004). Neste contexto, considerando que os alimentos infantis são recomendados aos lactentes e crianças de primeira

infância como parte de uma dieta diversificada, a Comunidade Europeia tem estabelecido restritos limites máximos para alguns desses contaminates em alimentos infantis, incluindo resíduos de agrotóxicos e HPAs (EC, 2006; EC, 2011).

Em contrapartida a alguns países da Comunidade Europeia, até o momento foram poucos os estudos conduzidos no Brasil sobre contaminantes em alimentos destinados ao público infantil, dentre os quais podemos ressaltar a análise das micotoxinas fumonisinas (CALDAS, SILVA, 2007; CASTRO et al., 2004) e os cloropropanóis 3-monocloropropano-1,2-diol e 1,3-dicloropropan-2-ol (ARISSETO et al., 2013) em cereais matinais, e do composto furano em papas para bebês (ARISSETO, VICENTE, TOLEDO, 2010).

O presente trabalho teve como objetivo principal disponibilizar novos métodos analíticos, empregando técnicas cromatográficas e espectrometria de massas, para a determinação de resíduos e contaminantes em alimentos infantis. Neste contexto, métodos de preparo de amostra foram desenvolvidos e otimizados para a análise de resíduos de agrotóxicos, análise simultânea de acrilamida e HDMF, análise de HMF, como também dos contaminantes ambientes HPAs e ftalatos, e das poliaminas espermina e espermidina em alimentos infantis a base de frutas. As características de desempenho dos métodos analíticos foram avaliadas, incluindo seletividade, linearidade, efeito de matriz, limites de detecção e quantificação, exatidão e precisão. Adicionalmente, todos os métodos analíticos desenvolvidos foram aplicados em amostras de alimentos infantis disponíveis comercialmente nos mercados da cidade de Campinas, SP, Brasil.

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CAPÍTULO 1 - Multiclass pesticide analysis in fruit-based baby food: A comparative study of sample preparation techniques previous to gas chromatography–mass spectrometry

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Abstract

With the aim to develop a new gas chromatography–mass spectrometry method to analyze 24 pesticide residues in baby foods at the level imposed by established regulation two simple, rapid and environment-friendly sample preparation techniques based on QuEChERS (quick, easy, cheap, effective, robust and safe) were compared – QuEChERS followed by dispersive liquid-liquid microextraction (DLLME) and QuEChERS followed by dispersive solid-phase extraction (d-SPE). Both sample preparation techniques achieved suitable performance criteria, including selectivity, linearity, acceptable recovery (70–120%) and precision (≤ 20%), under repeatability and within-laboratory reproducibility conditions. A higher enrichment factor was observed for QuEChERS-DLLME and consequently better limits of detection (LOD) and quantification (LOQ) were obtained. Nevertheless, QuEChERS-d-SPE provided a more effective removal of matrix co-extractives from extracts than QuEChERS-DLLME, which contributed to lower matrix effects while maintaining LOD and LOQ sufficient to meet the MRL for the investigated compounds. Twenty-two commercial fruit-based baby food samples were analyzed by the developed QuEChERS-DLLME procedure, and procymidone, a dicarboximide fungicide, was detected in one sample at a level above the European Community MRL.

Keywords: Pesticides; Baby food; QuEChERS; d-SPE; DLLME; Contaminants.

1. Introduction

Currently, a wide variety of ready-to-eat fruit- and cereal-based foods is available for consumption by infants (aged less than 12 months) and young children (between one and three years old). It is well known that fruits and cereals provide important nutrients, including vitamins and minerals, to humans, particularly in the early stage of growth. However, the presence of pesticide residues in baby food ingredients implies a potential risk to this vulnerable consumer group, since their metabolic pathways are still immature and food consumption rates per body weight is higher when compared to adults. Evidence suggests that early exposures to pesticides and others environmental toxicants increases the risk of developing chronic diseases, including certain cancers and neurodegenerative diseases, as well as dysfunctions in the endocrine and reproductive systems (Hulin et al., 2014; Labord et al., 2015; Landrigan, Garg, &Droller, 2003; Landrigan, Kimmel, Correa, &Eskenazi, 2004).

Considering that fruit- and cereal-based baby foods are used as part of a diversified diet for infants and young children, the European Community (EC) fixed a standard maximum residue limit (MRL) of 0.01 mg kg^{-1} for pesticides in these products, while more restrictive MRLs were established for certain pesticides of greatest concern, such as fipronil (0.004 $mgkg^{-1}$), cadusafos (0.006 $mgkg^{-1}$) and ethoprophos (0.008 $mgkg^{-1}$). The Commission Directive 2006/125/EC also states the pesticides that must not be used in agricultural production intended for the production of baby foods, such as the organophosphate insecticide omethoate (EC, 2006).

Notwithstanding the effort to control and minimize risk in the use of pesticides in fruit and cereal cultures, their presence has been reported in foods intended for infants and young children throughout the world. For instances procymidone, chlorpyrifos and phosalone were detected in apple-based baby foods during a monitoring program between 2001 and 2003 in the Czech Republic (Stepán, Tichá, Hajslová, Kovalczuk, & Kocourek, 2005). The fungicides carbendazim, imazalil and thiabendazole were detected by Gilbert-López et al. (2007) in more than 60% of the baby food samples analyzed, including juices, purée and follow-on formulae from Spain and the United Kingdom. In another study, the same compounds were detected in 18 of 25 fruit-based baby food samples collected in Spain (Gilbert-López, García-Reyes, & Molina-Díaz, 2012). Azoxystrobin (2.31 µg kg^{-1}) and thiabendazole (3.04 µg kg^{-1}) were also detected in cereal-based baby food and powdered milk-based infant formula samples, respectively, purchased from different markets in China (Jia, Chu, Ling, Huang, & Chang, 2014). In all these studies the pesticide levels were lower than the MRL established for baby foods.

To meet the restrictive European MRLs, several analytical methods have been developed for multiclass and multiresidue analysis of pesticides in foods intended for infants and young children. Selectivity and sensitivity are considered the two main analytical parameters for pesticide monitoring in baby foods (Hercegová, Dömötörová, & Matisová, 2007). Baby foods are complex matrices, composed of a mixture of fruit purées, milk, cereal flours and/or starch, and small amounts of fat (Sandra, Tienpont, & David, 2003). Therefore, sample preparation represents one of the most critical steps when dealing with pesticide analysis in these kinds of products. Most of the studies reported for pesticide monitoring in baby foods include sample preparation based on the QuEChERS (quick, easy, cheap, effective, robust and safe) method, which involves a liquid-liquid partitioning with acetonitrile and subsequent dispersive solid-phase extraction (d-SPE) cleanup, using a mixture of $MgSO_4$ and selected sorbents usually primary and secondary amine (PSA) and C_{18} (Gilbert-López, García-Reyes, & Molina-Díaz, 2012; Gilbert-López, García-Reyes, Ortega-Barrales, Molina-Díaz, & Fernández-Alba, 2007; González-Curbelo, Hernández-Borges, Borges-Miquel, & Rodríguez-Delgado, 2012; Leandro, Fussell, & Keely, 2005; Leandro, Hancock, Fussell, & Keely, 2006; Leandro, Hancock, Fussell, & Keely, 2007; Vukovic, Shtereva, Bursic, Mladenova, & Lazic, 2012; Wang & Leung, 2009). Although QuEChERS has been demonstrated to be very effective for the multi-residue analysis of pesticides, the procedure provides a low concentration factor which can result in higher limits of detection and quantification when compared to other sample preparation techniques. Other extractive techniques also based on low consumption of solvents, such as stir bar sorptive extraction (Sandra, Tienpont, & David, 2003), direct immersion solid-phase microextraction (Viñas, Campillo, Martínez-Castillo, & Hernández-Córdoba, 2009), multi-walled carbon nanotubes dispersive solid-phase extraction (González-Curbelo, Asensio-Ramos, Herrera-Herrera, & Hernández-Borges, 2012), and ultrasound-assisted extraction and hollow-fiber liquid-phase microextraction (González-Curbelo, Hernández-Borges, Borges-Miquel, & Rodríguez-Delgado, 2013) have like wise been applied for baby food analysis. Dispersive liquid-liquid micro-extraction (DLLME) is another recently developed extraction technique presenting unique features concerning simplicity of operation, amount of organic solvent extractor (only a few microliters) and high enrichment factor (Cunha, Pena, & Fernandes, 2015). Although more suitable for extraction of analytes from aqueous samples DLLME can be also applied to solid samples after previous extraction with acetonitrile similar to the first step of the QuEChERS procedure (Cunha & Fernandes 2011).

Considering the advantages and limitations of d-SPE and DLLME, a comparative

study between these two techniques was carried out, after previous QuEChERS extraction, for the simultaneous analysis of 24 pesticides belonging to 12 different chemical classes in baby foods. Once developed the entire GC-MS method was applied in the analysis of pesticide residues in baby foods commercially available in Brazil.To the best of our knowledge, this is the first pesticide monitoring in baby food from Brazil and a pioneer in comparing the selectivity and sensibility of QuEChERS-DLLME and QuEChERS-d-SPE techniques for multiclass analysis of pesticide in baby foods.

2. Materials and Methods

2.1. Chemicals and solutions

Pesticide standards were purchased from Fluka (Neu-Ulm, Germany), Dr. Ehrenstorfer (Augsburg, Germany) and Riedel-de Haën (Seelze, Germany). The purity of these analytical standards ranged from 95.1 to 99.9%. Individual stock standard solutions corrected by their purity (1200–5500 mg L^{-1}) were prepared, depending on the solubility, in acetonitrile, methanol or toluene, and were stored at –18 ºC. A multi-compound working solution was prepared in toluene at 100 mg L^{-1} by combining appropriate aliquots of individual stock standard solutions, and it was stored at 4 ºC. The internal standard (IS) triphenylphosphate (TPP; 99% purity) was obtained from Fluka, and an isotopically labeled solution of malathion-d_6 (98%) was obtained from Dr. Ehrenstorfer. Working standard solutions of IS and malathion-d_6 were prepared in acetonitrile (100 mg L^{-1}) and acetone (2 mg L^{-1}), respectively, and were stored at 4 ºC. Acetonitrile, methanol and toluene, for pesticide residue analysis, and acetone and carbon tetrachloride (CCl_4), high purity solvents for GC analysis, were obtained from Fluka. Analytical grade glacial acetic acid was obtained from Panreac. Anhydrous magnesium sulfate ($MgSO_4$) and anhydrous sodium acetate (NaOAc) were purchased from Sigma-Aldrich Chemie, and the sorbents primary secondary amine (PSA; particle size 50 µm) from Supelco (Bellefonte, PA, USA) and C_{18}-bonded silica (particle size 55–105 µm) from Waters (Milford, MA, USA).

2.2. Sampling

A total of twenty-two ready-to-eat baby food samples were randomly collected from five local markets in the city of Campinas, SP, located in the South-eastern region of Brazil, between August and September 2014. All the samples were packed in plastic bags (113 g each) or glass jars (120 g each), which included fruit juice or purée (apple, banana, grape, mango, orange, papaya, pear, pineapple, plum and/or strawberry), cereal flour (rice or oat),

starch, milk and/or yogurt in their composition. All the samples were kept in their original packing at room temperature until analysis.

2.3. Determination of the pesticides

2.3.1. Sample preparation techniques

The QuEChERS procedure, which uses acetate buffering and has been adopted by the AOAC as Official Method 2007.01 (Lehotay, 2007), was employed for pre-concentration of the pesticides with few modifications. Fifteen grams of homogenized baby food samples and 150 µL of TPP solution (100 mg L^{-1}) were added to a 50 mL polypropylene centrifuge tube and mixed by hand for 10 s. Then 15 mL of acetonitrile acidified with 1% acetic acid (v/v) was added and the mixture was vortexed for 1 min. After vortexing, 1.5 g of NaOAc and 6.0 g of anhydrous $MgSO_4$ were added to the tube, and this was again vortexed for 1 min, and subsequently centrifuged at 5000 rpm for 3 min at room temperature. From buffered QuEChERS extract, two procedures, dispersive solid-phase extraction (d-SPE) and dispersive liquid-liquid microextraction (DLLME), were carried out and then compared for the determination of 24 pesticides pertaining to 12 different chemical classes (Table 1).

2.3.2. Dispersive solid-phase extraction (d-SPE)

One milliliter of QuEChERS extract, 50 mg of C_{18}, 50 mg of PSA and 150 mg of $MgSO_4$ were added to a 15 mL polypropylene centrifuge tube, vortexed for 30 s, and then the mixture was centrifuged at 5000 rpm for 2 min at room temperature. The supernatant was collected and then injected into a GC-MS system.

2.3.3. Dispersive liquid-liquid microextraction (DLLME)

One milliliter of QuEChERS extract (acetonitrile – disperser solvent) was added to a 5 mL glass vial containing 75 µL of CCl_4 (extraction solvent) and this was manually shaken for 5 s. The mixture was rapidly injected into 4 mL of deionized water, placed into a 10 mL polypropylene centrifuge tube, and then this tube was gently shaken by hand for 1 min. A cloudy solution was formed, and the pesticides were extracted into micro-droplets of CCl_4 dispersed entirely into the aqueous phase. After shaking, the tube was centrifuged at 5000 rpm for 5 min at room temperature. The upper layer was discarded, and 55 µL of the bottom layer were collected for subsequent GC-MS analysis.

2.3.4. Chromatographic and mass-spectrometric conditions

A 6890 gas chromatograph (Agilent, Little Falls, DE, USA) equipped with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an electronically controlled split/splitless injection port, interfaced to a single quadrupole inert mass selective detector (5975B, Agilent) with electron ionization (EI) chamber, was used for pesticide analyses. GC separation was achieved on a ZB-5MS capillary column (30 m x 0.25 mm I.D. x 0.25 µm film thickness; Phenomenex, USA), which was maintained initially at 95ºC for 1.5 min, increased at 20ºC min^{-1} to 180ºC, then ramped to 230ºC at 5ºC min^{-1}, and finally increased to 290°C at 25°C min^{-1} and held for 11.85 min, with a total run of 30 min. The injector was maintained at 250°C and 1 µL of extract was injected in splitless mode (purge time of 1 min and purge flow of 100 mL min^{-1}). Ultra-high purity helium (99.999%) was used as carrier gas at constant flow 1 mL min^{-1}. The electron energy was 70 eV, and the temperatures of the transfer line, ion source and quadrupole mass analyzer were set to 280°C, 230ºC and 150ºC, respectively. Agilent Chemstation was used for GC-MS system control and data analysis.

2.3.5. Identification and quantification

Individual standard solutions (20 mg L^{-1}) were analysed in full-scan mode, from *m/z* 50 to 550, to establish the retention time and characteristic spectrum of each pesticide. One target ion and at least two qualifying ions were selected for each compound (Table 1), based on their abundance and specificity. Data acquisition was divided in 10 different ion groups, and the dwell times (20–30 ms) were set depending on the number of ions per group. The compounds were identified by the retention time of the target ion and by the qualifier to target ion ratios, as well as by using the Automated Mass Spectral Deconvolution and Identification System (AMDIS, version 2.64, NIST – National Institute of Standards and Technology, USA). Pesticides were quantified under selective ion monitoring (SIM) mode with a solvent delay of 3 min, employing seven-point matrix-matched calibration curves, and the obtained results were not corrected for recovery. The IS triphenylphosphate was added to the sample, at a level of 1 mg kg^{-1}, prior to the extraction procedure, in order to keep track of possible losses occurring during the sample preparation method and GC-MS analysis, and the IS malathion-d_6 was added to the final extract, at 0.2 mg kg^{-1}, prior to GC injection, as a quality control for the GC-MS system.

3. Results and Discussion

3.1. Sample preparation methods

In order to develop a method with the required selectivity, sensitivity and precision for pesticide evaluation in baby foods, two different cleanup/concentration procedures DLLME and d-SPE were compared after previous acetonitrile extraction in the QuEChERS mode. Taking into account the final goal of pesticide residues monitoring in baby food commercially available in Brazil twenty-four pesticides pertaining to 12 different chemical classes were chosen (Table 1).

The selection was based on previous studies in baby foods around the world (Gilbert-López, García-Reyes, & Molina-Díaz, 2012; Gilbert-López, García-Reyes, Ortega-Barrales, Molina-Díaz, & Fernández-Alba, 2007; Jia, Chu, Ling, Huang, & Chang, 2014; Stepán, Tichá, Hajslová, Kovalczuk, & Kocourek, 2005), as well as on the data obtained from the Brazilian and European monitoring programs in cereals, fruits and vegetables (Jardim & Caldas, 2012; EFSA, 2015). These 24 pesticides cover a wide range of physicochemical properties, including different polarities (*n*-octanol-water partition coefficient, log K_{ow}, ranging from 0.7 to 6.6). Some of the investigated pesticides, such as atrazine, chlorpyrifos, cypermethrin, deltamethrin, dimethoate, malathion, and parathion-methyl have been associated with endocrine-disrupting activity, which is of major concern in baby food (Mezcua et al., 2012).

Table 1. Pesticides evaluated in the present study, their chemical class, use type, molecular weight (MW), *n*-octanol-water partition coefficient (log K_{ow}), retention time (t_R), mass spectrometric conditions for the GC analysis, and limits of detection (LOD) and quantification (LOQ) obtained using dispersive liquid-liquid microextraction (DLLME) and dispersive solid-phase extraction (d-SPE).

Pesticide	**Chemical class/Use type** [a]	**MW** [a] (g mol^{-1})	**Log K_{OW}** [a]	**t_R** (min)	**Start time of window** (min)	**Data acquisition rate** (scans s^{-1})	**SIM ions *m/z*** [b]	**LOD** DLLME (d-SPE) mg kg^{-1}	**LOQ** DLLME (d-SPE) mg kg^{-1}
Dichlorvos	Organophosphate/Insecticide	221.0	1.42	3.59	3.40	3.36	**109**, 185, 79	0.01 (0.05)	0.05 (0.1)
Diuron	Urea/Herbicide	233.1	2.85	4.20			**187**, 124, 158	0.01 (0.05)	0.05 (0.1)
Dimethoate	Organophosphate/Insecticide	229.3	0.704	7.36	4.30	4.35	**87**, 125, 58, 143	0.01 (0.01)	0.05 (0.05)
Atrazine	Triazine/Herbicide	215.7	2.5	7.57			**200**, 215, 173	0.005 (0.01)	0.01 (0.05)
Chlorpyrifos-methyl	Organophosphate/Insecticide	322.5	4.24	8.84	7.67	3.74	**286**, 125, 79, 93	0.005 (0.01)	0.01 (0.05)
Parathion-methyl	Organophosphate/Insecticide	263.2	3.0	8.96			**109**, 263, 79	0.005 (0.01)	0.01 (0.05)
Malathion-d_6	*Internal Standard*	*336.4*		*9.70*			***99**, 131, 174*		
Malathion	Organophosphate/Insecticide	330.4	2.75	9.78	9.10	2.56	**125,** 173, 93, 158	0.005 (0.01)	0.01 (0.05)
Chlorpyrifos	Organophosphate/Insecticide	350.6	4.7	9.91			**314**, 97, 197, 258	0.01 (0.05)	0.05 (0.1)
Tetraconazole	Triazole/Fungicide	372.1	3.56	10.21	10.05	2.78	**336**, 171, 191, 254	0.005 (0.01)	0.01 (0.05)
Tolylfluanid	Sulfamide/Fungicide	347.3	3.9	10.95			**137**, 238, 181	0.005 (0.01)	0.01 (0.05)
Thiabendazole	Benzimidazole/Fungicide	201.3	2.39	11.10	11.02	2.25	**201**, 174, 129	0.01 (0.05)	0.05 (0.1)
Captan	Phthalimide/Fungicide	300.6	2.8	11.14			**79**, 149, 117	0.01 (0.05)	0.05 (0.1)
Procymidone	Dicarboximide/Fungicide	284.1	3.14	11.28			**283**, 96, 67	0.005 (0.01)	0.01 (0.05)
Folpet	Phthalimide/Fungicide	296.6	3.11	11.35			**104**, 260, 76	0.01 (0.05)	0.05 (0.1)
Methidathion	Organophosphate/Insecticide	302.3	2.2	11.49			**145**, 85, 125	0.005 (0.01)	0.01 (0.05)
Imazalil	Imidazole/Fungicide	297.2	3.82	12.28	12.00	2.56	**215,** 173, 159	0.01 (0.05)	0.05 (0.1)
Fenhexamid	Hydroxyanilide/Fungicide	302.2	3.51	14.86			**97**, 55, 177	0.005 (0.01)	0.01 (0.05)
Trifloxystrobin	Strobilurin/Fungicide	408.4	4.5	14.95			**116**, 132, 222	0.005 (0.01)	0.01 (0.05)
Triphenylphosphate	*Internal Standard*	*326.3*		*15.56*			***326**, 215, 170, 232*		
Phosalone	Organophosphate/Insecticide	367.8	4.01	17.08	16.00	3.03	**182**, 121, 367, 97	0.005 (0.01)	0.01 (0.1)
Azinphos-methyl	Organophosphate/Insecticide	317.3	2.96	17.12			**77**, 160, 132, 93	0.01 (0.05)	0.05 (0.1)
Cypermethrin	Pyrethroid/Insecticide	416.3	6.6	18.80	17.40	4.23	**163**, 181, 207	0.01 (0.05)	0.05 (0.1)
Difenoconazole	Triazole/Fungicide	406.3	4.4	19.78			**323**, 265, 202, 139	0.005 (0.01)	0.01 (0.05)
Deltamethrin	Pyrethroid/Insecticide	505.2	4.6	20.10	19.95	4.86	**181**, 253, 209	0.01 (0.05)	0.05 (0.1)
Azoxystrobin	Strobilurin/Fungicide	403.4	2.5	20.26			**344**, 388, 372	0.005 (0.01)	0.01 (0.05)

[a] Tomlin (2003);
[b] Ions used for quantification in bold.

An acetate-buffered version of the QuEChERS method was chosen as the first step for extraction of the analytes. According to the literature, this procedure provided slightly greater recoveries and more consistent results for some pesticides investigated in the present study, such as dimethoate, phosalone and thiabendazole, than the un-buffered or citrate-buffered QuEChERS procedures (Lehotay et al., 2010). Additionally, it can improve the extraction and stability of pH-dependent compounds like captan, folpet and tolylfluanid (Lehotay, Mastovská, & Lightfield, 2005). Thus, initially a blank sample of baby food (a mixture of fruit purées – apple, banana and papaya, milk, rice flour and starch) was spiked at a 5 mg kg^{-1} level with a multi-compound working solution (100 mg L^{-1}). After standing 1 h at room temperature, to promote a proper integration of the pesticide standards into the matrix, and further solvent evaporation, the pesticides were extracted with 15 mL of acetonitrile as referred in 2.3.1., and 1 mL of the extract was submitted to DLLME or d-SPE techniques.

The DLLME technique applied was based on our previous studies of pesticide residue analysis (Andrascíková, Hrouzková, & Cunha, 2013; Cunha & Fernandes, 2011; Cunha, Fernandes, & Oliveira, 2009). Therefore, acetonitrile extract obtained by acetate-buffered QuEChERS was used as the dispersive solvent and carbon tetrachloride was used as the extraction solvent. In this study the volume of carbon tetrachloride was optimized, being evaluated at 50, 75 and 100 μL. As can be observed in the Fig. 1, minimal or no difference was observed in average peak area response (n = 3) obtained for most of the studied compounds, demonstrating a constant distribution coefficient of compounds for the three volumes studied. As is known, low volume of extraction solvent results in higher enrichment factor, providing a lower volume of the sedimented phase, and consequently this can hamper the reproducibility of the procedure. Therefore, 75 μL was selected as the volume of extraction solvent, which resulted in 55 μL of sedimented phase total volume and relative standard deviations ≤ 20%, indicating good reproducibility of procedure.

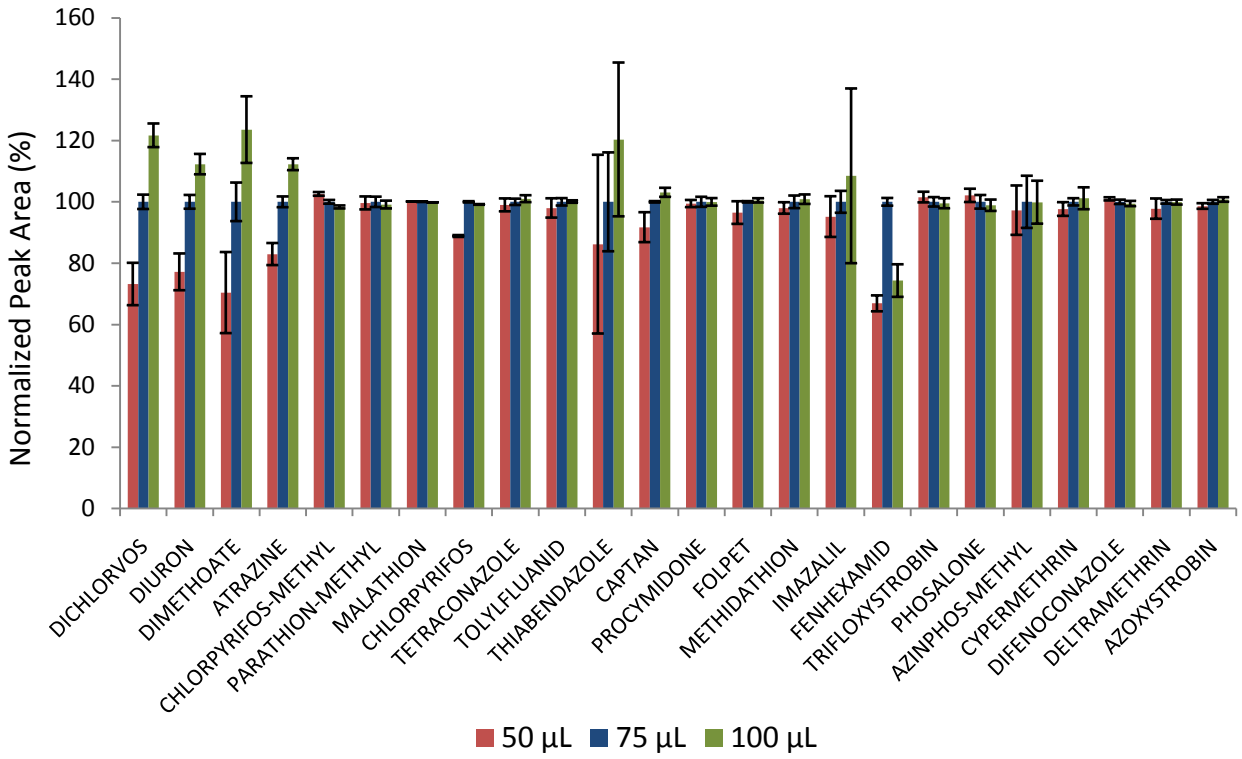

Fig. 1. Comparison of different volumes of DLLME extraction solvent with average peak area response normalized to 75 µL (n = 3).

d-SPE was performed using a mixture $MgSO_4$, C_{18} and PSA as described in several QuEChERS procedures for pesticides extraction (Cunha & Fernandes, 2011; Georgakopoulos et al., 2011; González-Curbelo, Hernández-Borges, Borges-Miguel, & Rodríguez-Delgado, 2012; Leandro, Fussell, & Keely, 2005).

The comparison of a blank sample extract obtained by DLLME or d-SPE is shown in Fig. 2A.Visually, d-SPE provided effectively better cleanup when compared to DLLME. Actually, d-SPE showed to be suitable to remove very non-polar interferences, such as fats, and polar interferences, including sugars and organic acids. The use of a non-polar extraction solvent in DLLME can contribute to an insertion of non-volatile and thermally unstable matrix compounds into the GC-MS system, which can adversely affect the limits and ruggedness of the method, as well as a source of pronounced matrix effects. The major interferences observed in both techniques did not overlap with the target pesticides, indicating that the quantification of analytes could be accurately performed, once the GC-MS chromatograms of the other baby food samples were similar.

In the figure 2B a comparison of average peak area response (n = 3) obtained with DLLME and d-SPE procedures is shown; for this purpose, the d-SPE response was normalized to the DLLME for each pesticide. As can be observed, DLLME provided a higher average peak area response than d-SPE for all studied pesticides, thus better meeting the restrictive MRL established by EC of 0.01 mg kg^{-1} for foodstuffs intended for infant consumption. Overall, the combination of acetate-buffered liquid-liquid acetonitrile partitioning with DLLME has demonstrated to be a simple, environmental-friendly and attractive alternative, in terms of analytical selectivity and sensitivity for pesticide analysis in fruit-based baby foods. However, the absence of an effective cleanup step in this procedure and the consequent analysis of crude complex extracts can affect the long-term GC-MS system performance during sample analysis, thus decreasing the sample throughput due to additional GC-MS system maintenance being constantly required. On the other hand, d-SPE offers practical benefits for pesticides monitoring, including an easy execution, less manual labor and no quantitative transferences of low volumes. However, its low enrichment factor can result in higher limits of detection and quantification when compared with DLLME.

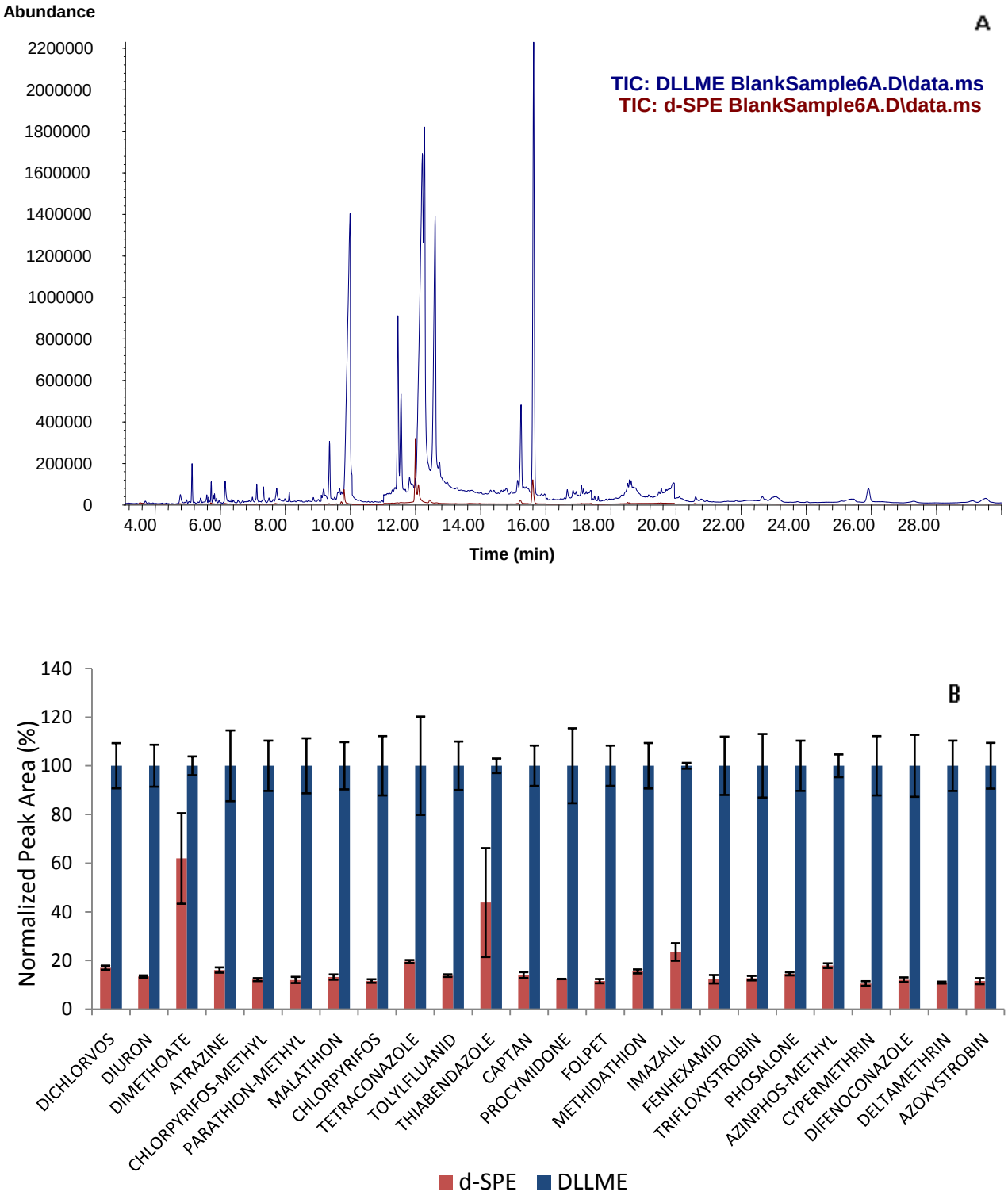

Fig. 2. (A) Total ion chromatograms obtained in selected ion monitoring (SIM) mode from a blank baby food extract using d-SPE and DLLME and; (B) Comparison of d-SPE and DLLME procedure with average peak area response normalized to DLLME (n = 3).

3.2. In-house method validation

The suitability of QuEChERS-DLLME and QuEChERS-d-SPE for multiclass determination of pesticides in baby foods was evaluated regarding performance criteria, including selectivity, linearity, matrix effects, recovery and precision, under repeatability and within-laboratory reproducibility, limits of detection (LOD) and quantification (LOQ).

The selectivity of the method was verified based on the total ion chromatograms obtained in SIM mode, demonstrating its ability to accurately quantify the pesticides of interest in the presence of matrix co-extractives (Thompson, Ellison, & Wood, 2002). In all analyzed samples, no interfering peaks were observed within the windows of retention time of each pesticide, including for those pesticides yielding low intensity ions, such as azinphos-methyl, captan, dimethoate, and fenhexamid.

Linearity was assessed in solvent and matrix-matched calibration curves (pesticide-free baby food, composed of a mixture of fruit purées, rice flour, starch and milk); each calibration curve included 7 points with concentrations ranging from 0.01 to 2.0 mg kg^{-1} for QuEChERS-DLLME and from 0.1 to 2.0 mg kg^{-1} for QuEChERS-d-SPE. Calibration curves were constructed by plotting the analyte/TPP peak area ratio obtained against the concentration values. A good linearity across the studied range was achieved for all studied compounds, with determination coefficients (R^2) higher than 0.99 in all cases (Table 2).

Table 2. Comparison of performance criteria obtained with dispersive liquid-liquid microextraction (DLLME) and dispersive solid-phase extraction (d-SPE) techniques for multiclass pesticides in a representative matrix of baby food [a].

Pesticide	**Linearity** [b] Determination coefficient R^2 DLLME (d-SPE)	**% Recovery** (n = 3) DLLME (d-SPE)			**% RSD Intra-day** (n = 3) DLLME (d-SPE)			**% RSD Inter-day (**n = 9) DLLME (d-SPE)		
		0.1 mg kg^{-1}	0.5 mg kg^{-1}	1.0 mg kg^{-1}	0.1 mgkg^{-1}	0.5 mg kg^{-1}	1.0 mg kg^{-1}	0.1 mg kg^{-1}	0.5 mg kg^{-1}	1.0 mg kg^{-1}
Dichlorvos	0.9952 (0.9984)	86 (70)	84 (95)	88 (97)	5.8 (12.7)	1.8 (3.2)	1.5 (1.0)	20.0 (14.3)	15.7 (17.0)	10.0 (4.1)
Diuron	0.9922 (0.9989)	84 (98)	89 (98)	93 (99)	9.6 (3.4)	2.0 (8.1)	4.7 (14.0)	14.5 (19.4)	15.5 (12.7)	13.2 (15.8)
Dimethoate	0.9934 (0.9969)	101 (109)	79 (119)	95 (117)	7.9 (1.5)	1.5 (1.3)	1.3 (2.5)	7.4 (6.0)	4.6 (1.8)	8.9 (5.7)
Atrazine	0.9942 (0.9980)	97 (106)	98 (109)	99 (114)	4.8 (2.8)	2.3 (2.0)	2.1 (1.2)	8.0 (5.9)	4.8 (4.9)	6.3 (4.8)
Chlorpyrifos-Methyl	0.9969 (0.9982)	108 (111)	103 (108)	110 (114)	3.6 (1.3)	2.0 (1.3)	1.7 (3.7)	5.3 (3.1)	3.7 (5.0)	3.4 (3.6)
Parathion-Methyl	0.9972 (0.9955)	104 (99)	97 (100)	97 (98)	4.2 (1.0)	1.4 (3.9)	1.3 (1.0)	5.6 (9.8)	4.0 (9.3)	4.7 (7.0)
Malathion	0.9960 (0.9988)	110 (114)	113 (105)	114 (118)	3.4 (1.0)	1.5 (3.9)	1.9 (5.3)	4.2 (6.5)	2.0 (2.0)	3.4 (5.0)
Chlorpyrifos	0.9965 (0.9981)	99 (95)	98 (99)	103 (104)	4.5 (1.7)	2.0 (1.7)	1.7 (2.5)	5.7 (6.3)	3.1 (3.0)	2.8 (3.2)
Tetraconazole	0.9964 (0.9980)	108 (105)	112 (106)	119 (108)	3.0 (1.9)	1.4 (1.5)	2.5 (1.2)	3.4 (3.5)	3.0 (3.5)	3.2 (5.8)
Tolylfluanid	0.9970 (0.9981)	83 (98)	99 (97)	101 (99)	4.4 (1.2)	4.6 (1.2)	2.8 (1.1)	8.5 (5.4)	5.8 (7.1)	7.5 (5.5)
Thiabendazole	0.9927 (0.9930)	110 (114)	105 (103)	99 (104)	2.0 (1.1)	2.5 (1.5)	1.5 (1.9)	6.0 (9.8)	5.0 (6.4)	5.5 (4.5)
Captan	0.9943 (0.9917)	94 (100)	98 (93)	99 (97)	3.7 (8.4)	3.3 (8.1)	1.8 (2.7)	7.7 (18.1)	6.3 (13.9)	6.2 (12.2)
Procymidone	0.9968 (0.9977)	101 (104)	104 (106)	113 (108)	2.8 (3.0)	2.1 (3.2)	1.0 (2.8)	4.0 (4.4)	2.2 (3.3)	2.6 (3.1)
Folpet	0.9973 (0.9966)	97 (118)	103 (104)	110 (109)	4.4 (2.8)	1.6 (2.8)	1.9 (3.6)	6.3 (12.2)	3.7 (13.6)	5.0 (9.3)
Methidathion	0.9975 (0.9978)	113 (101)	110 (89)	112 (118)	3.2 (1.9)	1.6 (1.5)	2.3 (1.1)	4.8 (9.0)	3.8 (3.1)	5.1 (4.7)
Imazalil	0.9904 (0.9959)	97 (112)	86 (91)	96 (97)	6.1 (5.1)	1.1 (4.5)	13.5 (3.1)	15.0 (16.8)	13.7 (7.8)	19.2 (5.9)
Fenhexamid	0.9951 (0.9976)	99 (107)	99 (105)	107 (108)	1.7 (2.6)	1.0 (2.0)	2.5 (3.3)	6.0 (3.6)	3.4 (5.0)	2.8 (5.2)
Trifloxystrobin	0.9972 (0.9977)	101 (105)	110 (108)	112 (110)	1.6 (3.2)	1.3 (1.2)	2.1 (1.1)	4.4 (2.9)	2.0 (1.4)	2.7 (3.1)
Phosalone	0.9974 (0.9962)	111 (115)	114 (120)	115 (118)	1.4 (2.4)	1.7 (2.7)	1.0 (2.2)	5.9 (6.5)	4.5 (5.3)	6.3 (6.2)
Azinphos-Methyl	0.9954 (0.9917)	105 (118)	99 (105)	102 (119)	2.5 (3.8)	2.7 (3.8)	3.6 (2.1)	8.4 (9.6)	9.4 (9.3)	13.3 (16.3)
Cypermethrin	0.9966 (0.9933)	101 (111)	102 (115)	116 (121)	3.2 (14.7)	1.1 (7.3)	2.0 (4.5)	15.2 (20.0)	5.0 (11.1)	3.7 (6.1)
Difenoconazole	0.9973 (0.9914)	100 (105)	106 (104)	104 (102)	2.4 (2.0)	1.2 (1.8)	2.5 (2.9)	5.2 (2.7)	4.3 (2.7)	2.8 (3.5)
Deltamethrin	0.9966 (0.9902)	99 (113)	110 (110)	115 (120)	3.8 (3.4)	1.4 (6.1)	1.7 (3.2)	8.3 (6.3)	3.8 (6.5)	3.2 (4.6)
Azoxystrobin	0.9961 (0.9966)	103 (121)	108 (115)	114 (118)	2.5 (2.0)	1.2 (2.4)	2.3 (3.7)	6.0 (3.3)	5.1 (3.0)	3.6 (3.8)

RSD: relative standard deviation;

[a] Baby food composed by a mixture of purée of fruits (apple, banana and papaya), rice flour, starch and milk;

[b] Seven-point matrix-matched calibration curve with concentrations ranging from 0.01 to 2.0 mg kg^{-1} for DLLME and from 0.1 and 2.0 mg kg^{-1} for d-SPE.

The slopes obtained for calibration standards of the same concentrations in solvent and matrix extracts were employed to evaluate the matrix-induced effects using the equation: Matrix Effect % (ME%) = [(matrix slope – solvent slope)/ solvent slope] x 100 (Sapozhnikova & Lehotay, 2013); for this purpose, no internal standard was used in the trials, once it could also be subjected to the effect (Lehotay et al., 2010). Signal suppression or signal enhancement was observed for all compounds, whose extent was dependent on each pesticide, as well as on the sample preparation method applied (Fig. 3). Higher matrix-induced effects were obtained with DLLME for most compounds, possibly due to an ineffective removal of matrix co-extractives achieved with this procedure. High matrix effect (ME% < –50% or ME% > +50%) was observed for cypermethrin (51.5%), methidathion (66.7%), thiabendazole (–68%), imazalil (85%), azinphos-methyl (95%), chlorpyrifos (95%) and fenhexamid (95%) when using the QuEChERS-DLLME technique. The ME% was lower when using QuEChERS-d-SPE with 61.6%, 84.8% and 85.5% for imazalil, azinphos-methyl and fenhexamid, respectively. Several factors can contribute to matrix-induced effects, including the nature of the pesticide, the type of matrix, the pesticide/matrix ratio, and the routine maintenance of the GC-MS system (Hajslová et al., 1998; Hajslová & Zrostlíková, 2003; Schenck & Lehotay, 2000). In order to obtain accurate and reliable results, matrix-matched calibration curves were used for quantification of pesticides (Gilbert-López, García-Reyes, Ortega-Barrales, Molina-Díaz, & Fernandéz-Alba, 2007; Jia, Chu, Ling, Huang, & Chang, 2014; Lehotay et al., 2010; Poole, 2007).

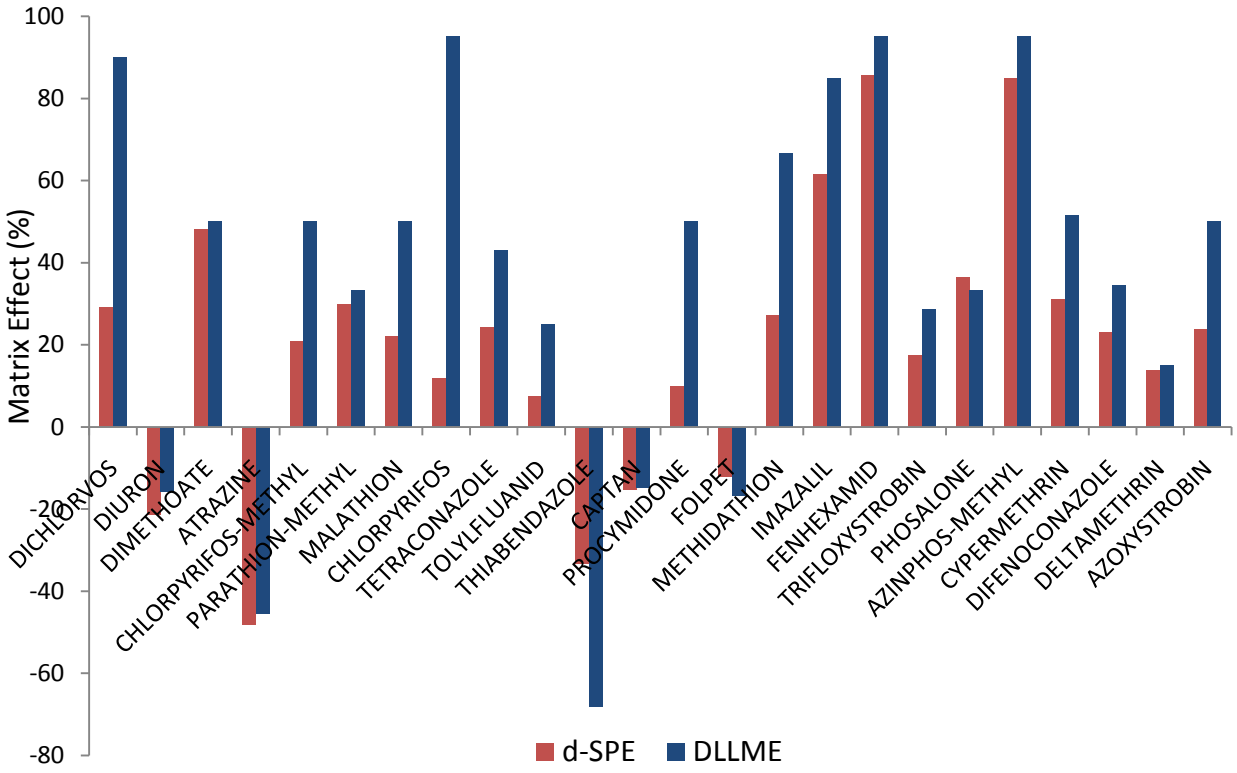

Fig. 3. Matrix effect (%) obtained for the selected pesticides employing d-SPE and DLLME procedures.

The extraction efficiency of the method was assessed by calculating the mean recovery (%) obtained from three independent replicates of spiked samples at three levels. For this purpose, blank baby food samples were spiked with a multi-compound working solution 1 h before extraction procedure at 0.1, 0.5 and 1.0 mg kg^{-1}. The recoveries achieved were between 79% (dimethoate) and 115% (phosalone and deltamethrin) for QuEChERS-DLLME, and from 70% (dichlorvos) to 121% (azoxystrobin and cypermethrin) for QuEChERS-d-SPE (Table 2).

The precision was evaluated under repeatability and within-laboratory reproducibility conditions, and it was expressed in terms of relative standard deviations (RSD) (Table 2). Under repeatability conditions, three independent replicates of spiked samples at each level were analyzed on the same day by the same analyst under the same chromatographic conditions, with RSD values ranging from 1 to 13.5%, and between 1 and 14.7% for QuEChERS-DLLME and QuEChERS-d-SPE, respectively. Under within-laboratory reproducibility conditions, three independent replicates of spiked samples at each level were analyzed on 3 different days by the same analyst under the same chromatographic conditions,

totaling nine independent replicates for each level; the RSD values varied between 2 and 20%, and from 1.4 to 20% for QuEChERS-DLLME and QuEChERS-d-SPE, respectively. According to the SANCO/12571/2013 guidance, mean recoveries within the range of 70–120% and RSD values ≤ 20%, under repeatability and within-laboratory reproducibility conditions are acceptable for pesticide residues analysis in foods (EC, 2014).

LOD was set as the smallest level of pesticidein the baby food extracts that was reliably detectable but not necessarily quantifiable, and which can be distinguished from noise, while LOQ was the smallest level that could be detected and quantified with acceptable precision (RSD ≤ 20%). These limits were established by successive chromatographic analysis of extracts spiked with decreasing concentrations of the compounds until obtaining the signal-to-noise ratios of 3:1 and 10:1, respectively. In general, better LOQ and LOQ values were achieved with DLLME when compared to the d-SPE procedure.

The LOD for most of the pesticides was 0.005 mg kg^{-1} using QuEChERS-DLLME, while, a LOD of 0.01 mg kg^{-1} was obtained for azynphos-methyl, captan, chlorpyrifos, cypermethrin, deltamethrin, dichlorvos, dimethoate, diuron, folpet, imazalil and thiabendazole, a level which meets the baby food safety requirements. Using QuEChERS-d-SPE, the LODs were between 2 and 5 times higher than those achieved with DLLME; for atrazine, azoxystrobin, chlorpyrifos-methyl, difenoconazole, dimethoate, fenhexamid, malathion, methidathion, parathion-methyl, phosalone, procymidone, tetraconazole, tolylfluanid and trifloxystrobin, the levels obtained are enough to ensure the monitoring of these compounds at the MRL established by the EC for baby foods. LOQs with QuEChERS-DLLME ranged from 0.01 to 0.05 mg kg^{-1}, while with QuEChERS-d-SPE the values were slightly higher, ranging from 0.05 to 0.1 mg kg^{-1} (Table 1).

3.3. Application to real samples

QuEChERS-DLLME was applied to twenty-two samples of the most popular baby food brands available on the Brazilian market. The dicarboximide fungicide procymidone was detected in one sample at 0.0688 ± 0.0027 mg kg^{-1}, a level which significantly exceeded the MRL (0.01 mg kg^{-1}) authorized for pesticide residues in baby foods. The positive sample was composed of a mixture of apple, banana, papaya, strawberry, rice flour, starch and yogurt, and it was intended for infant consumption over 1 year old. The sample was re-analyzed with QuEChERS-d-SPE to confirm the presence of the analyte, totaling another six determinations. Fig. 4 shows the positive detection and identification of procymidone (t_R = 11.28 min) in the baby food extract, including the extracted ion chromatogram and product ion scan mass

spectrum.

According to the results of Brazilian monitoring programs for pesticide residues in foods performed in the period between 2001 and 2010, apple, papaya and strawberry were among the crops with higher percentages of positive samples for the pesticides investigated by the programs, and procymidone was one of the fifteen pesticides most frequently detected in the analyzed samples. Furthermore, the presence of the non-authorized active ingredient for use in certain crops was the main irregularity found for the period under study (Jardim & Caldas, 2012). Specifically, in Brazil, the fungicide procymidone is authorized for use in apple and strawberry, among other crops, whose MRL is 2 and 3 mg kg^{-1}, respectively (ANVISA, 2015). Together with the organophosphorus insecticides chlorpyrifos and phosalone, procymidone was also detected in samples of fruit baby foods analyzed within a monitoring program in the period between 2001 and 2003 in the Czech Republic (Stepán, Tichá, Hajslová, Kovalczuk, & Kocourek, 2005).

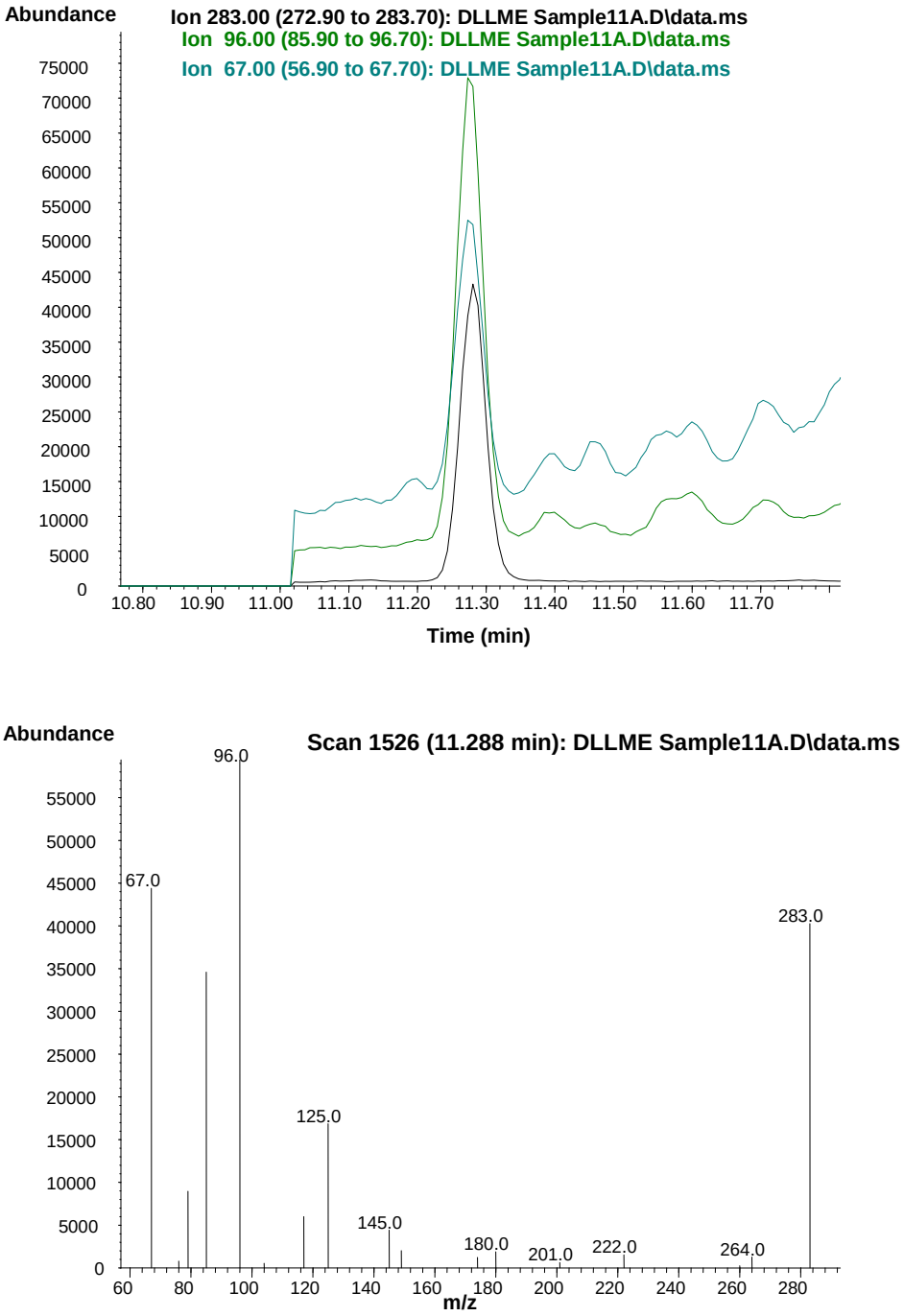

Fig. 4. Extracted ion chromatogram obtained in selected ion monitoring mode of the contaminated baby food sample with procymidone (ions *m/z* 283, 96 and 67) and corresponding product ion scan mass spectrum.

4. Conclusions

DLLME and d-SPE, combined with previous QuEChERS extraction, are simple, rapid and suitable techniques for residue analysis of the 24 pesticides investigated in baby foods. Both techniques, when allied with gas chromatography-mass spectrometry analysis provided good linearity, acceptable recovery and precision, under repeatability and within-laboratory reproducibility, and quantification and confirmation of pesticide residues at their respective MRLs. QuEChERS-DLLME, recognized by a low consumption of organic solvents, short extraction time and high enrichment factor, achieved better limits of detection and quantification, which were sufficiently low to meet the MRL of 0.01 mg kg^{-1} set by EC for pesticide residues in baby foods. However, high matrix effect, observed for all pesticide residues studied, was probably a result of an insufficient cleanup. On the other hand, QuEChERS-d-SPE with PSA and C_{18} sorbents provided an effective removal of matrix co-extractives from extracts, which contributed to lower matrix effects and method limits sufficient to meet the MRL for some of the pesticides investigated.

The detection of the fungicide procymidone in one of the 22 samples analyzed at a level above the established MRL demonstrates the necessity for constant residue monitoring of pesticides in foods intended for infants and young children in order to ensure baby food safety.

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CAPÍTULO 2 - Comparison of green sample preparation techniques in the analysis of pyrethrins and pyrethroids in baby food by liquid chromatography–tandem mass spectrometry

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Abstract

A new selective and sensitive liquid chromatography triple quadrupole mass spectrometry method was developed for simultaneous analysis of natural pyrethrins and synthetic pyrethroids residues in baby food. In this study, two sample preparation methods based on ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) and salting-out assisted liquid-liquid extraction (SALLE) were optimized, and then, compared regarding the performance criteria. Appropriate linearity in solvent and matrix-based calibrations, and suitable recoveries (75–120%) and precision (RSD values ≤ 16%) were achieved for selected analytes by any of the sample preparation procedures. Both methods provided the analytical selectivity required for the monitoring of the insecticides in fruit-, cereal- and milk-based baby foods. SALLE, recognized by cost-effectiveness, and simple and fast execution, provided a lower enrichment factor, consequently, higher limits of quantification (LOQs) were obtained. Some of them too high to meet the strict legislation regarding baby food. Nonetheless, the combination of ultrasound and DLLME also resulted in a high sample throughput and environmental-friendly method, whose LOQs were lower than the default maximum residue limit (MRL) of 10 μg kg^{-1} set by European Community for baby foods. In the commercial baby foods analyzed, cyhalothrin and etofenprox were detected in different samples, demonstrating the suitability of proposed method for baby food control.

Keywords: Baby food; Green chemistry; Food contaminants; LC-MS/MS; UA-DLLME; Pesticides.

1. Introduction

Pyrethrins are botanical chemicals, with insecticidal activity, naturally present in chrysanthemum flowers, including the species *Chrysanthemum cinerariaefolium* and *Chrysanthemum coccineum*. These natural insecticides comprise esters formed from the chrysanthemic (pyrethrin I, cinerin Iand jasmolin I) and pyrethic (pyrethrin II, cinerin II and jasmolin II) acids and the alcohols pyrethrolol, cinerolol and jasmolol [1]. Notwithstanding the insecticidal propriety against a large range of insect pests, the use of pyrethrins for insect control was widespread with the advent of their synthetic analogues, pyrethroids. In contrast to naturally occurring pyrethrins, the pyrethroids present better stability under air and light as well as higher selectivity for target species, which contribute to their extensive use in the agriculture, household products and formulations for pets, etc. [1,2]. Although the pyrethrins and pyrethroids are less toxicto mammalian cells and less persistent in the environment than organochlorines and organophosphates [1], dietary exposure to these natural compounds and their synthetic derivatives triggers safety concerns. Infants and young children are groups of special concern because their metabolic pathways are still immature and the food intake rate per body weight is higher than adults [3]. Pyrethroids act mainly on the nervous system[1], however, endocrine-disrupting activity has also been reported for some of them [4,5]. Studies have demonstrated higher sensitivity of neonatal rats to toxicity of the cypermethrin, deltamethrin and permethrin pyrethroids, when compared to adult animals [6,7].

Few reports on the occurrence of pyrethrins and pyrethroids in baby foods are available in literature. Bifenthrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin and etofenprox have been the main pyrethroids investigated in baby foods, being gas chromatography coupled to mass spectrometry (GC-MS) the analytical technique commonly employed for their identification and quantification [8–12]. Conversely, liquid chromatography (LC) has been less often employed. For instance, pyrethrin I and II were investigated in baby foods using LC with UV-Vis detection [13]. In other study, LC coupled to Orbitrap high resolution mass spectrometry was applied for multi-residue analysis, including some pyrethrins and pyrethroids, in fish-, meat- and vegetables-based baby foods [14]. Although the number of analytes that can be simultaneously monitored in one single run is theoretically unlimited by this technique, lower analytical sensitivity has been achieved when compared to LC triple quadrupole mass spectrometry. As a result, limits of quantification between 5- and 10-fold higher than the maximum residue limit (MRL) of 10 μg kg^{-1} set by European Community for baby foods were obtained for some of the investigated insecticides using this analytical technique [14].

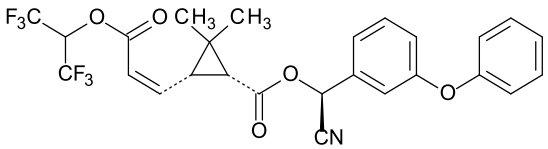
An important aspect of routine analytical methods is their environmental impact. Most of the methods used for baby food monitoring are time consuming and involve laborious sample preparation steps, including gel permeation chromatography (GPC) [8,11], solid-phase extraction (SPE) [14] and matrix solid-phase dispersion (MSPD) [9] and also subsequent liquid–liquid partitioning with hexane and methylene chloride [13]. Therefore, considerable amounts of sample and reagents, high volumes of extraction solvent, and SPE cartridges are required, which makes them expensive and generators of large residues. On the other hand, major advances have been achieved on sample preparation techniques within a green chemistry context, including minimal solvent and reagent consumption; elimination or reduction of the use of toxic substances; application of factors as pressure, microwave and ultrasound radiation focusing in high process effectiveness in a short time interval; and lower residues generation [15]. In this way, dispersive liquid-liquid microextraction (DLLME) and salting-out assisted liquid-liquid extraction (SALLE) can be attractive and environmental-friendly alternatives for application in routine analysis. DLLME is based on a ternary component solvent system, which involves the rapid injection of an appropriate mixture of disperser (water soluble solvent) and few microliters of extracting solvent into an aqueous solution. It results in the dispersion of water-immiscible extracting solvent throughout the aqueous phase as fine droplets and then the analytes are enriched into it [16]. SALLE involves addition of an electrolyte to aqueous sample matrix with the aim to increase the distribution ratio of solute by weakening or the disruption of the solvation forces between the solute and the solvent, as well as to provide separation of water-miscible solvents by formation of a biphasic system [17]. Although these techniques have already been applied for pesticide residues monitoring, there is a paucity of published research on their application for non-aqueous and complex matrices.

As an alternative to usual GC-MS, this paper reports a liquid chromatography triple quadrupole mass spectrometry (LC-QqQ-MS/MS) method for simultaneous analysis of 17 natural pyrethrin and pyrethroid residues in baby food (Fig. 1). Furthermore, two sample preparation methods, based on ultrasound-assisted DLLME and SALLE techniques, were optimized and compared. This study was focused on satisfying the following requirements (i) simplicityof operation and minimal solvent consumption, (ii) high sample throughput, and (iii) analytical selectivity and sensitivity for the monitoring of these insecticides at levels below the maximum residue limit (MRL) of 10 μg kg^{-1} established for baby foods by European Community.

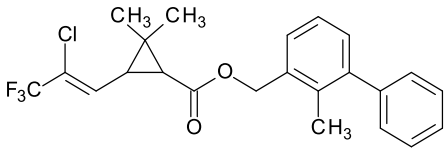
2. Experimental

2.1. Chemicals and standard solutions

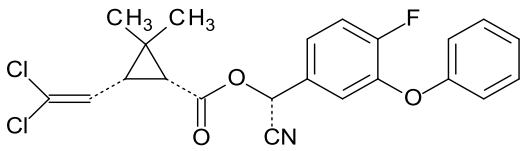
Analytical standards of pyrethroids (acrinathrin, bifenthrin, β-cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, esfenvalerate, tefluthrin, fluvalinate, flumethrin and etofenprox) and the internal standard (I.S.) etofenprox-D5 were obtained from Dr. Ehrenstorfer GmbH and Fluka, whose purity ranged from 95.7 to 99.8%. A pyrethrins standard mixture, including, cinerin I (4.8%), cinerin II (4.7%), jasmolin I (2.9%), jasmolin II (1.8%), pyrethrin I (51.7%) and pyrethrin II (33.7%), was purchased from Dr. Ehrenstofer GmbH. Stock standard solutions were prepared in methanol at 1000 μg mL^{-1}. A multi-compound working solution was prepared in methanol at 1μg mL^{-1} by combining appropriate aliquots of the individual stock solutions of the pyrethroids. Also, a pyrethrins working solution was prepared dissolving 10 mg of the standard mixture in 10 mL of methanol, which is equivalent to 48 μg mL^{-1} of cinerin I, 47 μg mL^{-1} of cinerin II, 29 μg mL^{-1} of jasmolin I, 18 μg mL^{-1} of jasmolin II, 517 μg mL^{-1} of pyrethrin I and 337 μg mL^{-1} of pyrethrin II. All standard solutions were stored at –18ºC and protected from light. LC-MS grade acetonitrile and methanol were obtained from Fisher Scientific and Panreac, respectively. Analytical grade carbon tetrachloride and chloroform were purchased from VWR International; and ammonium formate, glacial acetic acid and sodium chloride were from Sigma-Aldrich. Deionized water was obtained from a Milli-Q SP reagent water system (Millipore).



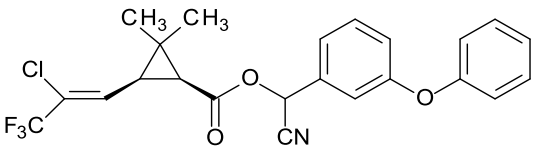
Acrinathrin



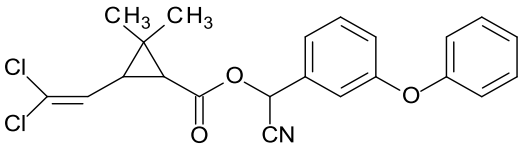
Bifenthrin



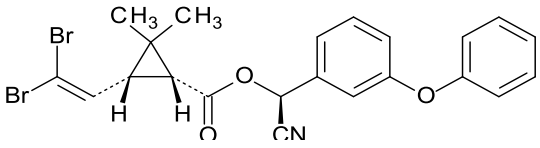
β-Cyfluthrin



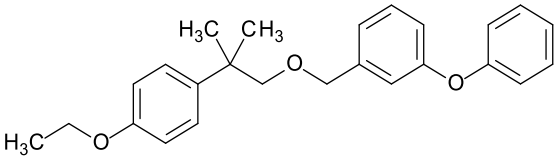
Cyhalothrin



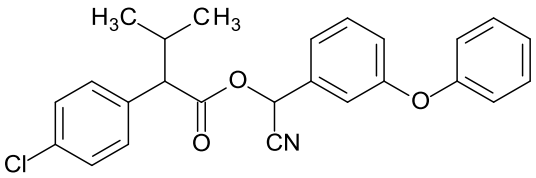
Cypermethrin



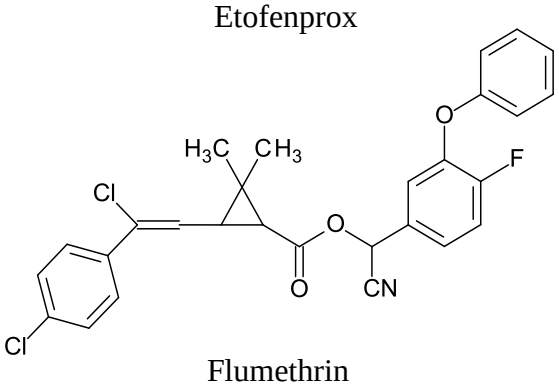
Deltamethrin



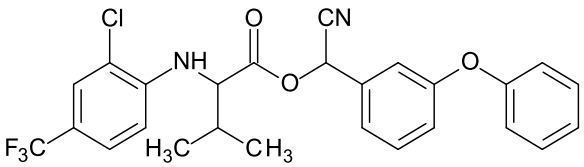
Etofenprox



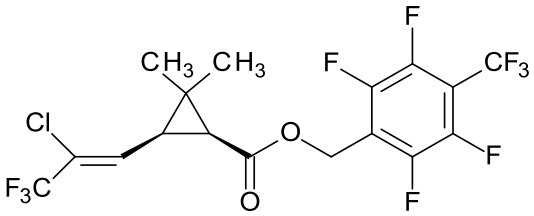
Esfenvalerate



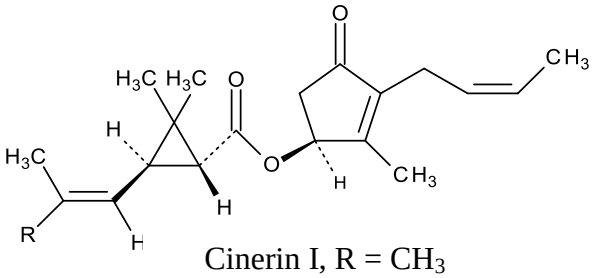
Flumethrin



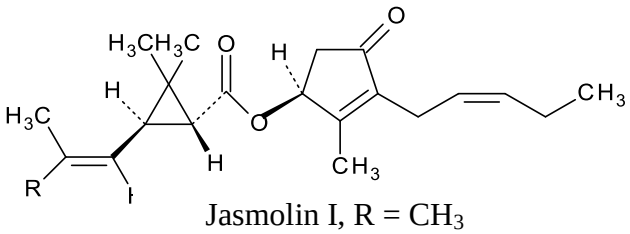
Fluvalinate



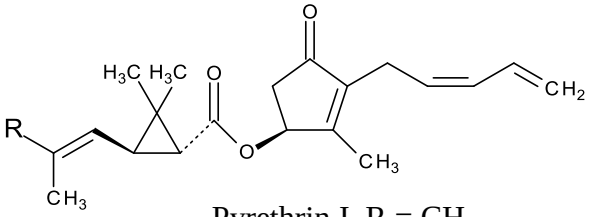
Tefluthrin



Cinerin I, R = CH_3
Cinerin II, R = $COOCH_3$



Jasmolin I, R = CH_3
Jasmolin II, R = $COOCH_3$



Pyrethrin I, R = CH_3
Pyrethrin II, R = $COOCH_3$

Fig. 1. Chemical structures of investigated natural pyrethrins and synthetic pyrethroids.

2.2. Sampling

Commercial baby foods were purchased in the city of Campinas, SP, located in the South-Eastern region of Brazil, between April and May 2016. A total of 15 ready-to-eat samples of the most popular brands were randomly collected from 3 local supermarkets, including 10 fruit and cereal-based baby foods, and 5 fruit and milk-based baby foods. All the samples were kept in their original packing, plastic bag (113 g each) or glass jar (120 g each), at 4ºC until analysis.

2.3. Simultaneous analysis of pyrethrins and pyrethroids by LC-MS/MS

2.3.1. Ultrasound-assisted dispersive liquid–liquid microextraction (UA-DLLME)

One gram of homogenized baby food sample and 4 mL of pre-extraction solvent (acetonitrile-water-glacial acetic acid 79:20:1, v/v/v) were added to a 50 mL polypropylene centrifuge tube, agitated in a mechanical shaker for 30 min and centrifuged at 1372 rcf for 15 min at 15°C. The supernatant was collected, added of 7 mL of deionized water and 100 µL of chloroform and gently shaken by hand for 30 s, and then, the tube was immersed in an ultrasonic water bath for 3 min at room temperature. A cloudy solution was formed and the analytes were extracted into micro-droplets of chloroform dispersed entirely into the aqueous phase. The tube was centrifuged at 1372 rcf for 15 min at 15°C, the upper aqueous phase was removed with a Pasteur pipette and the bottom phase (chloroform) was collected using an automatic micropipette. The chloroform extract was evaporated to dryness under a stream of nitrogen and finally, the residue was dissolved in 100 µL of methanol (10 g mL^{-1} of sample equivalent in the final extract) and then submitted to LC-MS/MS analysis.

2.3.2. Salting-out assisted liquid–liquid extraction (SALLE)

One gram of homogenized baby food sample and 4 mL of deionized water were placed in a 50 mL polypropylene centrifuge tube in an ultrasonic water bath for 3 min at room temperature to get an aqueous homogenous mixture. Then, 2 mL of acetonitrile and 1 g of NaCl were added and the tube was manually shaken for approximately 30 s to dissolve the salt. After agitation, the mixture was centrifuged at 1372 rcf for 15 min at 15°C and 500 µL of upper phase (acetonitrile) was collected using an automatic micropipette for subsequent LC-MS/MS analysis. A concentration of 2 g mL^{-1} of sample equivalent in the final extract was obtained.

2.3.3. Chromatographic and mass-spectrometric conditions

A 1260 Infinity ultra-high performance liquid chromatograph (UHPLC) interfaced to a 6410 triple quadrupole mass spectrometer (QqQ-MS/MS) with electrospray ionization (ESI) source of Agilent Technologies (Santa Clara, CA, USA) was employed for pyrethrins and pyrethroids analysis. MassHunter Workstation software (A GL sciences, Tokyo, Japan) was used for qualitative and quantitative data analysis. The chromatographic separation was achieved on a reversed phase column (150 mm x 2 mm i.d., 3 µm particle size; Luna C_{18}, Phenomenex) that was maintained at 30ºC. The mobile phase consisted of deionized water (A) and methanol (B), both containing 10 mM ammonium formate at a flow rate of 0.3 mL min^{-1}. The elution gradient was: 0 min, 50% B; 10–12 min, 83% B; 12–25 min, 98 % B; then, returned to the initial conditions and equilibrated for 15 min. The total run time was 40 min, the injection volume was 5 µL. Data acquisition was carried out in selective reaction monitoring (SRM) employing ESI in positive mode, whose conditions were: capillary voltage 4000V, nebulizer 25 psi, source temperature 300°C and gas flow 11 L min^{-1}. Nitrogen was used as collision, desolvation and nebulizing gas. The collision energy and cone voltage were optimized for each analyte and are shown in the Table 1.

2.3.4. Identification and quantification

From the ESI-MS/MS optimization, the two most abundant and characteristic product ions arising from ammonium adduct or protonated molecule species were selected for each compound. The most abundant precursor-to-product ion transition (SMR_1) was monitored for the quantification and one second (SMR_2), less intense, was used for the identification of each compound (Table 1). The I.S. etofenprox-D5 was added to sample at level of 10 µg kg^{-1} prior to the extraction procedure, and the pyrethrins and pyrethroids were quantified employing seven-point matrix-based calibrations constructed by plotting analyte/I.S. peak area ratio against concentration values.

Table 1. Selective reaction monitoring (SRM) transitions and LC-MS/MS operating parameters for simultaneous analysis of pyrethrins and pyrethroids using an electrospray interface (ESI) in positive ionization mode.

Analyte	MW (g mol^{-1}) [a]	t_R (min) [b]	Precursor ion	SRM_1 [c]	Frag (V) [d]	CE (V) [e]	SRM_2 [f]	Frag (V) [d]	CE (V) [e]
Cinerin II	360.40	15.94	361	149	76	10	107	76	14
Pirethrin II	372.40	16.13	373	161	76	10	133	76	14
Jasmolin II	374.45	16.92	375	163	25	20	107	20	20
Cinerin I	316.40	17.85	317	149	66	5	107	66	20
Cyfluthrin	434.29	17.85	451	206	66	50	191	66	10
Pirethrin I	328.40	17.89	329	161	76	10	133	76	20
Cyhalothrin	449.85	17.96	467	225	66	10	141	66	46
Cypermethrin	416.30	18.02	433	191	76	10	127	76	30
Acrinathrin	541.44	18.18	559	208	76	10	181	76	30
Deltamethrin	505.21	18.20	523	506	100	5	281	100	10
Esfenvalerate	419.91	18.25	437	181	66	38	167	66	10
Jasmolin I	330.40	18.35	331	163	76	10	107	76	20
Tefluthrin	418.74	18.36	436	177	100	20	---	---	---
Fluvalinate	502.92	18.40	503	208	50	10	181	50	26
Flumethrin	510.39	18.79	527	267	66	10	239	66	18
Etofenprox-D5	381.50	19.06	399	364	98	5	183	98	9
Bifenthrin	422.87	19.16	440	181	94	6	166	94	46
Etofenprox	376.50	19.20	394	359	66	10	177	66	10

[a] Molecular weight;
[b] Retention time;
[c] Precursor-to-product ion transition monitored for quantification;
[d] Fragmentor;
[e] Collision energy;
[f] Precursor-to-product ion transition monitored for qualification.

3. Results and discussion

3.1. Optimization of sample preparation method

3.1.1. Ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME)

Ultrasounds have been utilized to improve the extraction efficiency of the conventional DLLME technique [18–20] because enhances the contact surface area between the two immiscible phases, accelerates the mass transfer of analytes from the aqueous phase into the extracting micro-droplets and, consequently, reduces the extraction equilibrium time [21–24]. In this study, this positive effect was confirmed by appropriate recoveries for 17 pyrethrins and synthetic pyrethroids. Furthermore, stable and reproducible fine emulsions for all kinds of baby foods analyzed were obtained by using an ultrasonic water bath. Ultrasonication compared to usual injection of extracting and disperser solvents sample-to-sample provides other advantages as less manual labor and simultaneous extraction of a high number of samples in a short time interval.

As the baby foods analyzed were solid samples, extraction prior to UA-DLLME was mandatory. For this, a mixture of acetonitrile, water and glacial acetic acid (79:20:1, v/v/v) was tested at 4:1 ratio (extractor: sample, v/w) using a mechanical shaker under constant agitation. In addition, the time involved in this pre-extraction step was also evaluated at 30, 60 and 90 min. A comparison between the recoveries obtained from the different extraction times is shown in Fig. 2A. Acceptable average recoveries, between 71% (esfenvalerate at 30 min) and 120% (etofenprox at 90 min), were achieved for all compounds in the 3 studied conditions, demonstrating the fitness-for-purpose of the acetonitrile-based extraction mixture. Furthermore, considerable variations in the recoveries were observed, with standard deviations between 12 and 23%, 0.1 and 24%, and 0.2 and 17%, at 30, 60 and 90 min, respectively, suggesting little differences between the recoveries obtained for the 3 times evaluated. Therefore, 30 min was chosen for the pre-extraction step. An advantage, associated with the use of acetonitrile, is the extraction of less matrix components as fats, proteins and sugars, contributing to lower demand of further cleanups [25,26].

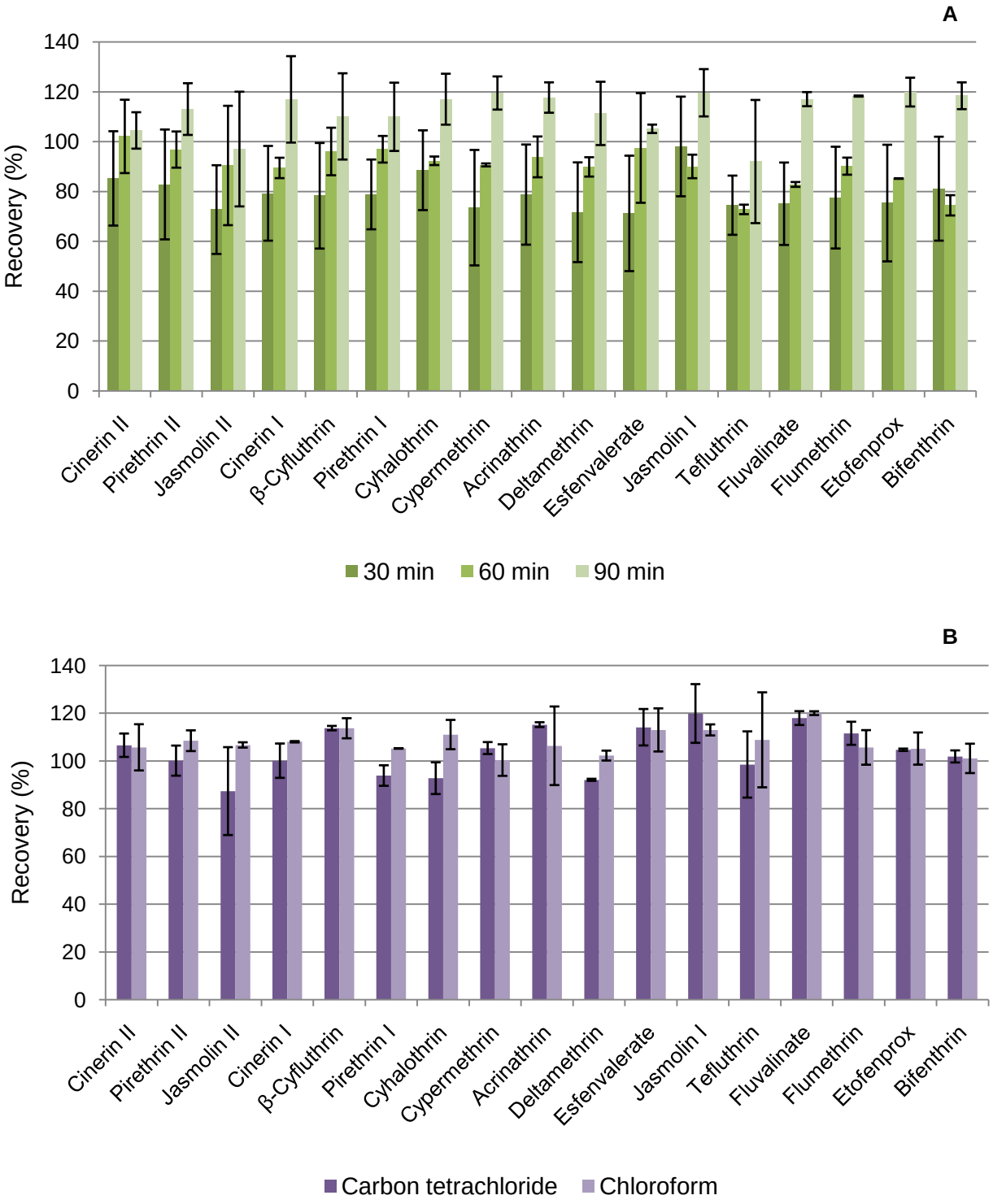


Fig. 2. Effect of different extraction times (A) and types of extraction solvent (B) on the recoveries of selected insecticides obtained with ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) using a representative baby food spiked at 10 µg kg^{-1} (n = 3). Conditions: baby food sample composed by fruit purées (apple and banana), apple juice, rice flour and starch; (A) solvent: carbon tetrachloride; (B) time: 90 min.

The UA-DLLME was carried out in the acetonitrile extract obtained after the pre-extraction step to provide high concentration factor. For this, 7 mL of deionized water and 100 µL of selected extracting solvent were added. The mixture was rapidly shaken by hand followed by sonication in ultrasonic water bath. A stable cloudy solution was formed (water–acetonitrile–extracting solvent), wherein the acetonitrile, initially employed as pre-extraction solvent, was used as disperser solvent. Selection of extracting solvent has been established as the major parameter to obtain maximum extraction efficiency with the DLLME technique, and it has been selected on the basis of higher density than water, high extraction affinity to the compounds and low solubility in aqueous solution [16]. In this way, 100 µL of either carbon tetrachloride ($d_{25°C}$=1.589 g mL^{-1}) or chloroform ($d_{20°C}$=1.484 g mL^{-1}) were tested as extracting solvent, which resulted in a final volume of 90 µL of organic phase at the bottom of the tube after centrifugation step. Lower extracting solvent volumes has been reported in literature for DLLME procedure [16]. Nonetheless, the difficult retrieval of resulting bottom layer can affect the reproducibility of the process. Therefore, during development of method, we fixed the extracting solvent volume at 100 µL.

As can be observed in the Fig. 2B, excellent average recoveries were obtained for all studied compounds using carbon tetrachloride (87–120%) and chloroform (100–120%) in UA-DLLME. These results indicate the suitability of ultrasonic energy for the dispersion of these extracting solvents as micro-droplets throughout the aqueous phase in absence of the conventional injection of the dispersive solvent, as well as an adequate and fast transfer of compounds from aqueous phase to extracting solvent in the studied conditions. In addition, the extraction time involved in this process was short.The surface area between the aqueous phase and extracting solvent is wide and the extraction equilibrium is rapidly obtained [16]. According to Fig. 2B, slightly better recoveries were achieved for most natural pyrethrins using chloroform, thus this organic solvent was selected as extracting solvent for the UA-DLLME.

3.1.2. Salting-out assisted liquid–liquid extraction (SALLE)

In parallel, a sample preparation based on salting-out was also studied. The method involves only 2 mL of extracting solvent and fast execution, which includes manual shaking-based extraction followed by centrifugation. Prior to extraction process, 4 mL of deionized water were added to 1 g of sample with the aim to obtain an aqueous homogenous mixture for further liquid-liquid extraction. Similar to DLLME technique, the type of extracting solvent plays a critical role in the process extraction efficiency. Taking into account the *n*-octanol-

water partition coefficients (logK_{ow}) of the compounds investigated in the present study, between 4 (esfenvalerate) and 7.65 (flumethrin), organic solvents with different water-solubilities, including acetonitrile and ethyl acetate, were evaluated focusing maximal extraction and a consistent organic upper layer after phase separation. According to literature acetonitrile [17,28,29] and ethyl acetate [30,31] have been successfully used in SALLE procedures. Other important parameters for the effectiveness of SALLE process are the salting-out agent and its amount. The choice of NaCl was based on their high solubility in aqueous solution, low cost and common presence in most of laboratories. Salting-out was successful using 1g of this salt to 5 g of aqueous mixture. Additionally, good recoveries (> 60%) were reported for polycyclic aromatic hydrocarbons combining 1 g this salt to acetonitrile in SALLE procedure [29].

To enhance the organic phase separation, a centrifugation step after manual-shaking based extraction was introduced. The final volume of upper organic layer (500 µL) was much lower than the remaining aqueous bottom phase for both organic solvents, contributing to higher concentration of analytes in the final extract. This finding is consistent with that reported by other authors [29]. We also observed a whitish middle layer at the interface between the organic and aqueous layers, possibly formed by polysaccharides and proteins, whose layer thickness was dependent on the composition of sample analyzed, especially for milk-based baby foods; however, this did not affect the retrieval of the upper organic layer. The same behavior was reported for honey [29,32,33]. The influence of different water-solubility solvents combined to NaCl on the extraction efficiency is shown in the Fig. 3. As can be observed adequate recoveries were achieved for all studied compounds using acetonitrile (81–113%) as well as ethyl acetate (75–120%). Compared to ethyl acetate, acetonitrile resulted in a less colored yellowish layer, indicating lower extraction of matrix components. Based on their advantages as also good compatibility with LC analysis, acetonitrile was selected as extraction solvent for the SALLE procedure.

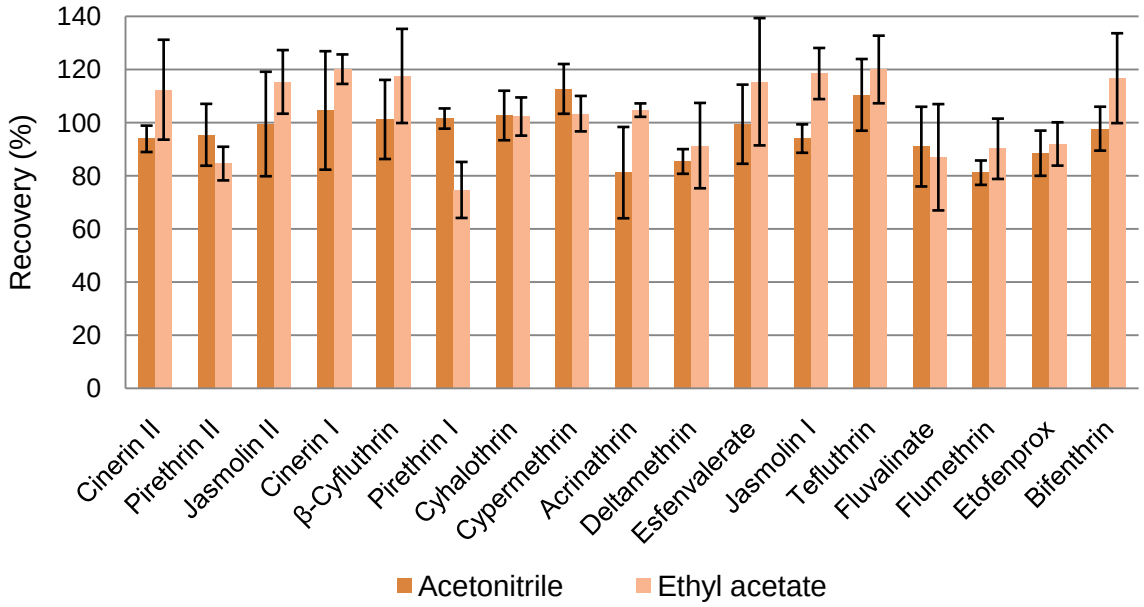


Fig. 3. Recoveries obtained with acetonitrile or ethyl acetate as the extraction solvent in salting-out assisted liquid-liquid extraction (SALLE) using a representative baby food composed by fruit purées (apple and banana), apple juice, rice flour and starch, spiked at 25 μg kg^{-1} (n = 3).

3.2. Performance criteria

The fitness-for-purpose of the optimized sample preparation methods was assessed establishing the selectivity, linearity, recovery, precision and sensitivity (limits of detection and quantification). For in-house validation, the SANTE/11945/2015 requirements were considered [34]. A representative blank matrix – baby food free of residues of target compounds, composed by a mixture of fruit purées (apple and banana), apple juice, rice flour and starch, was used for validation procedures as well as to prepare matrix-based calibrations for quantitative analysis.

Analytical selectivity was verified by analyzing blank baby food samples, and no matrix interferences were observed near the analyte retention time in the SRM chromatograms of individual compounds (Fig. 5), contributing to easy and fast integration of the analyte peaks. Linearity was assessed in solvent and matrix-based calibrations, which included seven concentration levels each. For most pyrethroids, this concentration range was between 10 and 500 μg L^{-1}, except for β-cyfluthrin, deltamethrin and tefluthrin that was from 25 to 500 μg L^{-1}. Taking into account the composition of the commercial pyrethrins standard

mixture employed, the linearity for pyrethrins was evaluated in different ranges, namely, cinerin I (4.8–240 μg L^{-1}), cinerin II (4.7–235 μg L^{-1}), jasmolin I (2.9–145 μg L^{-1}), jasmolin II (1.8–90 μg L^{-1}), pyrethrin I (51.7–2585 μg L^{-1}), and pyrethrin II (33.7–1685 μg L^{-1}). A good linearity across the studied ranges was achieved for both sample preparation methods with determination coefficients (R^2) higher than 0.99 for all compounds investigated (Table 2).

In the absence of certified reference material available for the compounds studied in baby food, the extraction efficiency was verified by recovery experiments. For this, five independent replicates of spiked baby foods at two levels were analyzed. As a result, average recoveries between 75% (acrinathrin) and 114% (β-cyfluthrin), and between 78% (bifenthrin) and 120% (β-cyfluthrin) were obtained for the pyrethroids using UA–DLLME and SALLE, respectively. The average recoveries for the natural pyrethrins ranged from 79% (pyrethrin I) to 94% (jasmolin I), and from 86% (cinerin I) to 104% (jasmolin I) for UA-DLLME and SALLE, respectively (Table 2). Considering the composition of the pyrethrins standard mixture, different spiked levels were obtained for the recovery studies, which are referred as level I and II in the Table 2. The method precision was assessed under repeatability conditions and it was expressed in terms of relative standard deviation (RSD_r). Under repeatability conditions, five independent replicates of spiked samples at each level were analyzed on the same day by the same analyst under the same chromatographic conditions, RSD_r values varied from 3% (cinerin I) to 15% (β-cyfluthrin and esfenvalerate) for UA-DLLME, and from 3% (cypermethrin) to 16% (acrinathrin) for SALLE (Table 2). Resulting recovery and precision values were consistent with the validation criteria established by the SANTE/11945/2015 guidance for pesticide residues analysis in food, with recoveries between 70 and 120%, and $RSD_r \leq 20\%$ [34].

Table 2. Performance criteria obtained with ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) and salting-out assisted liquid-liquid extraction (SALLE) procedures for pyrethrins and pyrethroids in a representative matrix of baby food [a].

Pyrethroid	UA-DLLME					SALLE				
	LOD µg kg^{-1}	LOQ µg kg^{-1}	Linearity [b], R^2	% Recovery [c] (% RSD_r)		LOD µg kg^{-1}	LOQ µg kg^{-1}	Linearity [b], R^2	% Recovery [c] (% RSD_r)	
				5 µg kg^{-1}	10 µg kg^{-1}				25 µg kg^{-1}	100 µg kg^{-1}
Cyfluthrin	1.7	5.0	0.9969	86 (15)	114 (7)	8.0	25.0	0.9998	120 (8)	99 (9)
Cyhalothrin	0.3	1.0	0.9989	78 (7)	90 (8)	1.7	5.0	0.9996	96 (10)	99 (11)
Cypermethrin	0.8	2.5	0.9990	101 (7)	114 (8)	4.0	12.5	0.9932	103 (3)	101 (12)
Acrinathrin	0.3	1.0	0.9991	75 (8)	85 (9)	1.7	5.0	0.9991	98 (11)	95 (16)
Deltamethrin	1.7	5.0	0.9989	78 (9)	84 (5)	8.0	25.0	0.9990	89 (7)	94 (14)
Esfenvalerate	0.8	2.5	0.9980	99 (15)	99 (9)	4.0	12.5	0.9993	88 (10)	90 (14)
Tefluthrin	1.7	5.0	0.9986	89 (13)	78 (7)	8.0	25.0	0.9989	91 (8)	84 (16)
Fluvalinate	0.3	1.0	0.9957	78 (9)	87 (10)	1.7	5.0	0.9981	92 (5)	87 (14)
Flumethrin	0.3	1.0	0.9970	86 (8)	83 (12)	1.7	5.0	0.9996	81 (12)	90(14)
Etofenprox	0.2	0.5	0.9981	79 (5)	87 (8)	0.8	2.5	0.9986	80 (10)	88 (9)
Bifenthrin	0.3	1.0	0.9982	84 (5)	91 (8)	1.7	5.0	0.9977	78 (9)	85 (12)

Pyrethrin	UA-DLLME					SALLE				
	LOD µg kg^{-1}	LOQ µg kg^{-1}	Linearity [b], R^2	% Recovery [c] (% RSD_r)		LOD µg kg^{-1}	LOQ µg kg^{-1}	Linearity [b], R^2	% Recovery [c] (% RSD_r)	
				Level I [d]	Level II [e]				Level I [f]	Level II [g]
Cinerin I	0.2	0.5	0.9993	89 (3)	86 (9)	0.8	2.5	0.9995	86 (5)	96 (11)
Cinerin II	0.2	0.5	0.9964	80 (7)	87 (12)	0.8	2.5	0.9996	99 (7)	97 (12)
Jasmolin I	0.2	0.7	0.9987	94 (7)	87 (6)	1.0	3.5	0.9995	104 (6)	91 (13)
Jasmolin II	0.2	0.5	0.9978	88 (12)	91 (9)	0.8	2.5	0.9969	97 (14)	99 (16)
Pirethrin I	1.7	5.0	0.9987	79 (7)	86 (10)	8.0	25.0	0.9997	95 (6)	95 (12)
Pirethrin II	1.0	3.0	0.9965	83 (9)	90 (4)	5.0	15.0	0.9987	96 (14)	97 (11)

R^2: determination coefficient; RSD_r: relative standard deviation under repeatability conditions; [a] Baby food composed by a mixture of fruits purée (apple and banana), apple juice, rice flour and starch; [b] Matrix-based calibration curves; [c] A total of five independent replicates;
[d] 2.4 µg kg^{-1}, cinerin I; 2.35 µg kg^{-1}, cinerin II; 1.45 µg kg^{-1}, jasmolin I; 0.9 µg kg^{-1}, jasmolin II; 25.85 µg kg^{-1}, pyrethrin I; 16.85 µg kg^{-1}, pyrethrin II;
[e] 4.8 µg kg^{-1}, cinerin I; 4.7 µg kg^{-1}, cinerin II; 2.9 µg kg^{-1}, jasmolin I; 1.8 µg kg^{-1}, jasmolin II; 51.7 µg kg^{-1}, pyrethrin I; 33.7 µg kg^{-1}, pyrethin II;
[f] 12 µg kg^{-1}, cinerin I; 11.75 µg kg^{-1}, cinerin II; 7.25 µg kg^{-1}, jasmolin I; 4.5 µg kg^{-1}, jasmolin II; 129.85 µg kg^{-1}, pyrethrin I; 84.25 µg kg^{-1}, pyrethin II;
[g] 48 µg kg^{-1}, cinerin I; 47 µg kg^{-1}, cinerin II; 29 µg kg^{-1}, jasmolin I; 18 µg kg^{-1}, jasmolin II; 517 µg kg^{-1}, pyrethrin I; 337 µg kg^{-1}, pyrethin II .

Sensitivity is one of the most critical analytical parameters for baby foods control due to the stringent MRL set by European Community [35]. In this context, UA-DLLME and SALLE procedures were compared with the aim to meet the default MRL of 10 μg kg^{-1} for pesticide residues in baby foods [36]. LOD and LOQ were established as the smallest analyte concentration in baby food extract that provided a signal 3 and 10 times, respectively, higher than the background signal, for both SRM_1 and SRM_2 transitions selected for each compound. In general, lower LOQs were achieved with UA-DLLME when compared to the SALLE procedure (Table 2). UA-DLLME provided LOQs lower than MRL of 10 μg kg^{-1} for the pyrethrins as also for the pyrethroids (from 0.5 to 5 μg kg^{-1}), demonstrating the high analytical sensitivity of the proposed method. On the other hand, higher LOQs (between 12.5 and 25 μg kg^{-1}) were obtained with SALLE for some analytes. However, for the pyrethrins cinerin I, cinerin II and jasmolin II (2.5 μg kg^{-1}), jasmolin I (3.5 μg kg^{-1}), and for the pyrethroids acrinathrin, bifenthrin, cyhalothrin, flumethrin and fluvalinate (5 μg kg^{-1}), and etofenprox (2.5 μg kg^{-1}), the LOQs achieved by SALLE allow the quantification of these compounds bellow the MRL specified by EC legislation.

Compared to published methods for the determination of pyrethroids using GC, the proposed UA-DLLME method presents adequate feature, including lower LOQs. For instance, LOQ of 50 μg kg^{-1} was obtained for cypermethrin and deltamethrin in fruit-based baby foods [12], and values between 12.5 and 50μg kg^{-1} were achieved for 5 pyrethroids in maize samples [37], both studies combining QuEChERS with DLLME and GC-MS analysis. In other work with apple juice, LOQs of 2.7 and 7.3 μg L^{-1} were obtained for λ-cyhalothrin and cypermethin, respectively, using DLLME and GC-MS [38]. Zhang et al. reported LOQs between 1 and 5 μg kg^{-1} for 10 pyrethroids in fruit juices employing DLLME and GC analysis with electron capture detection [27].

3.3. Matrix effects

Matrix effects are known sources of error in quantitative LC-MS/MS analysis, which lead to overestimation or underestimation of results [39,40]. Additionally, baby foods are complex matrices [41] because normally contain fruit purées, cereal flours, starch and milk or other dairy ingredient, which contribute to presence of potential analytical interferences in the final extract, such as pigments, organic acids, sugars and fatty acids. When co-eluted, these matrix components compromise the efficiency of the ionization process in the ESI source, resulting in enhancement or suppression of analyte signal [26,42,43]. These matrix effects

were investigated by using the slopes of the calibration curves at the same concentrations in solvent and matrix extracts (employing the same representative matrix as in performance criteria). For this, no internal standard was used in the trials in order to avoid misleading conclusions, once it could also be subjected to the effect [44]. Matrix effect (ME) was calculated employing the equation: ME% = [(matrix slope – solvent slope)/ solvent slope] x 100. Enhancement or suppression of the analytical signal was verified for analytes because the intensity of these matrix-induced effects was dependent on each compound and the sample preparation method employed (Fig. 4). For most compounds, higher matrix effects (ME% < –20% or ME% > +20%) were observed for UA-DLLME procedure when compared to SALLE, possibly due to high enrichment factor obtained with this procedure, thus resulting in concentration of co-eluting residual matrix components in the final extract. Matrix-based calibrations were employed for matrix effect compensation during quantitative analysis.

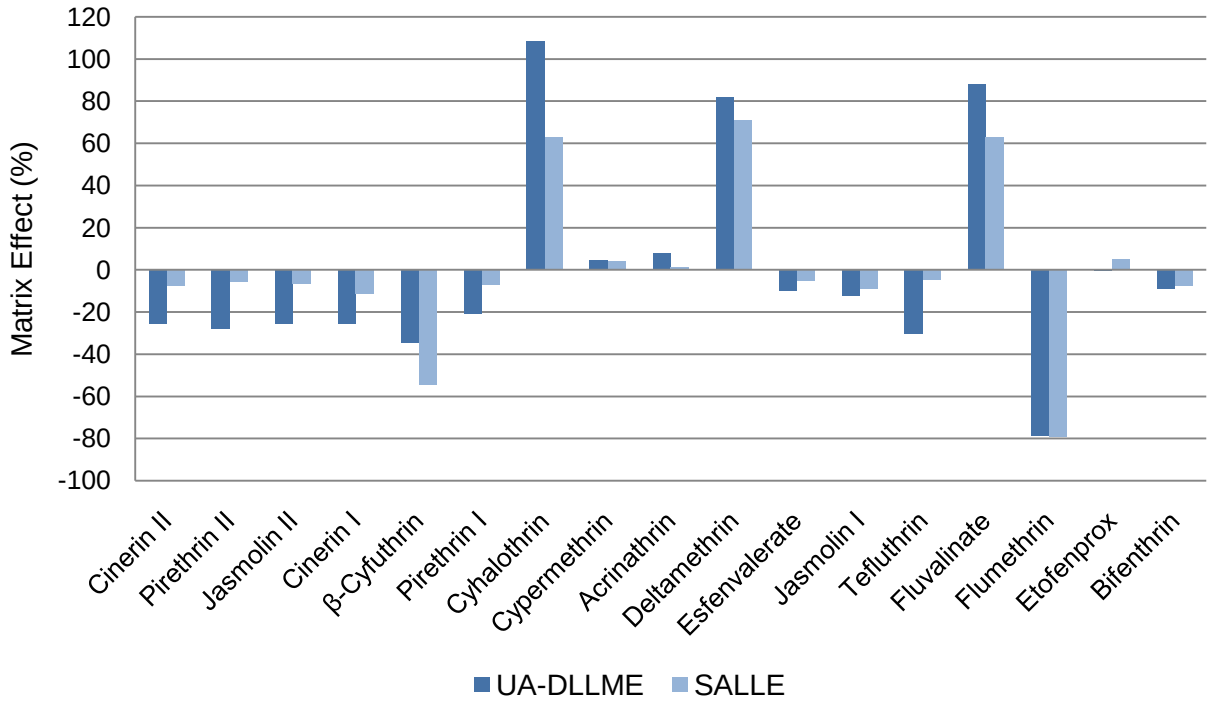


Fig. 4. Comparison of LC-MS/MS matrix effects obtained for the selected insecticides employing ultrasound-assisted dispersive liquid-liquid microextraction (UA-DLLME) and salting-out assisted liquid-liquid extraction (SALLE) procedures.

3.4. Application to commercial baby food samples

The occurrence of the target insecticides in baby foods intended for infants and young children commercially available on the Brazilian markets was investigated. Taking into account the high analytical sensitivity achieved with UA-DLLME, including LODs and LOQs lower than MRL of 10 μg kg^{-1}, this was the procedure applied to the 15 samples. Two pyrethroids were separately detected in baby food samples, whose levels found were lower than the MRL established for pesticide residues in baby food by the Commission Directive 2006/125/EC [36]. The positive identification of these analytes in the baby food samples involved the acquisition of the two SRM transitions for each compound. The identifcation was established by LC retention time (t_R) equal to that obtained in calibration standards within a maximum tolerance of ± 0.1 min, as well as the monitoring of ion ratios of the two selected SRM transitions, which was set as the relationship between the intensity of transitions used for identification and for quantification, SRM_2/SMR_1 [33]. Cyhalothrin and etofenprox were detected in different samples at levels around the LOQ achieved for these compounds (0.7 and 0.6 μg kg^{-1}, respectively) (Fig. 5).

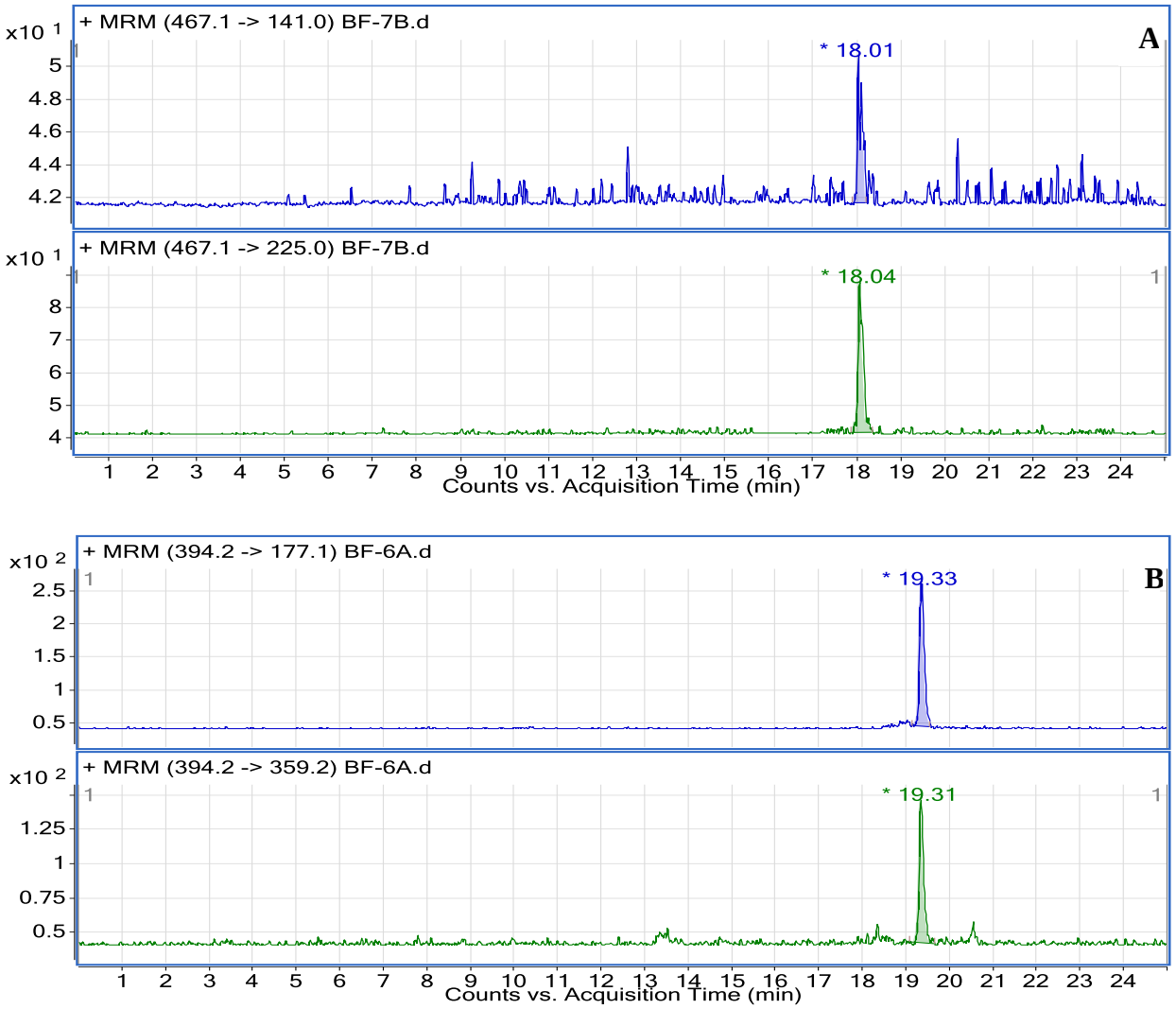


Fig. 5. LC-MS/MS chromatograms obtained in selective reaction monitoring (SRM) mode of baby food samples contaminated with cyhalothrin at a level of 0.7 µg kg^{-1}(A) and etofenprox at a level of 0.6 µg kg^{-1} (B).

4. Conclusions

LC-QqQ-MS/MS achieves simultaneous analysis of pyrethrins and pyrethroids in baby food, as an alternative to usual GC-MS that only works properly for synthetic pyrethroids. Furthermore, two sample preparation methods has been successfully optimized to provide suitable performance criteria, including linearity in solvent and matrix-based calibrations, recovery, precision and analytical sensitivity for the monitoring of the insecticides in complex matrices as fruit-, cereal- and milk-based baby foods. SALLE demonstrated to be an attractive technique for routine analysis, mainly with respect to cost-effectiveness, and simple and fast execution. Although the obtained LODs were below the

MRL of 10 μg kg^{-1} set by European Community for pesticide residues in baby foods, the lower enrichment factor achieved with this technique resulted in LOQs higher than the MRL for cyfluthrin, cypermethrin, deltamethrin, esfenvalerate, pyrethrin I, pyrethrin II and tefluthrin. On the other hand, the combination of ultrasound and DLLME techniques resulted in an analytical method highly sensitive, whose LOQs were well below the MRL fixed by the EC, satisfying baby food safety requirements. Furthermore, the use of ultrasonic bath in DLLME reduced the manual labor and allowed simultaneous extraction of high number of samples, contributing to a high sample throughput and environmental-friendly procedure for baby food control.

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CAPÍTULO 3 - A novel sample preparation approach for the trace determination of polycyclic aromatic hydrocarbons in baby food using gas chromatography–mass spectrometry: Application of Plackett-Burman screening design

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Abstract

This work reports the successful development of an accurate and sensitive gas chromatography-mass spectrometry method for the determination of twelve polycyclic aromatic hydrocarbons (PAHs) in baby food. The sample preparation involves QuEChERS-based extraction followed by analytes enrichment in the final extract by low-density extracting solvent-based dispersive liquid-liquid microextraction (LD-DLLME) combined with ultra-low temperature (–80ºC). In our method development, Plackett-Burman experimental design allowed identify the sample preparation variables that affect the PAHs extraction such as the amount of C_{18} sorbent in cleanup step and the toluene volume used in LD-DLLME. High co-extractives removal efficiency was achieved with the proposed cleanup. The fitness-of-purpose of the developed method was verified by analytical selectivity, linearity in solvent and matrix-matched calibrations and adequate recoveries (72–112%) and precision (RSDs ≤ 11%), under repeatability and within-laboratory reproducibility conditions. High analytical sensibility was achieved for the monitoring of target PAHs at the maximum limit of 1 μg kg^{-1} stated by European Commission for baby foods. The validated method was applied to commercial baby food samples, and the investigated PAHs were not detected in the samples analyzed.

Keywords: Polycyclic aromatic hydrocarbons; Baby Food; Food Contaminant; Plackett-Burman design; Sample preparation; Gas chromatography-mass spectrometry.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute environmental and food processing contaminants formed by two or more fused aromatic rings of carbon and hydrogen atoms, which present high hydrophobic and lipophilic nature [1-3]. These organic compounds have been classified as light (2 or 3 condensed aromatic rings) or heavy (4–6 rings) PAHs according to number of condensed aromatic rings in their chemical structure, being heavy PAHs considered more stable and toxic [1]. PAHs are produced during incomplete combustion or pyrolysis of organic matter and geological processes, at high temperatures (500–700°C) [1]. The diversity of emission sources, including natural (forest fires, volcanic eruptions, hydrothermal process) and anthropometric (combustion of fossil fuels and wood, and direct release of oil and oils products), have contributed to widespread distribution of PAHs in the environment [1, 2]. Additionally, the US Environmental Protection Agency has characterized these compounds as priority pollutants due to their high toxicity [4].

In 2005, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) on the basis of risk assessments of International Programme on Chemical Safety (IPCS) and European Union Scientific Committee on Food (SCF) concluded that the PAHs benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, chrysene, dibenz[*a,h*]anthracene, dibenzo[*a,e*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a,l*]pyrene, indene[*1,2,3-cd*]pyrene and 5-methylchrysene, are clearly genotoxic and carcinogenic to humans. Years later, the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM Panel) reviewed the available data on toxicity and occurrence of PAHs and concluded that the eight compounds benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*ghi*]perylene, chrysene, dibenz[*a,h*]anthracene and indene[*1,2,3-cd*]pyrene, either individually or in a combination, are actually possible indicators of carcinogenic potency of PAHs in foods [5]. Maximum levels have been established for PAHs in different foodstuffs by the European Commission, including 1 μg kg^{-1} for benzo[*a*]pyrene individually or combined with benz[*a*]anthracene, benzo[*b*]fluoranthene and chrysene in baby foods [6].

Dietary intake has been the major route for human exposure to PAHs, being vegetable oils and fats the main contributors to diet [1, 2]. In foods, the occurrence of these compounds is arising from environmental contamination; technological processes mainly those that involve combustion gases as smoking and drying, as also grilling and roasting; and due to direct contact of food with non-food-grade quality mineral oil and contaminated packaging [1, 2]. PAHs have been reported in wide range of food matrices including cereal-based products,

chocolate, coffee, dairies, meats, tea and others [3]. Nonetheless, few works on PAHs in foods intended for infant consumption have been found in the literature [7-9]. For instance, 11 PAHs were investigated in commercial milk formulae and infant cereals using liquid chromatography with fluorescence detection (LC-FLD) [7]. Infant formulae, follow-on formulae and baby foods commercialized in Poland were also analyzed employing LC-FLD [8]. On the other hand, 16 PAHs were investigated in infant formulae from Nigeria using gas chromatography coupled to mass spectrometry (GC-MS) [9].

Taking into account the complexity of baby food matrices, including carbohydrate- and lipid-rich samples, the sample preparation selection represents a critical parameter, particularly when trace compounds have to be determined. Different sample pre-treatments have been explored for PAHs in foodstuffs [10]. Liquid-liquid extraction followed by solid-phase extraction (SPE) using cartridges has commonly been employed for PAHs in oil [11]; in addition, gel permeation chromatography (GPC) has also been reported [12]. Pressurized liquid extraction (PLE) and microwave-assisted extraction have achieved adequate performance for the determination of these contaminants in complex matrices as roasted coffee [13] and fish [14], respectively. On the other hand, miniaturized extraction techniques have been successfully applied for PAHs analysis. QuEChERS (quick, easy, cheap, effective, rugged and safe) method, recognized by simplicity of operation and high sample throughput, has achieved good recoveries for PAHs in tea [15] and rice [16], although low final extract concentration represents their main disadvantage. Conversely, dispersive liquid-liquid microextraction (DLLME) has provided high analytes enrichment in final extract due to few microliters of extracting solvent used in the procedure, being mainly employed for PAHs analysis in aqueous matrices [17]. Additionally, salting-out assisted liquid-liquid extraction (SALLE), which involves little amount of an electrolyte (around 1 g) and few milliliters of organic solvent, has demonstrated adequate performance characteristics for the determination of PAHs in sugar-rich matrices as honey [18].

Currently efforts have been directed to the development of green sample preparation methods focusing minimal reagent consumption and lower residue generation. In this way, microextraction techniques combined with others environmental-friendly sample pre-treatments have demonstrated to be an useful strategy for cleanup and analytes enrichment purposes after target extraction from solid matrices [19]. In addition, the use of design of experiments (DOE) during method development has also contributed to reduced waste generation and less labour, time and final cost [20]. The main advantage of these factorial designs includes simultaneous multiple variable analysis employing a little number of trials or

repetitions [20]. The Plackett-Burman experimental design, introduced in 1946, has been an alternative to a preliminary assessment of the main variables that could significantly affect the studied response [20,21]. This screening methodology has been successful applied in several areas [22-24].

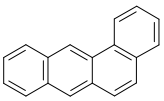
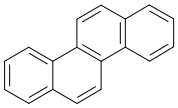
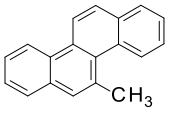
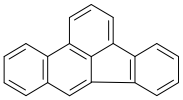
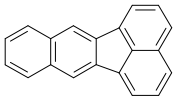
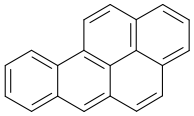
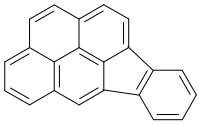
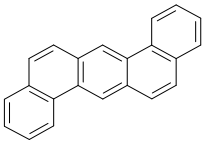
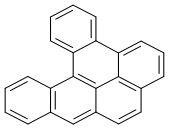
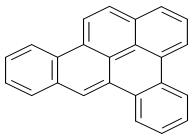
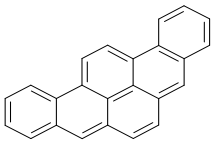
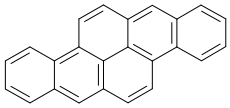
In the present work, an accurate and highly sensitive GC-MS method was developed for the trace determination of 12 PAHs in baby foods (Table 1). For first time, low-density extracting solvent-based DLLME combined with ultra-low temperature (–80°C) was described for analytes enrichment purpose after environmental-friendly QuEChERS extraction. In addition, Plackett-Burman design was employed in order to screen the main sample preparation variables that could affect the PAHs extraction. The developed method was in-house validated and then applied to commercial baby food samples.

2. Materials and methods

2.1. Standards and chemicals

Analytical standards of benzo[*a*]anthracene (99.5%, purity), benzo[*a*]pyrene (99.6%), benzo[*b*]fluoranthene (98.1%), chrysene (99.9%), chrysene-d12 (98.6%), dibenz[*a,h*]anthracene (99.6%), dibenzo[*a,i*]pyrene solution (100 µg mL^{-1} in dichloromethane, 99.9%) and indeno[*1,2,3-cd*]pyrene (99.7%) were purchased from Supelco, Sigma-Aldrich. Dibenzo[*a,l*]pyrene (99.4%) and dibenzo[*a,e*]pyrene (99.6%) were obtained from Cerilliant, Sigma-Aldrich; benzo[*k*]fluoranthene (99.5%) from Chem Service; 5-methylchrysene (99.5%) from Santa Cruz Biotechnology and dibenzo[*a,h*]pyrene (> 98%) from TCI. Stock standard solutions were prepared in toluene at concentrations between 9.99 µg mL^{-1} (dibenzo[*a,h*]pyrene) and 39.2 µg mL^{-1} (dibenzo[*a,e*]pyrene). A multi-analyte working solution was prepared in toluene at 1 µg mL^{-1} by combining appropriate aliquots of the individual stock solutions of PAHs. All standard solutions were prepared in amber glass flask and stored at –18ºC for 1 month from preparation. Acetonitrile was acquired from J.T. Baker and toluene from AlliedSigmal, Burdick & Jackson, all HPLC grade. Anhydrous magnesium sulfate ($MgSO_4$) was obtained from J.T. Baker, which was heated at 550°C at least 5 h in a muffle and stored in a desiccator before use; and sodium chloride (NaCl) was purchased from Synth. Amine primary and secondary (PSA) and C_{18}-bonded silica sorbents were supplied by Supelco. Deionized water was obtained from a Milli-Q SP reagent water system (Millipore).

Table 1. PAHs evaluated in the present study, their chemical structure, molecular weight (MW), retention time (t_R) and mass spectrometric conditions for the GC-MS analysis.

Analyte (abbreviation)	Chemical structure	MW (g mol^{-1})	t_R (min)	Start time of window, min (data acquisition rate, scans s^{-1})	SIM ions (m/z)*
Benzo[*a*]anthracene (BaA)		228.29	15.89	15.75 (3.11)	**228**, 226, 229
Chrysene (Chr)		228.29	16.00		**228**, 226, 229
5-Methylchrysene (5-MC)		242.31	17.85	17.75 (3.11)	**242**, 239, 241
Benzo[*b*]fluoranthene (BbF)		252.31	20.09	19.90 (3.11)	**252**, 250, 253
Benzo[*k*]fluoranthene (BkF)		252.31	20.18		**252**, 250, 253
Benzo[*a*]pyrene (BaP)		252.31	21.27	21.10 (3.11)	**252**, 250, 253
Indeno[*1,2,3-cd*]pyrene (IcdP)		276.33	25.20	25.10 (2.68)	**276**, 274, 277
Dibenz[*a,h*]anthracene (DahA)		278.35	25.31		**278**, 276, 279
Dibenzo[*a,l*]pyrene (DalP)		302.37	28.94	28.85 (2.74)	**302**, 300, 303
Dibenzo[*a,e*]pyrene (DaeP)		302.37	29.94		**302**, 300, 303
Dibenzo[*a,i*]pyrene (DaiP)		302.37	30.32		**302**, 300, 303
Dibenzo[*a,h*]pyrene (DahP)		302.37	30.53		**302**, 300, 303

*Ions used for quantification in bold.

2.2. Sample collection

Thirty-two samples of ready-to-eat baby foods, which included fruit purées, cereal flours, starch, milk or other dairy ingredient in their composition, were randomly collected from 5 markets in the city of Campinas, SP, located in the South-Eastern region of Brazil, betwen September and November 2016. The samples were stored at room temperature in their original packaging, glass jar (120 g each) or plastic bags (113 g each), until analysis.

2.3. Determination of PAHs in baby food

2.3.1. Sample preparation

The sample preparation strategy adopted in this report is presented in Fig. 1. The proposed method consists in two steps as follows:

(i) *QuEChERS-based extraction*: First, 10 g of homogenized baby food samples were vortexed with 10 mL of acetonitrile in a 50 mL polypropylene centrifuge tube for 1 min. Then, 1 g of sodium chloride and 4 g of anhydrous magnesium sulfate were added to mixture followed by agitation in vortex for 1 min. After centrifugation at 3500 rpm for 20 min, 4 mL of supernatant, 0.1 g of PSA, 0.1 g of C_{18} and 0.6 g of anhydrous magnesium sulfate were added to a 15 mL polypropylene centrifuge tube, vortexed for 1 min, and then the mixture was centrifuged at 3500 rpm for 10 min.

(ii) *Low-density extracting solvent-based dispersive liquid-liquid microextraction (LD-DLLME) combined with ultra-low temperature:* Two milliliters of obtained extract (acetonitrile as disperser solvent) were added to an Eppendorf tube containing 75 µL of toluene (extracting solvent) and this was manually agitated for 10 s. Then, the mixture was rapidly injected into 8 mL of deionized water, placed into a 15 mL polypropylene centrifuge tube, followed by delicate shaking by hand for 1 min and centrifugation at 3500 rpm for 5 min. Finally, the tube was maintained in ultra-low temperature freezer at –80 ± 2°C for 30 min. After this time, the remaining upper liquid phase (toluene) was transferred to glass insert placed into glass vial for subsequent GC-MS analysis.

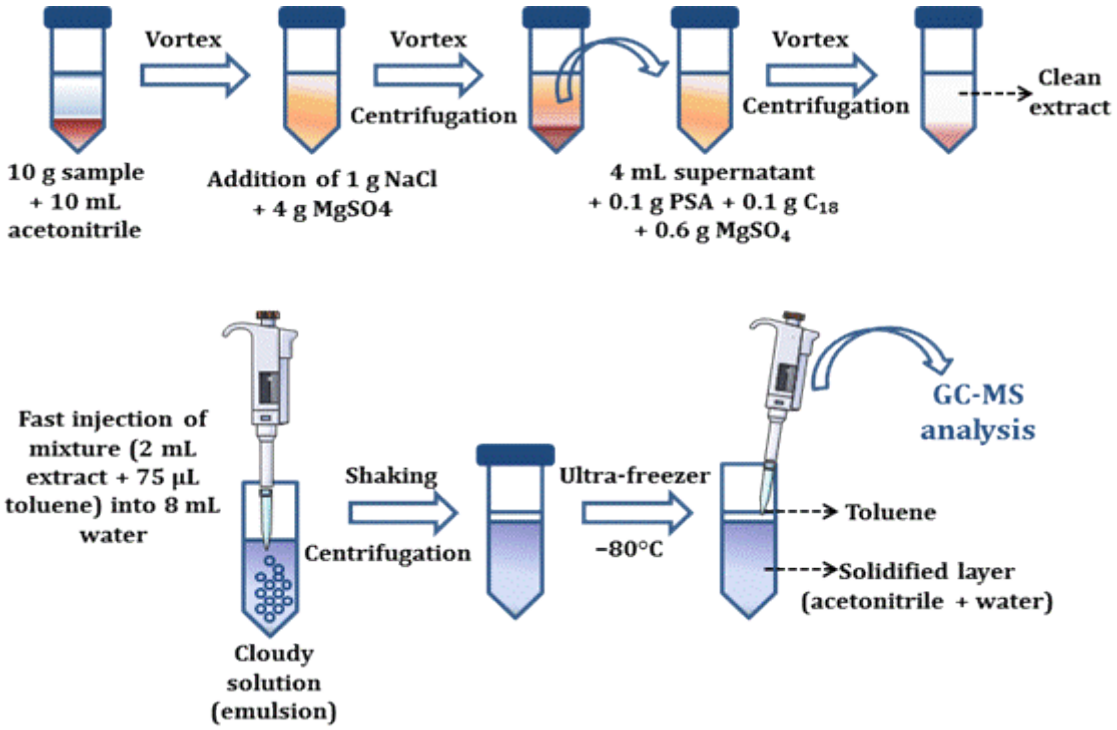


Fig. 1. Sample preparation scheme for the determination of PAHs in baby food.

2.3.2. Gas chromatographic and mass spectrometric conditions

A 7890A gas chromatography system interfaced to a single quadrupole inert mass selective detector (5975C) with electron ionization (EI) source of Agilent Technologies was employed for the PAHs analyses. ChemStation platform was used for qualitative and quantitative data analysis. GC separation was achieved on a ZB-5HT Inferno capillary column (20 m x 0.18 mm x 0.18 µm; Zebron, Phenomenex), which was maintained initially at 80°C, increased at 20°C min^{-1} to 200°C, then ramped to 300°C at 5°C min^{-1}, and finally increased to 320°C at 20°C min^{-1} and held for 5 min, resulting a total run of 32 min. A solvent delay of 3.5 min was applied. The injector was maintained at 300°C and 2 µL of extract were injected in splitless mode (purge flow of 100 mL min^{-1} at 1 min). Ultra-high purity helium (99.9999 %) was employed as carrier gas at constant flow of 1 mL min^{-1}. The electron energy was 70 eV, and transfer line and EI source were maintained at 300°C, while quadrupole mass analyzer at 180°C.

2.3.3. Identification and quantification

Individual standard solutions, all prepared in toluene at concentrations varying between 9.99 μg mL^{-1} (DahP) and 39.2 μg mL^{-1} (DaeP), were analyzed in full-scan mode (*m/z* 50–500) in order to set the retention time and characteristic spectra of each analyte. One target ion and two qualifying ions were selected for each PAH (Table 1). Data acquisition was divided in 6 different ion groups in selective ion monitoring (SIM) mode, and the dwell times (20–30 ms) were established depending on the number of ions per group. The identification of compounds was established by the retention time of the target ion as well as the qualifier to target ion ratios. The internal standard (IS) chrysene-d12 (*m/z* 240, t_R = 16 min) was added to sample at level of 4 μg kg^{-1} prior to the extraction procedure, and the PAHs were quantified employing seven-point matrix-matched calibration curves constructed by plotting analyte/IS peak area ratio against concentration levels.

2.4. Screening experimental design

A Plackett-Burman design was conducted in order to identify the sample preparation variables that could affect the determination of PAHs in baby food. Eight independent variables were evaluated at two levels, high (+1) and low (–1), according to procedure described by Rodrigues and Iemma [20]. The experimental design included 12 trials (4 trials more than the number of independent variables assessed in the study to ensure sufficient degrees of freedom for calculating the standard error) plus 3 central points (to evaluate the conditions in the central region of the studied range as well as the repeatability of the analyses), resulting in 15 independent trials [20]. Table 2 presents the design matrix with the coded and real values for each variable. From the recoveries obtained for each trial of design, the effect (%) of each variable on the recovery of PAHs was calculated, and its statistical significance was determined by *t* test. The significance level was set to 10% focusing minimize the risk of excluding any important variable. Statistica 8.0 software (Statsoft Inc., Tulsa, OK, USA) was used to analyze the data.

Table 2. Plackett-Burman design with the coded and real values for eight variables and recovery of PAHs.

Run	Variable								Recovery (%)											
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	BaA	Chr	5-MC	BbF	BkF	BaP	IcdP	DahA	DalP	DaeP	DaiP	DahP
1	1 (1.5)	–1 (2)	1 (3)	–1 (0.4)	–1 (0.1)	–1 (0.1)	1(3)	1 (75)	77.4	78.4	66.4	67.1	53.3	54.1	48.8	51.3	51.9	49.1	70.9	64.7
2	1 (1.5)	1 (6)	–1 (1)	1 (0.8)	–1 (0.1)	–1 (0.1)	–1 (1)	1 (75)	80.6	81.7	70.6	71.7	57.9	59.1	54.9	57.9	59.2	57.1	78.7	70.8
3	–1 (0.5)	1 (6)	1 (3)	–1 (0.4)	1 (0.3)	–1 (0.1)	–1 (1)	–1 (25)	22.4	22.3	17.8	19.2	13.8	13.8	13.1	13.6	14.4	13.5	21.1	19.5
4	1 (1.5)	–1 (2)	1 (3)	1 (0.8)	–1 (0.1)	1 (0.3)	–1 (1)	–1 (25)	24.3	24.5	19.8	20.6	15.6	15.4	14.1	15.5	15.2	14.2	21.5	19.7
5	1 (1.5)	1 (6)	–1 (1)	1 (0.8)	1 (0.3)	–1 (0.1)	1 (3)	–1 (25)	24.3	24.5	20.0	21.4	16.2	15.8	14.5	15.5	15.7	14.7	21.7	19.8
6	1 (1.5)	1 (6)	1 (3)	–1 (0.4)	1 (0.3)	1 (0.3)	–1 (1)	1 (75)	72.2	72.8	58.6	61.1	45.8	45.4	42.0	44.1	45.6	42.6	64.5	59.0
7	–1 (0.5)	1 (6)	1 (3)	1 (0.8)	–1 (0.1)	1 (0.3)	1 (3)	–1 (25)	24.2	24.2	19.5	20.6	15.3	15.2	13.8	14.7	14.9	13.9	21.3	19.6
8	–1 (0.5)	–1 (2)	1 (3)	1 (0.8)	1 (0.3)	–1 (0.1)	1 (3)	1 (75)	76.7	77.3	65.8	69.2	53.7	53.5	49.5	51.7	53.3	50.9	71.3	64.8
9	–1 (0.5)	–1 (2)	–1 (1)	1 (0.8)	1 (0.3)	1 (0.3)	–1 (1)	1 (75)	73.9	74.9	61.4	63.3	49.2	48.9	45.3	48.2	49.0	46.3	67.1	60.9
10	1 (1.5)	–1 (2)	–1 (1)	–1 (0.4)	1 (0.3)	1 (0.3)	1 (3)	–1 (25)	24.5	24.6	20.0	20.8	15. 6	15.3	13.7	14.4	14.8	13.9	21.2	19.7
11	–1 (0.5)	1 (6)	–1 (1)	–1 (0.4)	–1 (0.1)	1 (0.3)	1 (3)	1 (75)	76.8	77.7	65.2	66.7	52.2	51.9	47.3	50.5	49.4	47.6	69.3	63.1
12	–1 (0.5)	–1 (2)	–1 (1)	–1 (0.4)	–1 (0.1)	–1 (0.1)	–1 (1)	–1 (25)	22.6	22.6	18.2	19.6	14.4	14.3	13.7	14.4	15.1	14.3	21.8	19.9
13(CP)	0 (1)	0 (4)	0 (2)	0 (0.6)	0 (0.2)	0 (0.2)	0 (2)	0 (50)	56.6	57.4	50.6	50.2	41.7	42.4	36.3	38.9	36.7	35.1	48.4	44.3
14(CP)	0 (1)	0 (4)	0 (2)	0 (0.6)	0 (0.2)	0 (0.2)	0 (2)	0 (50)	59.1	59.9	53.5	52.4	43.8	44.9	37.8	41.4	38.4	37.5	50.1	45.7
15(CP)	0 (1)	0 (4)	0 (2)	0 (0.6)	0 (0.2)	0 (0.2)	0 (2)	0(50)	54.7	54.8	47.4	47.3	37.9	38.7	34.7	37.3	36.9	35.6	48.5	43.3

X_1: NaCl (g); X_2: $MgSO_4$ (g); X_3: Vortex (min); X_4: $MgSO_4$ (g); X_5: PSA (g); X_6: C_{18} (g); X_7: Vortex (min); X_8: Toluene (µL); CP: Central point.

3. Results and discussion

3.1. Sample preparation and screening of variables

A sample preparation strategy based on QuEChERS and DLLME techniques, with promising innovations, was proposed for the determination of twelve PAHs in baby food. Additionally, important variables that could influence the extraction of the analytes were screened using a Plackett-Burman design. For this, a representative blank matrix – baby food free of investigated PAHs composed by fruit purées (apple, banana, papaya and strawberry), rice flour, starch and heavy cream, was spiked at 4 μg kg^{-1} and then it was extracted according to conditions defined for each trial of experimental design (Table 2). From the recoveries obtained for each trial, the respective effect of the variables on the PAHs recovery was estimated, which ranged from –3 to 53% (Table 3). This previous screening revealed that the amount of C_{18} sorbent in cleanup step and the volume of toluene used as extracting solvent in DLLME were the most relevant variables for the extraction of PAHs from baby food.

Taking into account the high moisture content of samples analyzed (84 ± 2%, n = 12), a 1:1 sample to extractor solvent ratio was employed for the pre-extraction step promoting consistent homogenization between acetonitrile and matrix, and adequate reproducibility (RSD values ≤ 11%). In order to induce phase separation and force the analytes into acetonitrile layer, different amounts of NaCl (between 0.5 and 1.5 g) and $MgSO_4$ (from 2 to 6 g) were tested. Variations in the amount of NaCl and $MgSO_4$ did not affect the recovery of PAHs (p > 0.1), suggesting reduced use of these salts in the pre-extraction step. The vortexing time after the addition of salts was also evaluated. Increasing time from 1 to 3 min produced no statistically significant effect on the recoveries (p > 0.1), indicating that the time can be minimized to 1 min without impairing the extraction of the PAHs.

Dispersive solid-phase extraction (d-SPE) was adopted for the cleanup of acetonitrile extracts. Several advantages have been associated to d-SPE technique, including simplicity of execution, lower solvent consumption and time saving compared to SPE using cartridges. Therefore, a mixture of primary secondary amine (PSA) and C_{18} sorbents and $MgSO_4$ was tested. Increasing the amount of PSA from 0.1 to 0.3 g did not affect the recovery of PAHs (p > 0.1); however, this increment in the amount of C_{18} sorbent produced a statistically significant negative effect ($p \leq 0.1$) of –3% in the recovery of the compounds DaiP and DahP. Overall, negative effects were observed in the recovery of all PAHs increasing the amount of C_{18} sorbent. The hydrophobic nature of C_{18} sorbent might have contributed to lower recovery of the PAHs due to retention of these non-polar analytes. Therefore, minimal amount of both sorbents PSA and C_{18} was fixed for d-SPE cleanup. In addition, variations in the amount of

$MgSO_4$ (between 0.4 and 0.8 g) and in the vortexing time (from 1 to 3 min) resulted in no significant effects ($p > 0.1$) on the PHAs recoveries, indicating that minimal amount of salt and vortexing time can be used without affecting the extraction.

Dispersive liquid-liquid microextraction (DLLME) was carried out after QuEChERS procedure with the goal to obtain high enrichment factor in the final extract. In general, halogenated hydrocarbons, including carbon tetrachloride, chloroform, chlorobenzene and tetrachloroethylene, have been widely employed as extracting solvent in DLLME procedure due to their higher density than water facilitating the phase separation after centrifugation; while, acetonitrile, acetone and methanol, all miscible in both water and extracting solvent, as a disperser solvent [25]. In our method development, the acetonitrile extract obtained from QuEChERS was tested as disperser solvent and toluene, a lighter solvent than water ($d_{20°C}$ = 0.866 g mL^{-1}), was assessed as extracting solvent. Therefore, 2 mL of obtained acetonitrile extract and few microliters of toluene (25, 50 or 75 µL) were combined and this mixture was rapidly injected into 8 mL of deionized water resulting in the dispersion of toluene as micro-droplets throughout the aqueous phase, and a stable cloudy solution. Increasing the toluene volume from 25 to 75 µL we verified a statistically significant positive effect ($p < 0.1$) between 34 and 53% in the recovery of analytes, demonstrating a strong influence of this variable on the extraction efficiency. The higher volume of extracting solvent might have contributed to larger contact surface area between the aqueous acetonitrile phase and the extracting solvent which provided a fast and adequate transfer of analytes into toluene micro-droplets. Also, higher extracting volumes allow easy collection of them after centrifugation step. Although, high volumes result in lower enrichment factor, sufficient analytical sensibility was achieved with 75 µL, therefore this volume was fixed for further analyses. Due to extensive surface area between the aqueous acetonitrile phase containing the interest analytes and the extracting solvent phase, the extraction equilibrium time is achieved rapidly [25], thus shortly after emulsion formation (acetonitrile-toluene-water) the tubes were centrifuged resulting in floating extracting solvent on the aqueous acetonitrile phase (Fig.1).

Different procedures have been reported for the collection of extracting solvent for subsequent GC-MS analysis [26-30]. Solidification of floating extracting solvent phase has been the procedure commonly employed when lower density extracting solvents are used in DLLME [31-33]. In this report, we proposed a novel strategy for retrieval of extracting solvent on the basis of melting point (mp) of organic solvents used in DLLME, namely, acetonitrile (mp = –45°C) and toluene (mp = –95°C). Therefore, after centrifugation step and phase separation, the tubes were maintained for 30 min at –80 ± 2ºC in an ultra-low

temperature freezer resulting in complete solidification of bottom aqueous acetonitrile phase and, the upper liquid toluene phase was easily collected using an automatic micropipette and transferred to glass vial for subsequent GC-MS analysis. Additionally, this strategy contributes to an extra cleanup of the final extract, particularly for fatty baby foods as milk-based samples, promoting the freezing of fats.

Table 3. Main effects on the recovery of PAHs (%) estimated from the results of the Plackett-Burman design.

	Variable		BaA	Chr	5-MC	BbF	BkF	BaP	IcdP	DahA	DalP	DaeP	DaiP	DahP
QuEChERS	NaCl (g)	*Effect (%)*	1	1	1	0.7	1	1	0.9	1	1	0.9	1	0.1
		Standard Error	2.7	2.8	3.5	2.8	3.1	3.5	2.3	2.8	2	2.2	1.8	1.6
		t (6)	0.4	0.4	0.4	0.2	0.3	0.3	0.4	0.3	0.5	0.4	0.6	0.6
		p-value	0.697601	0.671171	0.734281	0.816003	0.768285	0.738953	0.72099	0.74243	0.618032	0.709181	0.566703	0.555536
	$MgSO_4$ (g)	*Effect (%)*	0.2	0.2	0.03	-0.01	-0.1	-0.1	0.1	0.2	-0.1	0.1	0.4	0.4
		Standard Error	2.7	2.8	3.5	2.8	3.1	3.5	2.3	2.8	2	2.2	1.8	1.6
		t (6)	0.1	0.1	0.01	-0.01	-0.03	-0.02	0.04	0.1	-0.02	0.04	0.2	0.2
		p-value	0.952586	0.954059	0.992336	0.994506	0.976427	0.98188	0.970516	0.955714	0.98129	0.966208	0.814013	0.826226
	Vortex (min)	*Effect (%)*	-0.9	-1	-1	-0.9	-1.3	-1	-1.3	-2	-1	-2	-1.5	-1
		Standard Error	2.7	2.8	3.5	2.8	3.1	3.5	2.3	2.8	2	2.2	1.8	1.6
		t (6)	-0.3	-0.4	-0.4	-0.3	-0.4	-0.4	-0.6	-0.6	-0.6	-0.7	-0.8	-0.7
		p-value	0.762495	0.703336	0.731848	0.748243	0.689420	0.7191	0.589444	0.571591	0.542991	0.500016	0.435522	0.491951
d-SPE	$MgSO_4$ (mg)	*Effect (%)*	1	1	2	2	2.1	2	2	3	3	3	2	2
		Standard Error	2.7	2.8	3.5	2.8	3.1	3.5	2.3	2.8	2	2.2	1.8	1.6
		t (6)	0.5	0.5	0.5	0.7	0.7	0.6	1	0.9	1	1	1	1
		p-value	0.633598	0.619493	0.621731	0.485805	0.523792	0.553787	0.373709	0.3938	0.224413	0.268665	0.283917	0.341137
	PSA (mg)	*Effect (%)*	-2	-2	-3	-1.9	-2	-3	-2	-3	-2	-2	-3	-2
		Standard Error	2.7	2.8	3.5	2.8	3.1	3.5	2.3	2.8	2	2.2	1.8	1.6
		t (6)	-0.7	-0.7	-0.8	-0.7	-0.8	-0.8	-1	-1	-1	-1	-1	-1
		p-value	0.500282	0.481718	0.46877	0.515551	0.474540	0.4441	0.337497	0.3479	0.315151	0.322113	0.179342	0.190412
	C_{18} (mg)	*Effect (%)*	-1	-1	-2	-3	-3	-3	-3	-3	-3	-3	-3	-3
		Standard Error	2.7	2.8	3.5	2.8	3.1	3.5	2.3	2.8	2	2.2	1.8	1.6
		t (6)	-0.5	-0.5	-0.7	-0.9	-0.8	-0.9	-1	-1	-2	-2	-2	-2
		p-value	0.640331	0.65011	0.515604	0.394924	0.441812	0.413432	0.238367	0.347566	0.134271	0.159747	0.108737	0.115989
	Vortex (min)	*Effect (%)*	1	1	2	2	2	1	1	1	0.3	0.3	0.1	0.3
		Standard Error	2.7	2.8	3.5	2.8	3.1	3.5	2.3	2.8	2	2.2	1.8	1.6
		t (6)	0.5	0.5	0.5	0.6	0.5	0.4	0.3	0.3	0.1	0.2	0.1	0.2
		p-value	0.649014	0.66011	0.632015	0.560115	0.629947	0.69411	0.756997	0.797728	0.900112	0.881239	0.939335	0.845635
DLLME	Toluene (µL)	*Effect (%)*	52	53	45	46	37	37	34	36	36	35	49	44
		Standard Error	2.7	2.8	3.5	2.8	3.1	3.5	2.3	2.8	2	2.2	1.8	1.6
		t (6)	19	19	13	17	12	11	15	13	18	16	27	28
		p-value	0.000001	0.000001	0.000013	0.000003	0.000023	0.00004	0.000007	0.000013	0.000002	0.000004	0.000000	0.000000

3.2. Cleanup efficiency and matrix effects

Co-extracted matrix components can adversely affect the GC-MS analysis by masking of analyte peak, overestimation or underestimation of quantitative results and contributing to false positives [34]. Therefore, the efficiency of d-SPE cleanup regarding co-extractives removal was assessed by gravimetric measurements [35-37]. For this, a representative baby food, composed by a mixture of fruit purées (apple, banana, papaya and strawberry), rice flour, starch and heavy cream, was extracted according to QuEChERS procedure (Item 2.3.1.) and then, the obtained extracts were submitted to 3 different procedures of d-SPE: (i) 0.1 g of PSA and 0.6 g of $MgSO_4$ per mL of extract; (ii) 0.1 g of C_{18} and 0.6 g of $MgSO_4$ per mL of extract; and (iii) 0.1 g of PSA, 0.1 g of C_{18} and 0.6 g of $MgSO_4$ per mL of extract.

After d-SPE procedure, acetonitrile extracts were transferred to pre-weighed glass tubes, which were previously heated at 110°C for at least 1 h to eliminate the moisture, and these extracts were evaporated to dryness under nitrogen stream. Then, the glass tubes containing the dried residues were again heated at 110°C for at least 1 h and weighed (Fig. 2A). Co-extractives removal efficiency was estimated as difference in weight of co-extractives before and after d-SPE procedure employing the equation: [(co-extractives weight before d-SPE – co-extractives weight after d-SPE)/ co-extractives weight before d-SPE] x 100 [37]. As a great result, PSA, an anion exchange sorbent used to remove polar matrix components such as sugars, organic acids, free fatty acids and some pigments [38], contributed to a reduction of 33% in the co-extractive amount compared to crude extract without d-SPE cleanup; while the sorbent C_{18}, often employed for fatty matrices to remove non-polar interferences as lipids [38], removed 53% of co-extractives present in the crude extract. In addition, the combination of PSA and C_{18} sorbents resulted in an expressive reduction of 84% in the co-extractives amount in the final extract compared to extract without cleanup, demonstrating the efficiency of the proposed d-SPE procedure (Fig. 2B). A comparison between acetonitrile extract before and after d-SPE cleanup with PSA and C_{18} sorbents is shown in the Fig. 2C. Sapozhnikova and Lehotay also verified a reduction of 80% in remaining co-extractives in fish extracts using a mixture of 0.05 g of PSA, 0.05 g of C_{18} and 0.15 g of $MgSO_4$ per mL of extract [37]. The great effect of C_{18} sorbent in cleanup efficiency was observed for apple-blueberry sauce and pea extracts, with reduction of approximately 90% in the co-extractives amount [36]. In addition, a reduction of 64% in co-extractives amount was achieved by using 0.05 g of PSA and 0.15 g of $MgSO_4$ per mL of extract obtained from olives [35].

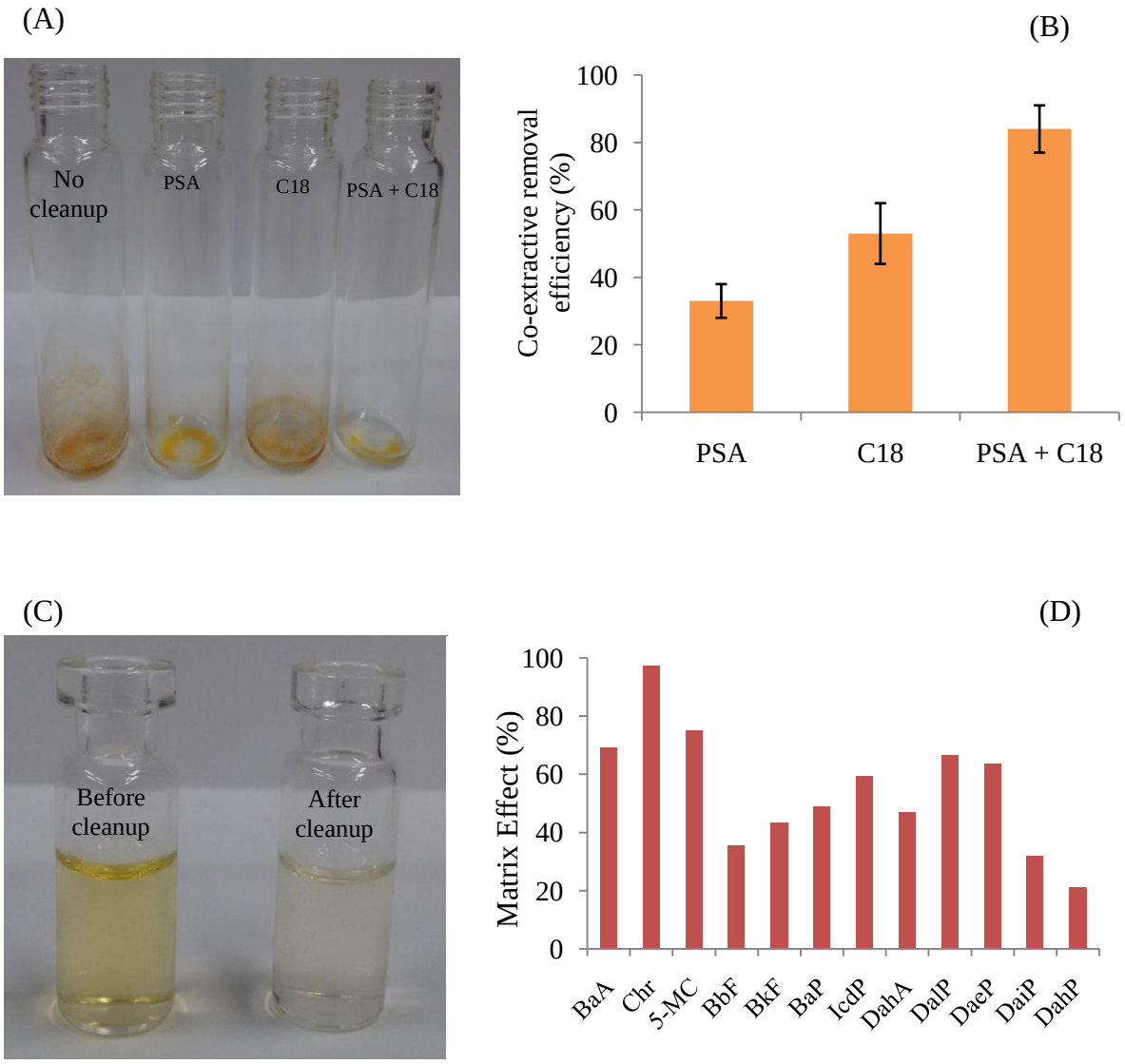


Fig. 2. Evaluation of cleanup efficiency: (A) comparison of matrix co-extractives content in dried extract after different d-SPE procedures; (B) co-extractive removal efficiency of d-SPE with different sorbents (%, n = 2); (C) comparison of acetonitrile extract before and after d-SPE cleanup using PSA and C_{18} sorbents; (D) GC-MS matrix effects (%) obtained for the selected PAHs employing the developed method.

Matrix effects were estimated using slopes obtained for calibration curves of same concentrations in solvent and matrix extracts, as indicated in the equation: ME (%) = [(matrix slope – solvent slope)/ solvent slope] x 100 [37]. In this trial, no internal standard was used because it could also be subjected to the effect leading to equivocal conclusions [36]. Enhancement of analytical signal was observed for all analytes, varying between 21% (DahP) and 97% (Chr) (Fig. 2D). Matrix-induced effects have been a critical point in GC-MS analysis, being chromatographic response enhancement commonly reported in the analysis of pesticides in food matrices [39,40]. This effect occurs because the co-extractives block the active sites in GC system, mainly in the injection line, resulting in higher efficiency of analyte transfer and consequently better analytical response in the presence of matrix when compared to solvent-only solutions [36, 40, 41]. The highest matrix-induced effects were observed for the lighter PAHs (first eluting), probably because the co-extracted matrix components were observed at the beginning of the chromatographic run thus affecting the first compounds to be eluted [42].

3.3. Performance characteristics

Selectivity, sensibility and accuracy of developed analytical method were verified by in-house validation procedure using a representative baby food sample. A blank baby food, free of investigated PAHs, composed by a mixture of fruit purées (apple, banana, papaya and strawberry), rice flour, starch and heavy cream, was used for recovery and precision experiments, and also to obtain matrix-matched calibration curves for quantitative analysis. Selectivity was demonstrated on the basis of total ion chromatograms (TIC) obtained in SIM mode from extracts of blank and spiked baby foods, including fruit-, cereal- and milk-based samples. No interfering peaks were observed within the windows of retention time of each PAH, allowing an unequivocal identification of investigated compounds.

Sensibility was established by limits of detection (LOD) and quantification (LOQ), which were obtained by successive analysis of baby food extracts spiked with decreasing concentrations of PAHs until to achieve the ratios signal-to-noise of 3:1 and 10:1, respectively. High analytical sensibility was achieved for all compounds with LODs ranging from 0.1 to 0.3 μg kg^{-1}, and LOQs between 0.25 and 1 μg kg^{-1} (Table 4). Overall, all obtained limits were suitable for routine monitoring of the PAHs at the maximum level of 1 μg kg^{-1} stated by the Commission Regulation (EU) N° 835/2011 for baby foods [6]. The use of higher temperatures in GC-MS system, particularly in transfer line and EI source at 300°C, might have contributed to greater quantitative transfers, minimum peak tailing and consequently

better MS detection, mainly for less volatile compounds [12]. Linearity was verified for solvent and matrix-matched calibration curves. For matrix-based calibrations, appropriate aliquots of standard solutions were added to blank baby food extracts in order to obtain 1, 2.5, 5, 7.5, 10, 12.5 and 15 μg kg^{-1} of standard equivalent in the sample, and for solvent calibrations, the same 7 levels were prepared in toluene. High coefficients of determination ($R^2 \geq 0.99$) were obtained for all analytes in solvent and matrix-matched curves (Table 4). In order to compensate the intense matrix-induced effect observed, matrix-matched calibration curves were employed for quantitative analysis, which were constructed by plotting the analyte/IS peak area ratio against the concentration levels.

The extraction efficiency of developed method was confirmed by recovery experiments. For this, blank baby foods were spiked with working standard solutions at 1, 5 and 10 μg kg^{-1} levels. A total of nine independent replicates at each level were analyzed on three days, with mean recoveries ranged from 72 to 112% (Table 4). According to performance criteria established by Commission Regulation (EC) N° 333/2007, recoveries between 50 and 120% are required for methods of analysis for benzo[*a*]pyrene in foodstuffs [43]. Under repeatability conditions, the precision was estimated from three independent replicates at each level which were analyzed on same day by same analyst under same chromatographic conditions, with relative standard deviation (RSD) values varying from 1 to 6% (Table 4). Within-laboratory reproducibility conditions, three independent replicates were analyzed on three days by same analyst under same chromatographic conditions, totaling nine determinations at each level, whose RSD values ranged between 4 and 11% (Table 4).

Additionally, the occurrence of target PAHs was investigated in thirty-two commercial baby foods, which were intended for infants and young children consumption. Samples of most popular baby food brands available on the Brazilian markets were analyzed, including fruit-, cereal- and milk-based baby foods, and the investigated PAHs were not detected in the samples.

Table 4. Performance characteristics of developed method for the determination of PAHs in baby food.

Analyte	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Linearity, R^2 (range 1–15 µg kg^{-1})		% Recovery (n = 9)			Precision, RSD% Intra-day, n = 3 (Inter-day, n = 9)		
			Solvent	Matrix	1 µg kg^{-1}	5 µg kg^{-1}	10 µg kg^{-1}	1 µg kg^{-1}	5 µg kg^{-1}	10 µg kg^{-1}
BaA	0.1	0.25	0.9972	0.9986	88	104	106	2 (11)	3 (10)	2 (10)
Chr	0.1	0.25	0.9993	0.9993	102	102	106	4 (10)	2 (8)	1 (11)
5-MC	0.1	0.25	0.9975	0.9988	88	102	102	4 (11)	3 (8)	2 (9)
BbF	0.3	1	0.9963	0.99	81	99	103	1 (10)	3 (7)	2 (10)
BkF	0.3	1	0.9917	0.9933	89	106	104	1 (9)	5 (10)	1 (9)
BaP	0.1	0.25	0.9908	0.9984	82	98	102	3 (10)	4 (7)	1 (10)
IcdP	0.3	1	0.991	0.997	94	96	99	3 (9)	2 (8)	5 (11)
DahA	0.1	0.25	0.9909	0.9964	107	102	103	4 (5)	4 (7)	3 (10)
DalP	0.2	0.5	0.9983	0.9934	88	100	99	1 (10)	4 (6)	3 (9)
DaeP	0.2	0.5	0.9983	0.9924	104	97	93	4 (6)	3 (6)	3 (9)
DaiP	0.3	1	0.9972	0.993	104	82	72	3 (5)	2 (4)	4 (9)
DahP	0.3	1	0.9975	0.9908	112	83	78	2 (5)	5 (6)	6 (9)

LOD: limit of detection; LOQ: limit of quantification; R^2: coefficient of determination; RSD: relative standard deviation.

4. Conclusions

Dispersive liquid-liquid microextraction technique combined with environmental-friendly QuEChERS achieves high analytical sensitivity and efficient matrix co-extractives removal for trace determination of twelve PAHs in baby food by GC-MS. This report introduces for first time low-density extracting solvent-based DLLME combined with ultra-low temperature for analytes enrichment after target extraction from solid matrices. Using Plackett-Burman design the main sample preparation variables that could significantly affect the PAHs extraction were identified, such as amount of C_{18} sorbent in cleanup step and toluene volume in LD-DLLME. Moreover, this experimental design contributed to lower waste generation and time and final cost involved in the method development due to reduced number of experiments carried out (only 15 trials). Overall, the developed method presents attractive features for routine analysis including small sample amount, low reagent consumption and easy operation. It also avoids time consuming SPE using cartridges; and negligible volume of extracting solvent (75 µL) and the same organic solvent used initially for analytes pre-extraction are employed in LD-DLLME procedure, contributing to a green sample preparation approach.

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CAPÍTULO 4 - Determination of polyamines in baby food by gas chromatography–mass spectrometry: Optimization of extraction and microwave-assisted derivatization using response surface methodology

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Abstract

The present work evaluates the feasibility of microwave-assisted acylation combined with previous ion-pair extraction for the determination of the polyamines spermidine and spermine in baby food by GC–MS. In this way, extraction and derivatization reaction times were simultaneously optimized using a central composite rotational design. From response surface analysis, we verified maximum analytical response for spermidine and spermine employing 1M hydrochloric acid solution as extraction solvent under shaking during 35 min, followed by acylation derivatization using a household microwave at 600 W for only 5 min. Limits of detection and quantification of 5 and 10 μg kg^{-1} were achieved, and recoveries between 72 and 112% and RSD values ≤ 16% were obtained under repeatability and within-laboratory reproducibility conditions for both polyamines, at levels of 250 and 500 μg kg^{-1}. The validated GC–MS method was applied to 20 baby food samples commercially available in Brazil, resulting in the first report on spermidine and spermine occurrence in baby foods.

Keywords**:** Biogenic amines; Baby foods; Central Composite Rotational Design; Microwave-assisted derivatization; Gas chromatography; Food Chemistry.

Introduction

Polyamines are aliphatic organic bases of low molecular weight formed by endogenous biosynthesis in all living organisms from the amino acids arginine, ornithine and methionine, which have been classified within biologically active amines group (Glória 2005; Kalac 2014). Therefore, these bioactive compounds are naturally present in animals and plant tissues and microbial cells or as products of enzymatic activities of some microbial agents (Standara et al. 2000). In humans, the diet contributes to greater daily quantity of polyamines than endogenous biosynthesis (Bardócz 1995); moreover, the polyamines spermidine (*N*-(3-aminopropyl)-1,4-diaminobutane) and spermine (*N,N'*-bis-(3-aminopropyl)-1,4-diaminobutane) are involved in important metabolic and physiological functions (Glória 2005). In the last decades, the scientific community has triggered a series of studies on the role of the polyamines in health as well as in the development of certain diseases (Dandrifosse 2009; Gugliucci 2004; Larqué et al. 2007; Moinard et al. 2005).

Spermidine and spermine are found at low millimolar to high micromolar intracellular levels, being essential for cell normal growth, function and proliferation (Kalac 2014; Soda 2011). These compounds are especially important during the growth stage (e.g. infants and children) and other situations that require a fast growth of human tissues, namely post-surgery recovery, scarring process, burns, etc (Igarashi and Kashiwagi, 2000). In intestinal tract, the polyamines play an important role for regeneration and maturation of the mucosal tissue cells. Furthermore, these compounds are able to scavenge oxygen reactive species, thus contributing to reduction of injuries in the cell components (Kalac 2014), as well as they have been involved in allergy prevention (Dandrifosse 2009). However, due to their participation in cell growth and proliferation, polyamines can increase the invasive and metastatic capability of cancer cells; in addition, these compounds have been associated with neurodegenerative diseases (Kalac 2014; Soda 2011). Specifically for polyamines spermine and spermidine, oral acute toxicity was observed in Wistar rats at 600 mg kg^{-1} of body weight, with no-observed-adverse-effect level (NOAEL) of 19 and 83 mg kg^{-1} of body weight per day, respectively (Til et al. 1997). Cytotoxicity on human retinal pigment ephithelial cells was also reported for spermine (Kaneko et al. 2007).

Considering the biological roles in human as well as the health risks associated to polyamines, some authors have strongly advocated the supplementation with polyamines of some types of diet, particularly those intended for infants and children; conversely, their complete removal of the diet of patients with certain types of diseases associated with improper growth of tissues, such as cancer, has been recommended. In this way, it is

necessary reliable information on polyamine content in the foods (Kalac 2009). However, as far as we know, there are no data on spermidine and spermine occurrence in ready-to-eat baby foods.

Analytical methods based on high-performance liquid chromatography (HPLC), with fluorescence (FL) (Adão and Glória 2005; Novella-Rodríguez et al. 2000; Vieira et al. 2010) or ultraviolet (UV) (Restuccia et al. 2011; Soufleros et al. 2007; Tang et al. 2009) detection, have been extensively used for the determination of polyamines in different food matrices. The most of these reports have employed pre-column (Restuccia et al. 2011; Soufleros et al. 2007; Tang et al. 2009) or post-column (Latorre-Moratalla et al. 2009; Rigueira et al. 2011; Santiago-Silva et al. 2011) derivatization technique, due to lack of a chromophore or fluorophore in the polyamines structure. For this purpose, reagents such as *o*-phthaldialdehyde, dansyl chloride, 4-chloro-3,5-dinitrobenzotrifluoride and 1-naphthylisothiocyanate have been chosen for the derivatization reactions (Jain et al. 2015; Önal et al. 2013). On the other hand, underivatized spermidine and spermine were analyzed in fish (Sagratini et al. 2012) and meat (Sirocchi et al. 2014) using HPLC-tandem mass spectrometry. Notwithstanding the high selectivity and sensibility achieved with this analytical technique, the applied sample preparation method includes solid-phase extraction (SPE) in cartridges, which makes them costly and time-consuming.

Additionally, gas chromatography (GC) has also been employed for the determination of amines in several matrices, taken into account their high resolution and sensitivity (Plotka-Wasylka et al. 2015). However, GC analysis of free amines presents some difficulties due to high polarity of these compounds (Kataoka 1996); therefore, acylation, silylation, dinitrophenylation, and carbamate formation have been the main derivatization reactions used to improve the selectivity, sensibility and resolution of GC analysis (Kataoka 1996; Plotka-Wasylka et al. 2015). Successful biogenic amine analyses have been achieved using GC coupled to mass spectrometry (GC-MS) (Almeida et al. 2012; Cunha et al. 2011; Cunha et al. 2017; Plotka-Wasylka et al. 2016). Nonetheless, GC-MS has been few explored for the determination of the polyamines spermidine and spermine in foods. A GC-MS method involving ion-pair extraction with bis-2-ethylhexylphosphate (BEHPA) and subsequent acylation derivatization using heptafluorobutyric anhydride (HFBA) reagent was successfully developed and validated for the determination of biogenic amines, including spermidine and spermine, in wine and grape juice (Fernandes and Ferreira 2000). Years later, this method was applied to the determination of aliphatic amines and aromatic amines in water (Akyüz and Ata 2006) and hair (Akyüz and Ata 2008). The acylation reaction was achieved at 80°C for 60

min on a heater block providing adequate performance criteria and low limits of detection and quantification; although, the time involved in the derivatization reaction has prevented widespread application to routine polyamines analysis of other matrices. In this context, the main objectives of this work were to (i) improve some features of the previously developed method, based on ion-pair extraction with acylation derivatization and GC-MS analysis, in order to reduce its time of analysis, and (ii) investigate the presence of the polyamines spermidine and spermine in baby foods commercialized in Brazil.

Materials and Methods

Chemicals and Standards

Analytical standards of spermidine and spermine and the internal standard (IS) nor-spermidine, all with purity > 99%, were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Individual stock standard solutions were prepared at 2 g L^{-1} in 0.1M HCl solution. A polyamine working standard solution was prepared at 100 mg L^{-1} in 0.1M HCl solution by combining appropriate aliquots of individual stock solutions. IS working standard solution was also prepared at 100 mg L^{-1} in 0.1M HCl solution. The ion-pair reagent BEHPA and the derivatization reagent HFBA were purchased from Sigma-Aldrich. Acetonitrile, dichloromethane and ethyl acetate, all of analytical grade, were obtained from Fluka (Neu-Ulm, Germany) and hydrochloric acid (HCl) was purchased from Merck (Darmstadt, Germany). Anhydrous sodium sulfate and potassium hydroxide were purchased from Alfa-Aesar (Karlsruhe, Germany).

Samples

A total of 20 commercial samples of baby food, including 13 fruit-based baby foods and 7 fruit and milk-based baby foods, were randomly collected from five local markets in the city of Campinas, SP, Brazil. All the samples were packed in plastic bags (113 g each) or glass jars (120 g each) and were maintained in their original packing at 4 °C and protected from light until analysis.

Determination of polyamines in baby foods

Polyamine extraction

Five grams of homogenized baby food sample and 10 mL of 1M HCl solution were added to a 50 ml polypropylene centrifuge tube, and this tube was agitated in a mechanical shaker for 35 min. After centrifugation at 4000 rpm for 10 min, the supernatant was collected

and the pH was adjusted to 7.4 ± 0.2 with concentrated potassium hydroxide solution, and 2 mL of phosphate buffer solution (pH 7.4) were added. Then, 5 mL of buffered mixture were extracted with 5 mL of 0.1M BEHPA solution in chloroform by agitation in a vortex for 1 min, followed by centrifugation at 3500 rpm for 5 min. Four milliliters of chloroform phase (bottom layer) were combined with 4 mL of 0.1M HCl solution and vortexed for 1 min, and subsequently centrifugated at 3500 rpm for 5 min. The aqueous phase (upper layer) was collected and then submitted to derivatization reaction (Fig. 1).

Microwave-assisted derivatization

One hundred microliters of aqueous hydrochloric acid extract were evaporated to dryness at 45°C under a stream of nitrogen. Then, the analytes were derivatized with a mixture of 200 µL of acetonitrile and 200 µL of HFBA using a household microwave at 600 W during 5 min. After cooling at room temperature, the solution was evaporated to dryness at 45°C under a stream of nitrogen. The residue was dissolved in 1 mL of phosphate buffer solution (pH 7.4), and the acylated analytes were extracted with 3 mL of dichloromethane by agitation in a vortex for 1 min. The dichloromethane phase (bottom layer) was collected and dehydrated with 100 mg of anhydrous sodium sulfate and evaporated to dryness at 45°C under a stream of nitrogen. Finally, the residue was dissolved in 100 µL of ethyl acetate containing 0.2% of Carbowax 1000 M, and 1µL was submitted to GC-MS analysis (Fig. 1).

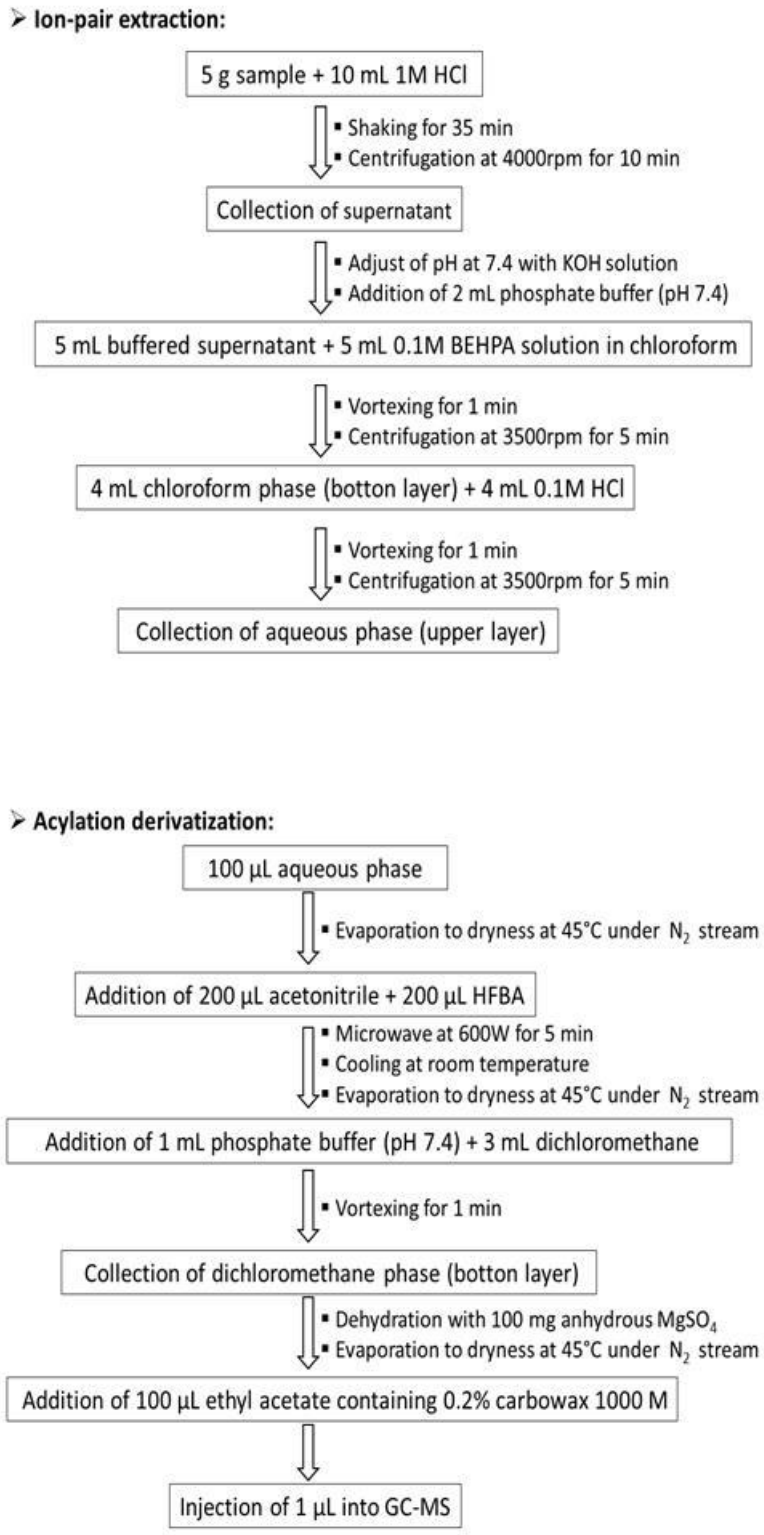

Fig. 1. General scheme of optimized sample preparation method for the determination of spermidine and spermine in baby food.

Gas Chromatography–mass spectrometric analysis

A gas chromatograph 6890 (Agilent, Little Falls, DE, USA) equipped with a Combi-PAL autosampler (CTC Analytics, Zwingen, Switzerland) and an electronically controlled split/splitless injection port, interfaced to a single quadrupole inert mass selective detector (5975B, Agilent) with electron ionization (EI) chamber, was used for polyamine analyses. GC separation was achieved on a DB-5MS capillary column (30 m x 0.25 mm I.D. x 0.25 µm film thickness; J&W Scientific, Folson, CA, USA), which was maintained initially at 80ºC for 1 min, increased at 15ºC min^{-1} to 210 ºC, then ramped to 290ºC at 20ºC min^{-1}, and held at 290°C for 5 min, with a total run of 18.7 min. The injector was maintained at 280°C, and 1 µL of extract was injected in splitless mode (purge-off time, 1 min). Ultra-high purity helium (99.999%) was used as carrier gas at constant flow of 1 mL min^{-1}. The electron energy was 70 eV, and the temperatures of transfer line, ion source and quadrupole mass analyzer were set at 280°C, 230ºC and 150ºC, respectively. Agilent ChemStation was used for GC-MS system control and data analysis.

The quantification was carried out in selected ion monitoring (SIM) mode, and the ions *m/z* 564 and 576 were used for the quantification of the spermidine (t_R = 10.7 min) and spermine (t_R = 13.2 min), respectively. Furthermore, the ions *m/z* 536 and 323 were monitored as confirmatory fragments for spermidine and *m/z* 479 and 309 for spermine. Nor-spermidine was used as IS (ion *m/z* 254 was used for quantification, and the ions *m/z* 226 and 309 were monitored for qualification; t_R: 10.2 min), which was added to sample at level of 500 µg kg^{-1} prior to the extraction procedure. The analytes were identified by the retention time and comparing the relative abundance ratios of quantification and qualification ions with those obtained in calibration standards.

Experimental design

A central composite rotatable design (CCRD) was conducted in order to optimize extraction and derivatization reaction times, according to Rodrigues and Iemma (2014). Statistica 8.0 software (Statsoft Inc., Tulsa, OK, USA) was employed for processing the data and to obtain response surface plots. The statistical significance of second-order models was assessed by *F* test (analysis of variance – ANOVA), and the quality of fit of these models was expressed by the coefficient of determination (R^2) and relative errors. The significance level used was set to 10%.

Results and Discussion

Optimization of sample preparation method

Sample preparation was based on the method previously described by Fernandes and Ferreira (2000) for the determination of polyamines in wine and grape juice, which consists of ion-pair extraction followed by acylation derivatization with HFBA and GC-MS analysis. An important improvement in terms of analysis time was achieved using microwave-assisted derivatization instead of the conventional reaction with a block heater. Therefore, extraction and acylation reaction times were simultaneous optimized to baby food matrix.

Initially, the acylation of polyamines obtained with a block heater was compared to those obtained with a household microwave. For this, 100 µL of a standard solution containing spermidine and spermine at concentration of 20 mg L^{-1} was evaporated to dryness at 45°C under a stream of nitrogen and derivatized with a mixture of 200 µL of acetonitrile and 200 µL of HFBA using a household microwave at 600 W for 5 min or a block heater at 80ºC for 60 min as proposed by Fernandes and Ferreira (2000). The analytical signals obtained with both equipments were similar; nevertheless, a significant reduction in the derivatization time was achieved with microwave-assisted reaction, making it a good choice. This result was compared with those obtained by our team for other analytes (Amaral et al. 2013; Cunha et al. 2015).

As baby foods are semi-solid samples, a pre-extraction of the polyamines was mandatory before ion-pair extraction procedure. In this way, 1M hydrochloric acid solution was tested as extraction solvent using an extractor: sample ratio of 2:1 (v/w) under constant agitation in a mechanical shaker. In the literature, acidified aqueous solutions have been used for the extraction of polyamines from different food matrices, such as 5% trichloroacetic acid for fish (Sagratini et al. 2012) and meat (Sirocchi et al. 2014), or 1N hydrochloric acid solution for tropical fruits (Santiago-Silva et al. 2011).

The pre-extraction time and the time of acylation reaction using microwave were optimized simultaneously employing a CCRD. The use of CCRD for optimization studies shows many advantages compared to one-factor-at-time approach, including simultaneous multiple variable analysis, assessment of possible interactions between these variables, reduced number of trials or repetitions, and consequently less labor, time and final cost (Rodrigues and Iemma 2014). In this study, a representative fruit-based baby food, composed by a mixture of fruit purées, milk, rice flour and starch, was spiked at 1000 µg kg^{-1} 1 h before the pre-extraction step, and then, it was extracted and derivatized according to the time defined for each trial of CCRD (Table 1).

The CCRD included a 2^k factorial design (where k is the number of independent variables), four axial points ($\pm\alpha = (2^k)^{1/4}$), and four central points (in order to assess the repeatability of the analyses and determine the pure error of statistical model) resulting in 12 trials (Rodrigues and Iemma 2014). The matrix used in the experimental design, with coded and real values for the two independent variables studied, namely extraction time (X_1) and derivatization time (X_2), is shown in Table 1. The time involved for pre-extraction of polyamines ranged between 10 and 60 min, while the derivatization reaction time using microwave at 600 W varied from 2 to 8 min. From obtained chromatographic peak areas, the regression coefficients were calculated, and then, these were used to obtain second-order models (Eq. 1) in order to predict the analyte response as a function of the studied variables.

$$y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i<j} \sum_{j} \beta_{ij} x_i x_j + \varepsilon \qquad \text{(Eq. 1)}$$

where y is the predicted response (chromatographic peak area), β_0 is the global mean, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the coefficient of interaction, ε is the error of the model, x_i and x_j are coded values of the independent variables.

Table 1. Central composite rotatable design (CCRD) matrix with coded and respective real values for extraction and derivatization times and the polyamine analytical responses.

Trial	Coded		Real		Spermidine			Spermine		
	X_1	X_2	Extraction (min)	Derivatization (min)	Analytical response *		Relative error (%)	Analytical response *		Relative error (%)
					Experimental	Predicted		Experimental	Predicted	
1	–1	–1	20	3	23294	20141.8	13.5	7011	6053.5	13.7
2	+1	–1	50	3	23264	22838	1.8	6740	6670.5	1.0
3	–1	+1	20	7	24244	22505.2	7.2	7347	6836.1	6.9
4	+1	+1	50	7	23508	24495.4	–4.2	7205	7582.3	–5.2
5	–1.41	0	10	5	16834	19913.4	–18.3	5048	5979.5	–18.5
6	+1.41	0	60	5	24003	23217.2	3.3	7268	6940.5	4.5
7	0	–1.41	35	2	19986	22124.6	–10.7	5918	6533.7	–10.4
8	0	+1.41	35	8	24828	24959.2	–0.5	7748	7728.3	–0.3
9 (CP)	0	0	35	5	35164	32330.5	8.1	7922	8446.3	–6.6
10 (CP)	0	0	35	5	29201	32330.5	–10.7	9140	8446.3	7.6
11 (CP)	0	0	35	5	27014	32330.5	–19.7	8761	8446.3	3.6
12 (CP)	0	0	35	5	37943	32330.5	14.8	7962	8446.3	–6.1

*Chromatographic peak area; CP: central point; Relative error (%) = [(Experimental – Predicted)/ Experimental x 100]

The statistical significance of second-order model for each polyamine was checked by *F* test (ANOVA), whose confidence level was set to 90%. As a great result, the obtained models were considered predictive because *F* test value for the regression was significant ($p < 0.1$) while the lack of fit was not significant ($p > 0.1$) (Table 2). Moreover, the percent of variation explained by the model was appropriate with determination coefficients (R^2) higher than 70% for both polyamines, and the relative error values ranged from –19.7 to 14.8%, demonstrating a close relationship between the response predicted by the model and the experimental response (Table 1). Therefore, the models obtained for spermidine (Eq. 2) and spermine (Eq. 3) were employed to generate response surface plots (Fig. 2).

$$y = 32330.5 + 1171.6X_1 - 5414.8X_1^2 + 1005.2X_2 - 4420.6X_2^2 - 176.5X_1X_2 \quad \text{(Eq. 2)}$$

$$y = 8446.3 + 340.8X_1 - 999.1X_1^2 + 423.6X_2 - 661.6X_2^2 + 32.3X_1X_2 \quad \text{(Eq. 3)}$$

Table 2. Analysis of variance (ANOVA) of the regression model for spermidine and spermine.

Response *	Source	Regression model					R^2
		SS	DF	MS	*F* value	*p* value	
Spermidine (y_1)	Regression	2.8×10^8	5	5.6×10^7	3.2	0.09529	72.7%
	Residual	1.0×10^8	6	1.8×10^7			
	Lack of fit	2.8×10^7	3	9.4×10^6	0.4	0.78579	
	Pure error	7.8×10^7	3	2.6×10^7			
	Total	3.9×10^8	11				
Spermine (y_2)	Regression	1.0×10^7	5	2.0×10^6	3.3	0.09098	73.1%
	Residual	3.7×10^6	6	6.2×10^5			
	Lack of fit	2.6×10^6	3	8.8×10^5	2.4	0.24229	
	Pure error	1.1×10^6	3	3.6×10^5			
	Total	1.4×10^7	11				

*Chromatographic peak area; SS: sum of squares; DF: degree of freedom; MS: mean square; R^2: coefficient of determination.

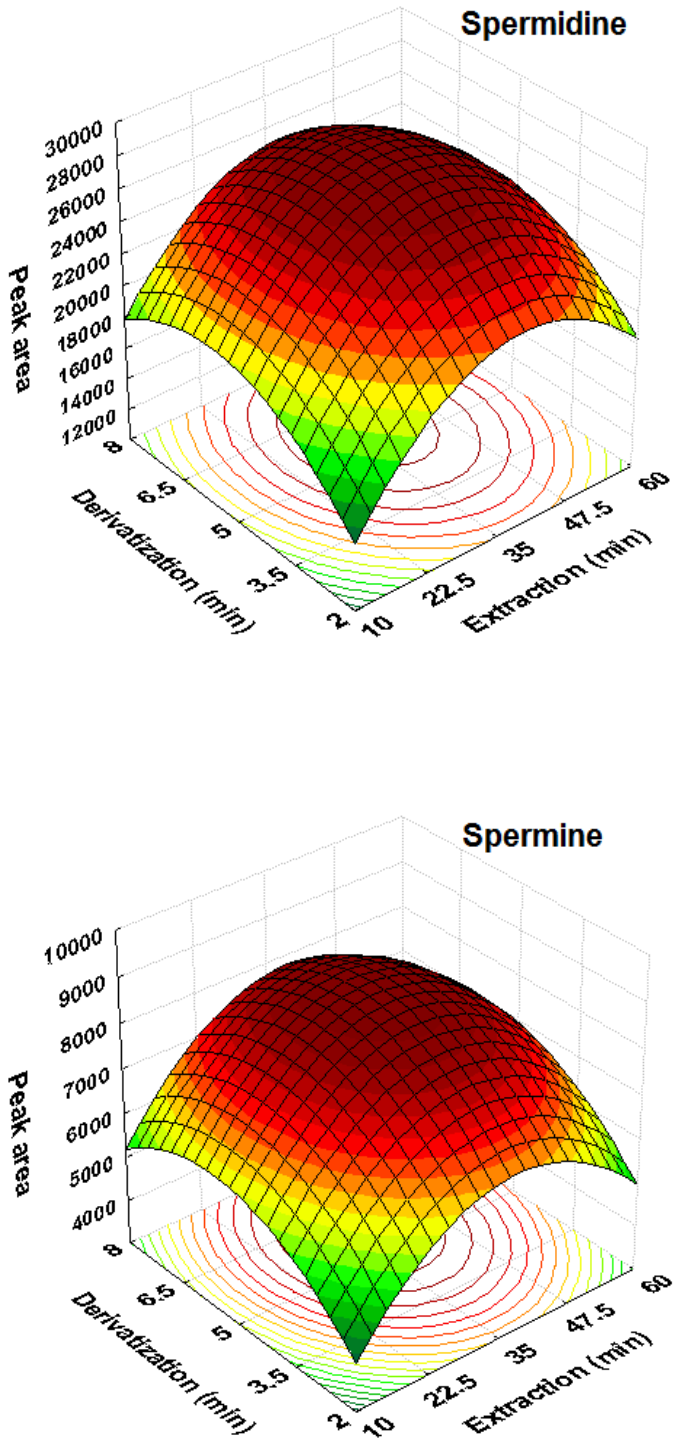

Fig. 2. Response surface plots for chromatographic peak area of spermidine and spermine as a function of the derivatization and extraction times.

Based on response surface plots (Fig. 2), the optimal time for the pre-extraction of polyamines from baby food samples as well as for microwave-assisted acylation was defined. Among different times of agitation evaluated for pre-extraction – 10, 20, 35, 50 and 60 min, maximum analytical response was achieved at 35 min for spermidine and spermine. For microwave-assisted acylation, 2, 3, 5, 7 and 8 min were tested, but 5 min were enough to achieve the maximum analytical response for both polyamines, using a household microwave at 600 W.

In summary, the modified sample preparation method reduced in 12-fold the time involved in the acylation reaction compared to the previous method (Fernandes and Ferreira 2000), resulting in a faster and less laborious analytical method for routine analyses. Furthermore, the additional pre-sample extraction step using 1M HCl solution demonstrated to be very efficient for the extraction of the polyamines from baby foods.

Method performance

The selectivity of the method was assessed based on its ability to determine the polyamines accurately in the presence of matrix co-extractives. A complete separation of the polyamines spermidine (t_R = 10.7 min) and spermine (t_R = 13.2 min) from matrix interferences was observed in total ion chromatograms (TICs) obtained in SIM mode for all baby food samples analyzed, demonstrating the selectivity of the proposed method (Fig. 3).

Linearity was assessed in solvent calibration curves prepared in 0.1M HCl solution in the range between 50 and 1000 μg L^{-1}, including six concentration levels. The calibration curves were constructed by plotting the analyte/nor-spermidine peak area ratio against the concentration values. High determination coefficients (R^2) were obtained for spermidine and spermine, being always greater than 0.99, confirming method reliability (Table 3). The limits of detection (LOD) and quantification (LOQ) were established by successive analysis of baby food extract until obtaining signal-to-noise ratios of 3:1 and 10:1, respectively. LOD of 5 μg kg^{-1} and LOQ of 10 μg kg^{-1} were achieved for spermidine and spermine. These analytical limits are lower than those obtained for spermidine and spermine in tropical fruits (Santiago-Silva et al. 2011) and banana (Adão and Glória 2005), with determination limits ranging between 100 and 400 μg kg^{-1}, using post-column derivatization with *o*-phthaldialdehyde followed by HPLC-FL analysis. On the other hand, very low LOD (0.18 μg L^{-1}) and LOQ (0.62 μg L^{-1}) were obtained for spermidine in fruit juices, using a salting-out assisted liquid–liquid extraction followed by pre-column derivatization with 1-naphthylisothiocyanate and

HPLC-UV analysis (Jain et al. 2015). However, no results were reported for spermine, probably due to difficulties in the derivatization step.

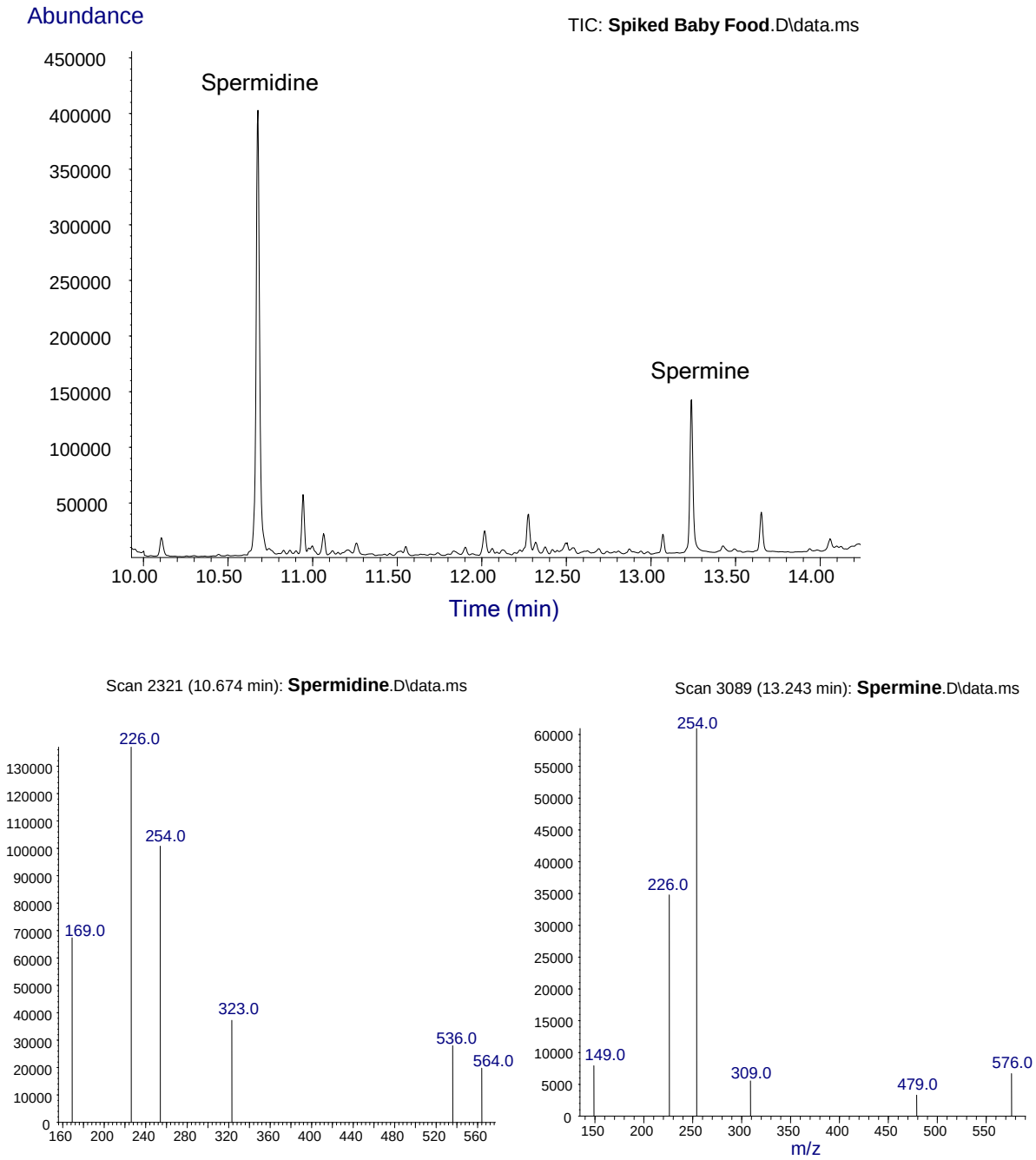

Fig. 3. Total ion chromatogram obtained in selected ion monitoring (SIM) mode of a spiked baby food sample and corresponding product ion scan mass spectra.

The extraction efficiency of the method was examined by recovery experiments using two spiked levels, 250 and 500 µg kg^{-1}. A representative fruit-based baby food was spiked 1 h before extraction procedure and, in parallel, the same baby food sample, added only of IS nor-spermidine at 500 µg kg^{-1}, was also submitted to analysis in order to determine the natural polyamine content in this spiked sample. Six determinations were carried out on two different days, totaling 12 replicates for each level. The mean recovery (%) varied between 78 and 102% for spermidine, and from 72 to 112% for spermine, assisting the efficiency of the method (Table 3).The precision, under repeatability and within-laboratory reproducibility conditions, was also evaluated and expressed in terms of relative standard deviations (RSD). Under the repeatability conditions, six independent replicates of spiked samples at each level were analyzed on the same day by the same analyst under the same chromatographic conditions. The RSD values ranged from 1 to 6% for spermidine, and from 0.3 to 8% for spermine. Under the within- laboratory reproducibility conditions, 12 independent replicates of spiked samples at each level were analyzed on two different days by the same analyst under the same chromatographic conditions; RSD values obtained ranged between 7 and 12% for spermidine, and from 5 to 6% for spermine (Table 3).

Table 3. Performance characteristics of optimized method for the determination of spermidine and spermine in baby food.

Parameters	Spermidine	Spermine
LOD (µg kg^{-1})	5	5
LOQ (µg kg^{-1})	10	10
Linearity (50–1000 µg L^{-1}, R^2)	0.9984	0.995
Recovery (%, *n = 6*)		
250 µg kg^{-1}	102	112
500 µg kg^{-1}	78	72
Precision (RSD %)	*Intra-day, n = 6 (Inter-day, n = 12)*	
250 µg kg^{-1}	1 (7)	0.3 (6)
500 µg kg^{-1}	6 (12)	8 (5)

LOD: limit of detection; LOQ: limit of quantification; R^2: coefficient of determination; RSD: relative standard deviation.

Polyamines in baby food

The applicability of the optimized method was assessed by analyzing 20 commercial baby foods intended for consumption by infants (aged less than 12 months) and young children (aged between one and three years old), which included samples of the most popular baby food brands available in Brazilian market. The contents of spermidine and spermine detected in the baby food samples are shown in the Table 4. Spermidine was detected in all baby foods analyzed, with levels varying between 27 ± 1.8 and 253.7 ± 46.5 μg kg^{-1}. On the other hand, the spermine levels were lower compared to spermidine, ranging from not detected to 97 ± 12.5 μg kg^{-1}.

Table 4. Polyamines content in baby food samples.

Baby food [a]	Polyamines (mean ± standard deviation, μg kg^{-1}) [b]	
	Spermidine	Spermine
Apple *Brand A*	27 ± 1.8	13.1 ± 0.9
Apple *Brand B*	50.2 ± 0.7	n.d.
Apple *Brand C*	117.6 ± 2.9	37.4 ± 2.8
Apple, Banana, Papaya and Yogurt	94 ± 3.6	20.8 ± 1.9
Apple, Banana and Pear	253.7 ± 46.5	n.d.
Apple and Yogurt *Brand A*	34.3 ± 8	n.d.
Apple and Yogurt *Brand B*	30.8 ± 2.3	n.d.
Banana	101.2 ± 4.7	10.6± 0.5
Banana and Apple	117.8 ± 16.3	27.7 ± 0.9
Banana, Mango and Pineapple	229.3 ± 36.4	97 ± 12.5
Banana and Milk	130.3 ± 4.6	31.6 ± 2.4
Banana and Oat	181.3 ± 29.5	53.7 ± 0.4
Banana, Orange and Pineapple	40.4 ± 1.2	n.d.
Banana and Yogurt *Brand A*	53 ± 4.4	12.3 ± 1.7
Banana and Yogurt *Brand B*	76 ± 0.7	10 ± 0.6
Papaya and Orange	152 ± 3.8	n.d.
Pear	100.5 ± 39	37.3 ± 5.7
Pear and Yogurt	30.3 ± 2.2	n.d.
Plum *Brand A*	45.5 ± 1	n.d.
Plum *Brand B*	30.4 ± 0.2	n.d.

[a] Principal ingredients; [b] Mean results from duplicate portions; n.d.: not detected.

Overall, the levels of spermine in fruit- and vegetable-based foods are typically lower than spermidine, which is an inverse relation to those levels observed in foods of animal origin (Kalac 2009). The mean level of spermidine in fruit-based baby food samples (111.3 μg kg^{-1}) was higher than those detected in samples with milk or yogurt in their composition (64.1 μg kg^{-1}); the same was observed for spermine levels, with mean value of 39.5 μg kg^{-1} for fruit-based baby foods and 18.7 μg kg^{-1} for samples elaborated with milk or yogurt.

As far as we know, there are no published data from other sources with respect to spermidine and spermine contents in baby food samples. In any case, we can find in the literature reports regarding spermidine and spermine levels in raw materials such as fruits (Kalac et al. 2005; Nishibori et al. 2007; Santiago-Silva et al. 2011; Vieira et al. 2007) and dairy products (Novella-Rodríguez et al. 2000), which are the main ingredients of the analyzed baby foods. Table 5 presents spermidine and spermine contents reported in selected fruits and dairy products.

Table 5. Contents of spermidine and spermine in selected foods.

Foods	Spermidine	Spermine	Reference
Fruits			
Apple	n.d. – 2100 μg kg^{-1}	n.d.	Kalac et al. (2005)
Banana	4200 – 8300 μg kg^{-1}	n.d. – 1600 μg kg^{-1}	Nishibori et al. (2007)
Orange (juice)	1800 – 4200 μg L^{-1}	70 – 340 μg L^{-1}	Vieira et al. (2007)
Papaya	3800 – 7600 μg kg^{-1}	n.d. – 2400 μg kg^{-1}	Santiago-Silva et al. (2011)
Pineapple	3100 – 16300 μg kg^{-1}	700 – 4400 μg kg^{-1}	Santiago-Silva et al. (2011)
Dairy products			
Milk	160 – 180 μg kg^{-1}	n.d.	Novella-Rodríguez et al. (2000)
Yogurt	n.d. – 430 μg kg^{-1}	n.d. – 340 μg kg^{-1}	Novella-Rodríguez et al. (2000)

n.d.: not detected

As can be seen, polyamine levels are remarkably higher than those reported herein, which might be a strong indication that baby food processing has a decisive role in the polyamines content. The effect of processing on polyamine content was reported in previous studies in milk infant formula (Buts et al. 1995; Gómez-Gallego et al. 2014; Pollack et al. 1992; Romain et al. 1992); results revealed that polyamine content in infant formula is around 10 times less than in human milk. Additionally, Vieira et al. (2010) verified a significant reduction in the spermidine level during the processing of concentrated orange juice. Lower

spermidine level was also observed in commercial mango juice, 6.8 μg L^{-1} (Jain et al. 2015), when compared to fresh mango, 4600 μg kg^{-1} (Santiago-Silva et al. 2011). Polyamines play an important role in the development of immune system and intestinal microbiota of children; therefore, the influence of baby food processing on polyamine content should be investigated in order to improve their quality in the future.

Conclusions

In this study, a sample preparation method combining ion-pair extraction with microwave-assisted acylation was introduced for spermidine and spermine analysis in baby food by GC-MS. The microwave-assisted reaction using heptafluorobutyric anhydride reagent demonstrated to be suitable for the derivatization of the polyamines, contributing to a reduction in time of the reaction from 60 to 5 min. Using a factorial design associated to response surface analysis, the extraction and acylation reaction times were simultaneously optimized, resulting in a faster and less laborious method for routine analysis. Adequate performance criteria were achieved, including selectivity, linearity, reliable limits of detection and quantification, and acceptable recoveries and precision, under repeatability and within-laboratory reproducibility conditions. The application of the optimized method to commercial samples demonstrated their feasibility for quantitative analysis of spermidine and spermine in baby food. The low levels found for both polyamines may be attributed to the baby food processing.

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CAPÍTULO 5 - Simultaneous determination of acrylamide and 4-hydroxy-2,5-dimethyl-3(2H)-furanone in baby food by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatography triple quadrupole mass spectrometry method was developed and validated for simultaneous determination of acrylamide and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF) in baby food. The sample preparation involves acetonitrile-based extraction combined with dispersive PSA cleanup and cation-exchange solid-phase extraction, which promotes efficient removal of matrix interferences. Analytical selectivity and sensitivity was achieved for the quantification of acrylamide and HDMF in complex matrices such as fruit, cereal and milk-based baby foods; furthermore, adequate linearity (10–300 µg kg^{-1}) in solvent and matrix-matched calibrations, and appropriate recoveries (94–110%) and precision (RSD ≤ 10%), under repeatability and within-laboratory reproducibility conditions, were also obtained. Expanded measurement uncertainty was estimated at 20 µg kg^{-1} level (limit of quantification) on the basis of data obtained from in-house validation, with values of 25.5 and 16.5% for acrylamide and HDMF, respectively. The fitness-for-purpose of developed method was assessed by analyzing 15 commercial baby foods available in Brazilian markets. Acrylamide was detected in one plum-based baby food (35µg kg^{-1}) while HDMF in 67% of the samples analyzed (25–262 µg kg^{-1}).

Keywords: Acrylamide; Baby food; Food contaminant; 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone; LC-MS/MS; Uncertainty estimation.

1. Introduction

Recently, the European Food Safety Authority (EFSA) published its scientific opinion about acrylamide in foods [1]. On the basis of evidences from animal studies and analytical results reported by 24 European countries and six food associations, it was concluded that acrylamide potentially increases the cancer development risk for the consumers. Although evidences from human studies between cancer and dietary acrylamide exposure has been limited and inconclusive [1,2]. Additionally, acrylamide has been classified under group 2A as a probably carcinogenic to humans by International Agency for Research on Cancer (IARC) [3]. It is known that *in vivo* this compound is metabolized to the epoxide glycidamide, a reactive metabolite with potential genotoxicity [4]. Animal experimental studies have also demonstrated adverse effects of acrylamide on male reproduction as well as its neurotoxicity [4]. The Maillard reaction between the amino acid asparagine and reducing sugars is the major chemical process described for acrylamide formation in foods during heating at temperatures above 120°C; however, other mechanisms involving acrolein, acrylic acid, wheat gluten and 3-aminopropionamide have also been proposed [5,6]. Carbohydrate-rich foods have been the main products investigated for acrylamide occurrence, being the highest levels found in solid coffee substitutes and coffee, and potato fried products [1]. Nonetheless, few reports on acrylamide in baby foodare available in literature [7-9].

Other compound generated by the Maillard reaction is 4-hydroxy-2,5-dimethyl-3(2H)-furanone (HDMF), an aroma chemical with attractive sensory properties [10]. In addition, HDMF is biosynthesized by certain microorganisms and plants, thus occurring naturally in some fruits and, wines, cheeses, soy sauce, cocoa and chocolate [10,11]. HDMF has also been added as flavouring agent in some foodstuffs [10,12]. However, positive results for genotoxicity tests have attracted attention for the levels this compound in foods [12,13]. In 2011, the European Food Safety Authority (EFSA) Panel on Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) reviewed HDMF studies and concluded that there are evidences for genotoxicity *in vitro* and *in vivo*, although absence of carcinogenic potential was observed in a chronic study with rats [14]. *In vitro*, HDMF induced reverse gene mutations, whose genotoxic effects have been associated with the generation of reactive oxygen species as a result of redox cycling in the presence of metal ions and oxygen, contributing to an increase in cellular oxidative stress and DNA-breaking activity [12-15]. Genotoxicity was also observed *in vivo* after HDMF administration via intraperitoneal and oral in mice [12-14,16]. A no-observed-adverse-effect level (NOAEL) of 200 mg kg^{-1} of body weight per day was reported for HDMF from a study with rodents [12,14].

Due to the increasing knowledge on health risks associated to compounds generated during high temperature cooking, several analytical methods have been proposed for food control. Nonetheless, very little works have explored simultaneous determination of these heat-induced compounds in foodstuffs. For instance, Nielsen et al. [17] and Zhang et al. [18] described simultaneous liquid chromatography–mass spectrometry (LC-MS) analysis of acrylamide and its precursors asparagine and sugars in carbohydrate-rich matrices. In other study, acrylamide and 3-monochloropropane-1,2-diol were simultaneously analyzed by gas chromatography–mass spectrometry (GC-MS) in bread, powered milk, soy sauce and others [19]. High polarity, low molecular weight, the lack of sufficiently strong chromophore group in chemical structure, and low volatility has been critical factors for acrylamide analysis [20, 21]. Furthermore, complex and time-consuming sample preparation methods have been reported in the literature, including steps of defatting by liquid-liquid partition with hexane or petroleum ether [8,9], rotary evaporation [8], solvent exchange [8,22], filtration on paper [23], extract clarification using Carrez reagents [20,24] and derivatization reactions [25]. Therefore, development of simpler and faster sample pre-treatments has been encouraged, particularly focusing minimal reagent consumption and lower residue generation.

Liquid chromatography (LC) coupled to mass spectrometry (MS) achieves adequate performance characteristics for the identification and quantification of acrylamide in foodstuffs [20]; however, some authors have demonstrated the applicability of LC with diode array detection (DAD) [23,26]. The most of LC-MS methods includes electrospray interface (ESI) and triple quadrupole (QqQ) mass analyzer, which contributes to acrylamide detection at low levels [21]. In addition, atmospheric pressure chemical ionization (APCI) and ion trap mass analyzers have also been related [24]. An advantage associated to LC methods includes acrylamide determination without derivatization step resulting in a shorter sample preparation [21]. On the other hand, underivatized acrylamide analysis by GC-MS presents some drawbacks such as unselective detection of the analyte due to lack of characteristic ion in mass spectrum as well as high interference caused by matrix composition and high limits of detection [21]. For HDMF analysis purpose, a LC-MS/MS method, with APCI ionization and QqQ mass analyzer, was successfully applied for the determination of HDMF in apple cider and wines [27]; while other LC method with diode array detector was described for strawberries [28].

In the present report, an accurate and selective method based on solid phase extraction and LC-QqQ-MS/MS was developed and validated for simultaneous determination of acrylamide and HDMF in baby food. The proposed method meets the Commission Decision

2002/657/EC criteria for quantitative method of analysis as well as the Commission Recommendation 2010/307/EU for the monitoring of acrylamide in foods. In addition, the expanded measurement uncertainty was estimated for both compounds from in-house validation data, and the developed method was successfully applied to commercial samples of baby food available in Brazilian markets.

2. Materials and methods

2.1. Chemicals and consumables

Analytical standards of acrylamide (99.9%) and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (99%) were purchased from Fluka, and the internal standard (IS) methacrylamide (99.5%) from Aldrich. Stock standard solutions at 1000 µg mL^{-1} were prepared by dissolving 10 mg of standard in 10 mL of deionized water, and then working standard solutions were obtained by diluting of stock solutions with deionized water to concentrations of 10 and 100 µg mL^{-1}. And, a multi-compound standard solution was prepared in deionized water at 1 µg mL^{-1} by combining appropriate aliquots of individual working solutions. All standard solutions were stored at –18ºC and protected from light. Acetonitrile and methanol, HPLC grade, were acquired from J.T. Baker and Panreac, respectively; and formic acid (85%) from Synth. Primary and secondary amine (PSA) sorbent was purchased from Supelco. Deionized water was obtained from a Milli-Q system (Millipore). PVDF syringe filters (0.22 µm pore size, 13 mm i.d.) were purchased from Millipore, and the glass vials, all LC-MS certified, from Waters. Bond Elut SCX (100 mg) solid-phase extraction (SPE) cartridges were supplied by Agilent.

2.2. Baby food samples

A total of fifteen samples of ready-to-eat baby foods, which included fruit purées, cereal flour, starch, milk and/or other dairy ingredient in their composition, was purchased in the city of Campinas, located in the South-Eastern region of Brazil, between January and February 2016.The samples were randomly collected from 3 supermarkets and all they were kept in their original packaging, glass jars (120 g each) or plastic bags (113g each), at room temperature until analysis.

2.3. Simultaneous determination of acrylamide and HDMF by LC-MS/MS

2.3.1. Sample preparation

Five grams of baby food sample, previously homogenized, were weighed into a polypropylene centrifuge tube and 5 mL of acetonitrile were added. The mixture was shaken in a vortex for 2 min. After centrifugation at 4000 rpm for 30 min, the supernatant was transferred to a volumetric flask and the volume was completed to 10 mL with acetonitrile. Then, 7 mL of acetonitrile extract were added to a polypropylene centrifuge tube containing 350 mg of PSA sorbent, and each tube was vortexed for 1 min and centrifuged at 4000 rpm for 15 min. Five milliliters of the clean extract were transferred to a graduated glass tube and this was carefully evaporated to 2 mL under a stream of nitrogen, followed by addition of 1 mL of 0.01% formic acid solution. Finally, 1 mL of the acidified extract was passed through a Bond Elut SCX (100 mg) cartridge in a vacuum manifold 20-port model at approximately 15 drops per minute. The final extract was collected, filtered through a 0.22 µm PVDF syringe filter and transferred into glass vials for LC-MS/MS analysis. Prior to use, the SPE cartridges were conditioned with 1 mL of methanol followed by 1 mL of deionized water.

2.3.2. Liquid chromatographic and mass spectrometry conditions

A Waters ACQUITY ultra-performance liquid chromatography (UPLC) system interfaced to a Quattro Premier XE triple quadrupole mass analyzer with electrospray ionization (ESI) source was employed. MassLynx4.1 software was used for qualitative and quantitative data analysis. The chromatographic separation was achieved on a C_8 analytical column (150 mm x 2.1 mm i.d., 5µm particle size; Zorbax Eclipse XDB, Agilent) maintained at 30ºC. The mobile phase consisted of deionized water (A) and acetonitrile (B), both containing 0.01% formic acid, and the elution gradient used was as follows: 0 min, 5% B; 5 min, 50% B; 5.1 min, 100% B; 6–10 min 5% B, at a flow rate of 0.2 mL min^{-1}, resulting in a total run time of 10 min. The injection volume was 20 µL. Data acquisition was carried out in selective reaction monitoring (SRM) mode employing ESI in positive mode. The optimal mass spectrometry conditions were: source and desolvation temperatures, 120°C and 400°C, respectively; capillary voltage, 3 KV; cone voltage, 25 V; gas flow, 500 L h^{-1} (desolvation), 54 L h^{-1} (cone), and 0.2 mL min^{-1} (collision). For all SRM transitions the dwell time was 0.15 s. Nitrogen was used as desolvation and nebulizing gas, and argon as collision gas.

2.3.3. Identification and quantitative analyses

Acquisition of two precursor-to-product ion transitions (SRM) was established for each compound (Table 1). The positive identification of acrylamide and HDMF in baby food samples included retention time (t_R) equal to that obtained in calibration standards within a maximum tolerance of ± 0.1 min, as well as the monitoring of ion ratios of the two selected SRM transitions, which was set as the relationship between the intensity of transitions used for identification (SRM_2) and for quantification (SRM_1), SRM_2/SRM_1. The IS methacrylamide was added to sample at level of 20 µg kg^{-1} prior to the extraction procedure. The quantification was carried out employing seven-point matrix-matched calibration curves (10–300 µg kg^{-1}) constructed by plotting analyte/IS peak area ratio against concentration values, and the obtained results were not corrected for recovery.

2.4. Estimation of measurement uncertainty

The expanded measurement uncertainty was calculated by using the data obtained during in-house validation according to procedure described by Boleda et al. [29]. Data on recovery, within-laboratory repeatability and reproducibility experiments were used, as indicated in the Eq. (1):

$$U = k \times \sqrt{u_s^2 + u_{SD}^2 + u_{RV}^2 + u_{corr}^2} \qquad \text{Eq. (1)}$$

where U is the expanded measurement uncertainty (%), k is the coverage factor, u_s is the uncertainty of (im)precision of the measurement in terms of repeatability, u_{SD} is the uncertainty of (im)precision of the measurement in terms of reproducibility, u_{RV} is the uncertainty estimate for the reference value used, and u_{corr} is the uncertainty of the corrected analyte concentration.

3. Results and discussion

3.1. Development of analytical method

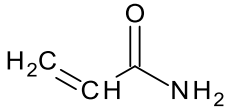
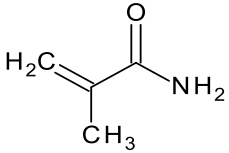
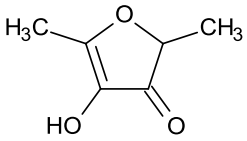
3.1.1. LC–MS/MS optimization

The ESI-MS/MS operating conditions were optimized by infusing individual solutions of analytes at 10 µg mL^{-1} prepared in 0.01% formic acid: acetonitrile (50:50, v/v), at flow rate of 0.05 mL min^{-1}. High sensitivity was achieved in ESI positive ionization mode for all

compounds. The fragmentation profile was studied between 10 and 30V for collision energy, and from 10 to 40V for cone voltage; therefore, the most abundant and specific product ions arising from protonated molecular ion $[M+H]^+$ were selected for each analyte. Moreover, product ions resulting from common losses (e.g. H_2O) were not selected for SMR monitoring in order to avoid equivocal identification of the compounds in complex matrices as baby foods [30].

The most intense precursor-to-product ion transition (SRM_1), such as *m/z* 55 assigned as $[C_3H_3O]^+$ and *m/z* 69 as $[C_4H_5O]^+$, corresponding to loss of NH_3, was monitored for the quantification of acrylamide and methacrylamide, respectively; while, *m/z* 101 designated as $[C_5H_9O_2]^+$, corresponding to loss of CO, was employed for the quantification of HDMF. One second precursor-to-product ion transition (SRM_2), less intense, was used for the confirmation of the compounds as shown in the Table 1. Therefore, a total of four identification points (IPs), namely, 1 IP for the precursor ion and 1.5 IP for each SRM transition, was used as identification criteria fulfill the requirements set by the Commission Decision 2002/657/EC for MS detection [31].

Table 1. Selective reaction monitoring (SRM) transitions and LC-MS/MS operating conditions for simultaneous analysis of acrylamide and HDMF using an electrospray interface (ESI) in positive ionization mode.

Analyte	Chemical struture	MW [a] (g mol^{-1})	t_R [b] (min)	Precursor ion $[M+H]^+$	SRM_1 [c]	CE [d] (V)	SRM_2 [e]	CE [d] (V)
Acrylamide		71.08	2.51	72	55	10	44	12
Methacrylamide		85.10	3.41	86	69	12	58	12
HDMF		128.13	4.27	129	101	10	83	10

[a] Molecular weight;
[b] Retention time;
[c] Precursor-to-product ion transition monitored for quantification;
[d] Collision energy;
[e] Precursor-to-product ion transition monitored for qualification.

The analysis of polar acrylamide is particularly difficult due to their poor retention and chromatographic separation on conventional LC columns [20, 21]. During our method development, two analytical columns with thinner film than in traditional columns, including C_{18} (100 x 2.1 mm i.d., 1.7 µm particle size; Acquity UPLC BEH, Waters) and C_8 (150 x 2.1 mm i.d., 5 µm particle size; Zorbax Eclipse XDB, Agilent) stationary phases, were tested using gradient elution. The composition of the mobile phase consisted of several ratios of deionized water: organic solvent (acetonitrile or methanol), with percentage of organic solvent ranging from 0 to 100%. An important improvement in terms of analytical performance was achieved on C_8 column, especially regarding separation between an intense interference peak and acrylamide, as can be seen in the Fig. 1A. Also, acceptable retention time (t_R = 2.5 min) was reached for acrylamide taking into account the void volume of the column. The minimum acceptable t_R for an analyte is two-fold the retention time corresponding to the void volume of column [31].

The gradient elution that started with high percentage of water (95%) and acetonitrile (5%) resulted in better peak shape for all analytes and it was more sensible for MS detection. On the other hand, the addition of higher volumes of formic acid in the mobile phase, between 0.05 and 0.1%, provided a significant reduction in the acrylamide signal, when compared to 0.025 and 0.01%. Therefore, minimum formic acid volume (0.01%) was added in both mobile phases. This same phenomenon was related by Mastovska and Lehotay [32] with addition of 0.1% formic or acetic acid in the mobile phase during analyses of acrylamide in cereal, chocolate, coffee and peanut butter. Effects of flow rate (0.1–0.5 mL min^{-1}), column temperature (20–40°C) and injection volume (5–20 µL) on the analytical performance were also examined; and reproducible retention time and sufficient MS sensitivity were achieved with 20 µL of injection volume and a flow rate of 0.2 mL min^{-1} with C_8 column maintained at 30°C.

3.1.2. Sample preparation

Regarding the sample preparation strategy adopted, our main goal was to obtain a simplified pre-treatment with low residue generation that effectively removes matrix co-extractives and ensures sufficient analytical sensitivity and adequate recoveries for all compounds. Additionally, the presence of a co-extractive with same precursor-to-product ion transition as acrylamide (*m/z* 72 > 55) contributed to achieve a selective sample preparation method prior to LC-MS/MS analysis.

Taking into account the high solubility of the acrylamide in water (2155 g L^{-1} at 30°C)

[1], in our first trials, this solvent was preferably employed for the extraction of analytes. However, a dirtier yellowish extract was obtained for all types of samples analyzed, when compared to acetonitrile or methanol extracts. An advantage associated with use of acetonitrile is very little extraction of common matrix components such as fats and proteins [33], furthermore, this solvent contributes to proteins precipitation [21], factors particularly important for analyses of milk-based baby foods. Thus, acetonitrile was chosen as extraction solvent.

Dispersive solid-phase extraction (d-SPE) using primary secondary amine (PSA) sorbent was carried out in the acetonitrile extract obtained. Therefore, 50 mg of PSA were used to each 1 mL of extract, resulting in a cleaner solution than the crude extract. Due to high chelating ability, PSA removes mainly free fatty acids and polar matrix components such as sugars, organic acids and some pigments [34], contributing to lower insertion of co-extractives into LC-MS/MS system, which could adversely affect the sensibility and reproducibility of analytical method. Nonetheless, d-SPE did not avoid the presence of intense matrix interference within the acrylamide retention window (Fig. 1B). Additionally, the use of triple quadrupole mass analyzer was not efficient to overcome this inconvenient because acrylamide and the matrix interference present the same precursor-to-product ion transition (*m/z* 72 > 55) [20, 24]. According to literature, this matrix interference is the amino acid valine, which was firstly identified in Turkish foods [35], and potato chips and cereal-based foods [20] by using retention time and MS spectra of analytical standards and, years later, confirmed by Bermudo et al. [24] in typical Spanish products using a time-of-flight MS analyzer.

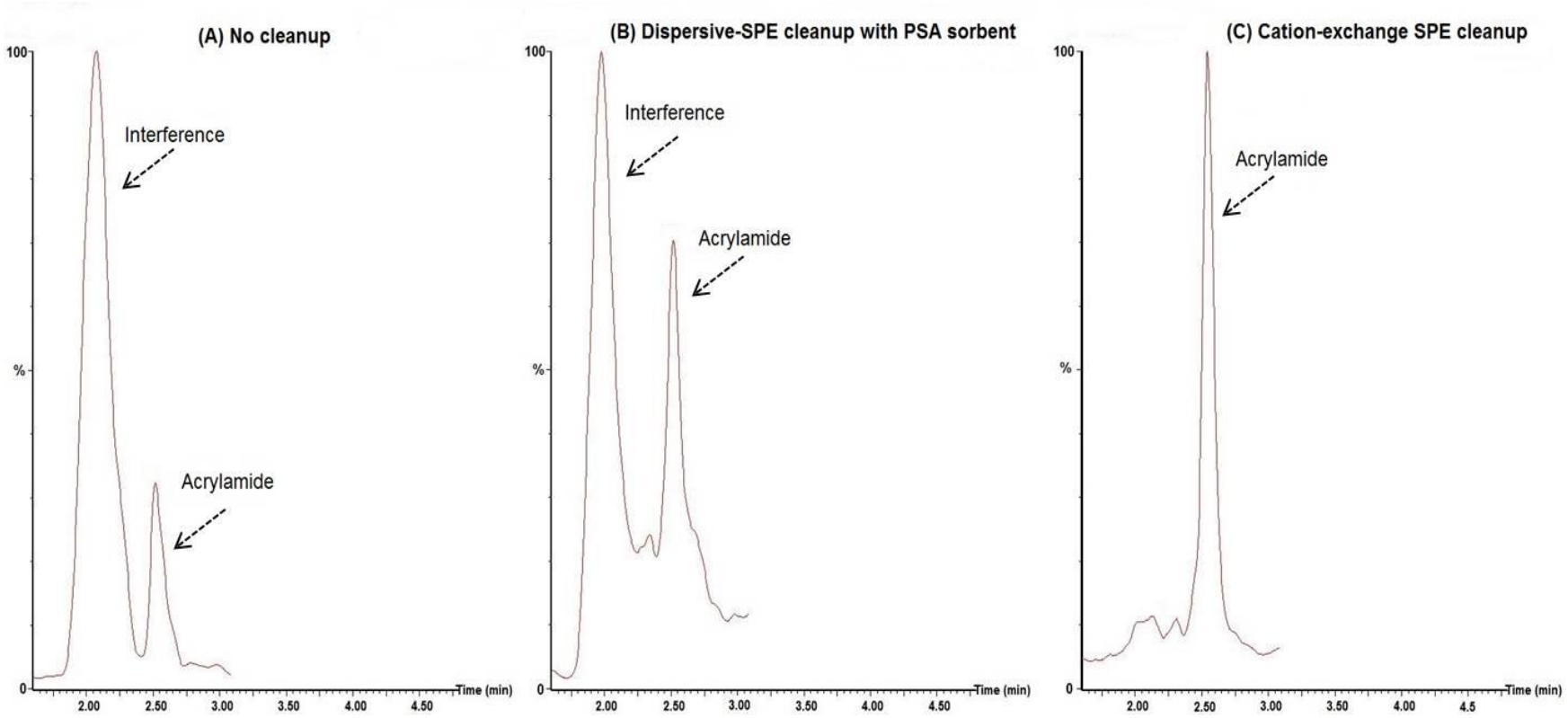


Fig. 1. Total ion chromatograms (TIC) of a representative baby food sample (mixture of banana purée, rice flour and starch) spiked at 200µg kg^{-1}. (A) Crude acetonitrile extract; (B) extract obtained after dispersive-SPE cleanup using PSA sorbent; and (C) extract obtained after cation-exchange SPE cleanup.

Notwithstanding the complete chromatographic separation between the matrix interference and acrylamide (Fig. 1A), the intensity of this interfering peak adversely affected the quantification of acrylamide at low levels. As a compromise between MS sensitivity and free-interference analysis, solid-phase extraction (SPE) was tested in order to remove this co-extractive. Therefore, the obtained acetonitrile extract was evaporated to 2 mL under a stream of nitrogen and 1 mL of 0.01% formic acid was added with the aim to attain positively charged amino acid molecules in solution. Then, the acidified extract was passed through a Bond Elut SCX (100 mg) SPE cartridge, containing a cation-exchanger benzenesulfonic acid-based polymeric sorbent, resulting in the retention of matrix interference without affecting the recovery of acrylamide as can be seen in the Fig. 1C. This same strategy was adopted by Şenyuva and Gökmen [20] during analysis of acrylamide in potato and cereal-based foods, where Oasis MCX SPE cartridges (30 mg; Waters) were employed.

3.2. Method validation

The performance characteristics of proposed method were established by in-house validation, including selectivity, sensitivity (limits of detection and quantification), linearity, matrix effect, recovery and precision, under repeatability and within-laboratory reproducibility conditions. A blank baby food, sample free of target compounds, composed by fruit purée (banana), rice flour and starch, was employed as a representative matrix for validation procedure as also to obtain the matrix-matched calibration curves for quantitative analysis.

Analytical selectivity was verified based on SRM chromatograms of extracts obtained from different samples, including fruit-, cereal- and milk-based baby foods, demonstrating the ability of the method to accurately quantify the target compounds in the presence of other matrix components [36] (Fig. 2).

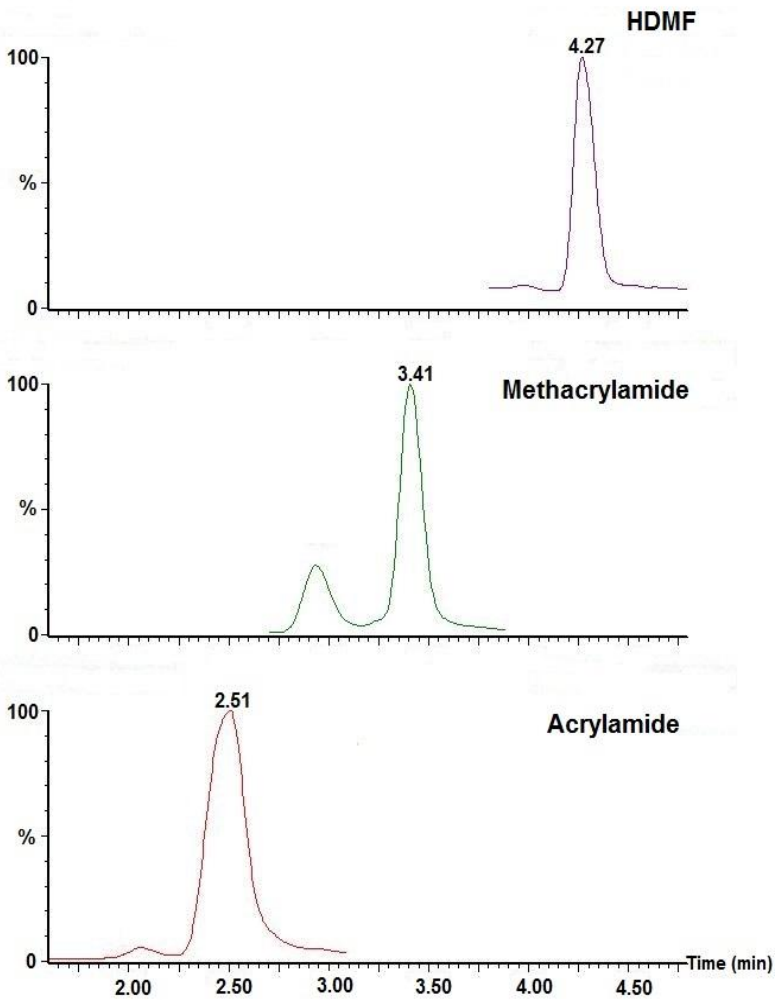


Fig. 2. Total ion chromatograms (TIC) of a representative baby food sample (composed by banana purée, rice flour and starch) spiked with acrylamide and HDMF at 50 µg kg^{-1} and methacrylamide (IS) at 20 µg kg^{-1}.

Limits of detection (LOD) and quantification (LOQ) were established by successive analysis of blank baby food extracts spiked with decreasing amounts of acrylamide and HDMF until obtaining the signal-to-noise ratios of 3:1 and 10:1, respectively. At 10 µg kg^{-1}, acrylamide and HDMF were reliably detected but not necessarily quantified, thus representing the LOD of method. On the other hand, both compounds were detected and quantified in baby foods spiked at 20 µg kg^{-1}, with acceptable mean recoveries (107% for acrylamide and 97% for HDMF) and precision (coefficients of variation ≤ 3%) (Table 2); therefore, this level was set as LOQ, the lowest level that allowed unequivocal identification and quantification of the compounds. Similar LOQ was reported for acrylamide in different food matrices using LC-

MS system [22,35,37,38]. Additionally, the obtained LOQ complies with the Commission Recommendation 2010/307/EU that establishes a LOQ of 30 μg kg^{-1} for analytical methods designed for the monitoring of acrylamide in foods intended for infants and young children [39].

Linearity was assessed in solvent and matrix-matched calibration curves, which included 7 concentration levels each. To obtain matrix-matched calibrations, appropriate volumes of working standard solutions were added to blank baby food extracts to obtain 10, 50, 100, 150, 200, 250 and 300 μg kg^{-1} of standard equivalent in the sample, and for solvent calibrations, the same levels were prepared in 2 mL of acetonitrile: water containing 0.01% formic acid (1:1, v/v). Adequate linearity was verified for acrylamide and HDMF, in solvent and matrix-matched calibrations, with determination coefficients (R^2) ≥ 0.9934 (Table 2). From obtained slopes, matrix effects (ME) were calculated using the equation: ME (%) = [(matrix slope – solvent slope)/ solvent slope] x 100, which no internal standard was used in order to avoid misleading results [40]. Suppression of analytical signal was observed for acrylamide (–48%) and HDMF (–11%). Several factors may have contributed to these matrix-induced effects, including the nature of analyte, the kind of matrix, and the sample preparation applied. Matrix-matched calibrations were used to compensate the effect of co-extracted matrix components in electrospray ionization, which were constructed by plotting the analyte/IS peak area ratio against the concentration levels.

The extraction efficiency and precision were assessed by recovery experiments because no certified reference materials are available for acrylamide and HDMF in baby foods. For this, blank baby foods were spiked with working standard solutions to yield concentrations equivalent to 1-, 5- and 10-fold the LOQ of the method. Before the extraction procedure, these fortified samples were allowed to stand for 1 h for better interaction between the analytes and matrix. A total of six independent replicates at each level were analyzed and mean recoveries varied between 107 and 110% for acrylamide, and from 94 to 100% for HDMF (Table 2). According to requirements for quantitative methods of analysis set by the Commission Decision 2002/657/EC, recoveries within the range 80–110% are acceptable for samples spiked at levels ≥ 10 μg kg^{-1} [31]. Under the repeatability conditions, the precision was calculated from three independent replicates of spiked samples at each level analyzed on the same day by the same analyst under the same chromatographic conditions, with coefficient of variation (CV) values ranging from 3 to 8 % for acrylamide, and between 1 and 5% for HDMF (Table 2). Under the within-laboratory reproducibility conditions, the precision was obtained from three independent replicates of spiked samples analyzed on two

different days by the same analyst under the same chromatographic conditions, totaling six independent replicates for each level, whose CV values varied from 3 to 10% for acrylamide, and between 3 and 10% for HDMF (Table 2). All these values not exceed the CV recommended for 100 µg kg^{-1} (22.6%) and 200 µg kg^{-1} (20.4%) levels, which were calculated by using the Horwitz equation: $CV = 2^{(1 - 0.5 \log C)}$, where C is the studied analyte concentration [31]. In addition, for levels lower than 100 µg kg^{-1}, the CV values shall be as low as possible, while, under repeatability conditions, the values shall be between one-half and two-thirds of the recommended CV [31].

Table 2. Method validation parameters for the determination of acrylamide and HDMF in baby food.

Parameters	Acrylamide	HDMF
LOD (µg kg^{-1})	10	10
LOQ (µg kg^{-1})	20	20
Linearity (10–300 µg kg^{-1}, R^2)		
Solvent calibration	0.9934	0.9975
Matrix-matched calibration	0.9957	0.9984
Matrix effect (%)	–48	–11
Recovery (%, *n = 6*)		
20 µg kg^{-1}	107	97
100 µg kg^{-1}	110	94
200 µg kg^{-1}	107	100
Precision (CV %)	*Intra-day, n = 3 (Inter-day, n = 6)*	
20 µg kg^{-1}	3 (3)	1 (3)
100 µg kg^{-1}	8 (10)	5 (8)
200 µg kg^{-1}	8 (9)	4 (10)

LOD: limit of detection; LOQ: limit of quantification; R^2: coefficient of determination; CV: coefficient of variation.

3.3. Estimation of measurement uncertainty

The expanded measurement uncertainty (U) was estimated at level of 20 µg kg^{-1}, set as the LOQ of the method. A coverage factor (k) of 2 was applied to calculate the expanded uncertainty, which is often used for approximately normally distributed data and it yields an expanded uncertainty that can be employed to construct a 95% coverage interval [41]. As

indicated in the Eq. (1), four individual contributions were used to estimate the expanded uncertainty, which are summarized in the Table 3.

Table 3. Estimation of expanded measurement uncertainty (%) for acrylamide and HDMF in baby food at a 20 µg kg^{-1} level.

Terms	Acrylamide	HDMF
u_S	8	3
u_{SD}	7	7
u_{RV}	0.8	0.9
u_{corr}	7	3
U	25.5	16.5

u_S: uncertainty of (im)precision of the measurement in terms of repeatability; u_{SD}: uncertainty of (im)precision of the measurement in terms of reproducibility; u_{RV}: uncertainty estimate for the reference value used; u_{corr}: uncertainty of the corrected analyte concentration; *U:* expanded measurement uncertainty.

The uncertainty associated with the (im)precision of the measurement in terms of repeatability (u_s) was calculated from standard deviation (*SDr*) obtained from three independent replicates of spiked baby foods (n = 3), which were analyzed on same day by the same analyst under the same chromatographic conditions, as follows: $u_s = SD_r/\sqrt{n}$. While, the uncertainty associated with the (im)precision of the measurements in terms of reproducibility (u_{SD}) was obtained from standard deviation of replicate measurements (SD_R) carried out on two different days by the same analyst under the same chromatographic conditions, totaling 6 independent replicates (N = 6), using the equation: $u_{SD} = SD_R/\sqrt{N}$.

The uncertainty for the reference value used (u_{RV}), namely, the analyte concentration in the spiked sample, was estimated from the equation: $u_{RV} = u_{assoc}/k$. For this, the uncertainty related with the preparation of the standard solution (u_{assoc}) was obtained by combining the uncertainties associated with the standard mass (u_m), the dilution volume (u_{Vdil}) and the standard purity (u_P) as indicated in the Eq. (2). Furthermore, the resolution (10^{-4} g) of the analytical balance (Sartorius CP 225D) used, sensibility derive (10^{-6} g) and the uncertainty obtained on the calibration of the balance (10^{-5} g) were taken into account in this uncertainty estimation, as detailed by Díaz, Vàzquez, Ventura and Galceran [42].

$$u_{assoc} = \sqrt{u_m^2\left(\frac{P}{V_{dil}}\right)^2 + u_{V_{dil}}^2\left(m\frac{P}{V_{dil}^2}\right)^2 + u_P^2\left(\frac{m}{V_{dil}}\right)^2} \quad (2)$$

Finally, the uncertainty of corrected analyte concentration (u_{corr}), which is associated with the recovery, was obtained by difference between the spiked concentration and the average concentration obtained from reproducibility experiments carried out on two different days.

The resulting expanded measurement uncertainty (U) was 25.5% for acrylamide and 16.5% for HDMF. The largest contribution to the final expanded measurement uncertainty was for that associated with the (im)precision of the measurements in terms of repeatability (u_S) for acrylamide and in terms of reproducibility (u_{SD}) for HDMF (Table 3). Additionally, the obtained U values were lower than the maximum standard uncertainty (Uf) of 32% estimated at 20 µg kg^{-1} level by using the Eq. (3) [43], confirming the reliability of the validated method.

$$Uf = \sqrt{\left(\frac{LOD}{2}\right)^2 + (\alpha \times C)^2} \quad \text{Eq. (3)}$$

where Uf is the maximum standard uncertainty (µg kg^{-1}), LOD is the limit of detection of the method (10 µg kg^{-1}), α is a constant (0.2) depending on the value of C, and C is the concentration of interest (20 µg kg^{-1}).

3.4. Analysis of commercial baby food samples

The applicability of developed method was verified by analyzing 15 samples of the most popular baby food brands available in Brazilian markets. Table 4 presents the levels of acrylamide and HDMF in the baby food samples, which are reported as mean (n = 3) ± expanded measurement uncertainty (U). Acrylamide was detected in one baby food at a level of 35 µg kg^{-1}, which was intentioned for infant consumption over six months old. Results from the European acrylamide monitoring, including twenty-two Member States and Norway, indicated mean values of acrylamide in jarred baby foods between 22 and 44 µg kg^{-1} in the year 2007, from 16 to 35 µg kg^{-1} in 2008, and between 32 and 47 µg kg^{-1} in 2009 [44]. A mean level of acrylamide of 55 µg kg^{-1} was also reported in jarred baby foods commercialized

in Poland [9].

On the other hand, HDMF was present in 67% of samples analyzed with mean levels ranging from 25 to 262 μg kg^{-1}. In the literature, high HDMF levels have been reported in fruits such as strawberry (20.9 mg kg^{-1}), mango (2 mg kg^{-1}) and pineapple (0.7 mg kg^{-1}) [45]. Although such investigation was not the major aim of this work, this is the first report on the occurrence of HDMF in baby food. Therefore, future studies, including samples of other foodstuffs, are needed to assess the infant dietary exposure to HDMF.

Table 4. Acrylamide and HDMF levels in commercial baby foods.

Baby food [a]	Mean [b] ± *U* (μg kg^{-1})	
	Acrylamide	HDMF
Apple *Brand A*	n.d.	83.9 ± 13.4
Apple *Brand B*	n.d.	121.1 ± 19.4
Apple *Brand C*	n.d.	64.7 ± 10.4
Apple *Brand D*	n.d.	262.4 ± 41.9
Apple, papaya and orange	n.d.	201.9 ± 32.3
Apple, peach and pear	n.d.	n.d.
Banana *Brand A*	n.d.	31.4 ± 5.0
Banana *Brand B*	n.d.	n.d.
Banana and apple	n.d.	166.9 ± 26.7
Banana and oat	n.d.	96.8 ± 15.5
Banana, orange and pineapple	n.d.	n.d.
Orange and papaya	n.d.	212.2 ± 33.9
Pear and yogurt	n.d.	n.d.
Plum *Brand A*	35.1 ± 8.8	25.1 ± 4.0
Plum *Brand B*	n.d.	n.d.

[a] Principal ingredients; [b] $n = 3$; *U*: expanded measurement uncertainty; n.d.: not detected.

4. Conclusions

Simultaneous liquid chromatography-tandem mass spectrometry analysis of acrylamide and 4-hydroxy-2,5-dimethyl-3(2H)-furanone is described for first time. Acetonitrile-based extraction combined with dispersive PSA cleanup and cation-exchange SPE achieves efficient removal of matrix interferences, without affecting the accuracy and sensibility of the final method. The proposed method provides other benefits such as small amount of sample, reduced reagent consumption, and consequently low residue generation.

The analytical figures of merit were adequate and the developed method meets the criteria specified by the Commission Decision 2002/657/EC for quantitative analysis, as well as the Commission Recommendation 2010/307/EU for the monitoring of acrylamide in foods. In addition, expanded measurement uncertainty, estimated from recovery and precision experiments, was lower than the maximum acceptable uncertainty for both compounds. The detection of acrylamide and HDMF in commercial baby foods demonstrates the suitability of the proposed method for routine analysis.

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CAPÍTULO 6 - 5-Hydroxymethylfurfural in baby food: Validation of dilute-and-shoot liquid chromatography method and estimation of dietary intake

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Abstract

A simple and environmental-friendly method is described for the determination of 5-hydroxymethylfurfural (HMF) in baby food by liquid chromatography with diode array detection. The developed method includes acetonitrile-based extraction followed by dilution of the extract with water in order to reduce the matrix effect. The fitness-for-purpose of proposed method was verified by in-house validation procedure, including analytical selectivity and high sensitivity, with limits of detection and quantification of 0.02 and 0.04 mg kg^{-1}, respectively. Linearity was achieved for solvent and matrix-matched calibration curves in the range from 0.04 to 4 mg kg^{-1}. Adequate mean recoveries of 102, 98 and 101% were obtained at spiked levels of 0.04, 2 and 4 mg kg^{-1}, respectively, with RSD values between 1 and 4%, under repeatability and within-laboratory reproducibility conditions. The validated method was applied to twenty-five commercial baby foods and HMF was detected in all samples analyzed with mean levels varying from 2.3 to 195.4 mg kg^{-1}, demonstrating its suitability for routine analysis. For the first time, HMF dietary exposure was evaluated through fruit-based baby food consumption in Brazil, with daily intake estimations between 30 and 2800 μg $kg^{-1}$of body weight for infants aged 6–11 months.

Keywords: 5-Hydroxymethylfurfural; Baby food; LC-DAD; Food processing contaminant; Dietary intake estimation.

1. Introduction

Food processing has been employed mainly to ensure microbiological and chemical safety as well as to improve the palatability of foods [1]. Moreover, heating treatment contributes to desired sensory qualities as aroma, taste, texture and color; however, it can result in the formation of new compounds of relevant health impact, such as the neo-formed contaminants (NFC) [1, 2]. 5-Hydroxymethylfurfural (HMF), a heterocyclic aldehyde, is one of the most important heat-induced contaminant occurring in carbohydrate-rich products [2]. This furanic compound is an intermediate product in the Maillard reaction; it is also formed from dehydration of reducing sugars under acidic conditions, via 1,2 enolisation and cyclization reactions, whose formation in foods depends on the temperature, type of sugar, pH, water activity and other factors [2, 3]. In addition, HMF is arising from degradation of hexoses and has been reported as one of the products of decomposition of the ascorbic acid. Therefore, the HMF levels detected in certain foods have been employed as an indicator of deterioration, severity of thermal processing and storage conditions [2], with maximum HMF levels established for honey per example [4].

HMF has been found in several foodstuffs including bakery products, breakfast cereals, dried fruits, fruit juices and jam [2], with levels can reach to 3251.3 mg L^{-1} in balsamic vinegar [5], being bread and coffee the major contributors to HMF dietary intake [2]. Data on infant dietary exposure to HMF are scarce in the literature. A mean intake of 5.08 mg HMF per day was estimated for Spanish adolescents as measured in the whole diet after 24 h recall, with cereal and related products being the most important contributing food items [6]. Rufián-Henares and de la Cueva described a dietary intake of 9.7 mg HMF per day (median value) for the Spanish population [7]. In addition, a daily intake of 5.6 mg (mean value) and 27.6 mg (95^{th} percentile) was estimated for HMF taking into account 53 Norwegian volunteers after a 24 h recall [8].

The toxicological relevance of HMF has been associated to its metabolite 5-sulfooxymethylfurfural (SMF) [9, 10]. Studies *in vitro* and *in vivo* have demonstrated HMF biotransformation to SMF through sulfonation of their allylic hydroxyl group by sulfotransferases (SULT) resulting in a highly reactive intermediate that can interact with cellular macromolecules such as DNA, RNA and proteins, promoting structural damages and genotoxic and mutagenic effects [9-13]. Furthermore, HMF has been shown to be bioactivated *in vitro* to 5-chloromethylfurfural (5-CMF) in the presence of chloride ions of gastric juice, a compound that has demonstrated to be more mutagenic than SMF in bacterial test systems [9, 10]. Additionally, animal toxicological studies have associated HMF with

induction and promotion of colon and skin cancers as well as with nephrotoxicity [12].

High performance liquid chromatography (HPLC) has been the analytical technique commonly employed for the determination of HMF in foodstuffs, although capillary zone electrophoresis has also been reported [14]. Furthermore, mass spectrometry with electrospray or atmospheric-pressure chemical ionization, or diode array detector (DAD), has been the main detection systems used [3, 14-16]. Gas chromatography coupled to mass spectrometry has also been reported for HMF analysis after derivatization reaction with N,O-*bis*-trimethylsilyltrifluoroacetamide (BSTFA) [17]. In the literature, the sample pre-treatments applied for HMF analyses involves analyte extraction with polar organic solvents, such as acidified aqueous solutions (acetic acid, oxalic acid or trichloroacetic acid), and subsequent purification of the extract with Carrez solutions (potassium hexacyanoferrate and zinc acetate solutions) and/or solid-phase extraction (SPE) in cartridges [18-20]. Sample hydrolysis step have also been employed during analysis of certain food matrices (Table 1).

Currently the sample preparation methods have been highly focused in the reduction of organic solvents and toxic reagents, low cost, simplicity of execution and prevention of residue generation [21]. In this context, dilute-and-shoot procedure, which involves dilution of aqueous matrix or extract containing the interest analytes and subsequent LC analysis, has attracted interest due to its easy application and high sample throughput [22, 23]. Dilute-and-shoot LC methods have mainly been employed for toxicological analysis including urine analysis for doping control [22]. Aditionally, some authors have successfully applied this sample preparation approach for the determination of mycotoxins in food matrices [23-25]. In the present work, a simple and environmental-friendly sample preparation method based on dilute-and-shoot approach was developed and validated for the determination of HMF in baby foods using HPLC with diode array detection. The proposed method was applied to commercial baby food samples, contributing to first data on HMF in Brazilian baby foods. Furthermore, HMF dietary intake was estimated for infants aged 6–11 months through fruit-based baby food consumption.

Table 1. Comparison of analytical methods for HMF analysis in foods intended for infant consumption and the detected HMF levels.

Sample	Sample preparation	Quantification system	Total (positive)	Range (mg kg^{-1})	Reference
Fruit-based baby food	Extraction with water followed by extract cleanup with Carrez solutions.	HPLC-DAD set at 283 nm	18 (15)	0.3 – 8	[29]
Fruit-based baby food	Extraction with water followed by extract cleanup with Carrez solutions.	HPLC-DAD set at 283 nm	10 (10)	1 – 65	[30]
Milk- and cereal-based baby food	Extraction with Carrez solution plus 0.2 mM acetic acid, and cleanup with Oasis HLB SPE cartridges.	HPLC-DAD set at 285 nm and mass spectrometry	16 (15)	0.17 – 57.2	[19]
Cereal-based baby food	Extraction with 1% trichloroacetic acid.	HPLC-DAD set at 284 nm	4 (3)	0.26 – 9.62	[14]
Milk powder-based baby formulae	Extraction with 0.01 mol L^{-1} NaOH and Carrez solutions, followed by dispersive liquid-liquid microextraction with ethanol and 1-octanol.	HPLC-DAD set at 284 nm	5 (5)	0.017 – 15.7	[16]
Infant formulae	Extraction with 0.01 mol L^{-1} NaOH and Carrez solutions.	HPLC-DAD set at 284 nm	3 (3)	1 – 3.5	[34]
Infant formulae	Extraction with Carrez solutions.	HPLC-DAD set at 284 nm	11 (9)	1.25 – 23	[35]
Infant formulae	Extraction with 0.15 M oxalic acid and trichloroacetic acid.	HPLC-DAD set at 284 nm	2 (2)	0.38 – 6.2	[36]
Infant formulae	Extraction with 0.15 M oxalic acid and trichloroacetic acid.	HPLC-DAD set at 284 nm	2 (2)	0.62 – 11.3	[37]

2. Material and methods

2.1. Chemicals and standard solutions

Analytical standard of 5-hydroxymethylfurfural was purchased from Carbosynth Ltd., UK (98% of purity). A stock solution was prepared by dissolving 0.017 g of standard in 10 mL of deionized water (1710 μg mL^{-1}). Working standard solutions were prepared by diluting the stock solution with deionized water to obtain concentrations of 10 and 100 μg mL^{-1}. All standard solutions were stored at 4°C and protected from light. Acetonitrile HPLC grade was acquired from J.T. Baker. And, deionized water was obtained from Milli-Q system (Millipore). PVDF syringe filters (0.45 μm pore size, 13 mm i.d.) were supplied by Millipore.

2.2. Sampling

Samples of ready-to-eat baby foods, which included fruit purées, cereal flour, starch, milk or other dairy milk ingredient in their composition, were purchased in the city of Campinas, located in the South-Eastern region of Brazil, between January and February 2016. A total of twenty-five samples were randomly collected from 3 supermarkets and all they were maintained in their original packing, glass jars (120 g each) or plastic bags (113 g), at room temperature until analysis.

2.3. Determination of HMF in baby foods

2.3.1. Dilute-and-shoot sample preparation method

Five grams of homogenized baby food were weighed into a 50 mL polypropylene centrifuge tube and 5 mL of acetonitrile were added. The mixture was vortexed by 2 min, followed by centrifugation at 5000 rpm for 15 min. The supernatant was transferred to a volumetric glass flask and then the volume was completed to 100 mL with deionized water. Prior to LC-DAD analysis, the extracts were filtrated through a 0.45 μm PVDF syringe filter.

2.3.2. Liquid chromatography analysis

An Agilent 1260 Infinity Quaternary liquid chromatography system with diode array detector (DAD) was employed for the analyses. The chromatographic separation was achieved on a reserved phase column (250 mm x 4.6 mm i.d., 5 μm particle size; Vydac C_{18}) that was maintained at 25°C. The mobile phase consisted of deionized water (A) and acetonitrile (B), and the elution gradient employed was as follow: 0–6 min, 5% B; 6.1–9 min, 100% B; 9.1–14 min, 5% B, at a flow rate of 1 mL min^{-1}, resulting in a total run time of 14

min. The DAD was set at wavelength of 284 nm, and the injection volume was between 1 and 100 μL according to HMF concentration of the extract.

3. Results and discussion

3.1. Method performance characteristics

A simple and high sample throughput pre-treatment method was proposed for the determination of HMF in baby food. The method involves only 5 mL of extracting solvent and easy execution, which includes vortexing-based extraction followed by centrifugation, and subsequent dilution of the extract with deionized water in order to minimize the matrix effect. Compared to other methods for HMF analysis in foodstuffs (Table 1), the proposed method avoids time-consuming solid-phase extraction (SPE) in cartridges and Carrez solutions, contributing to lower final cost and residue generation. Acetonitrile recognized by little extraction of proteins and lipids from food matrices was used as extracting solvent [26] focusing minimal matrix components extraction and no further cleanup step. In addition, acetonitrile resulted in a less colored yellowish extract compared to aqueous extract, demonstrating lower co-extractives content.

A homemade baby food composed by fruits (apple, banana, orange and papaya) and milk, which was not submitted to heating, was employed as a representative blank baby food (matrix free of HMF) for validation procedure as well as to obtain the matrix-matched calibration curves for quantitative analysis. Analytical selectivity was observed for HMF in all types of baby food analyzed. On the basis of chromatograms of extracts obtained from blank baby food, samples spiked with HMF standard solution and baby foods naturally contaminated with HMF, no interference peaks were observed in retention time of the HMF (4.9 min) allowing an unequivocal identification of the analyte and easy manual integration of chromatographic peak (Fig. 1).

High analytical sensibility was achieved using the proposed method with limits of detection (LOD) and of quantification (LOQ) of 0.02 mg kg^{-1} and 0.04 mg kg^{-1} (Table 2), respectively, demonstrating that the dilution step used in the sample preparation did not affect the detection of HMF at low levels. These limits were established by successive chromatographic analysis of blank baby food extracts spiked with decreasing concentrations of standard until obtaining the signal-to-noise ratios of 3:1 and 10:1, respectively. A LOD of 0.067 mg kg^{-1} was reported for HMF in infant formulae, while values of 0.157 mg kg^{-1} in honey, 0.276 mg kg^{-1} in breakfast cereals, 0.141 mg kg^{-1} in orange juice, 0.235 mg kg^{-1} in

biscuits and 0.216 mg kg^{-1} in jam were obtained by Teixidó et al. [3] using liquid chromatography coupled to mass spectrometry system.

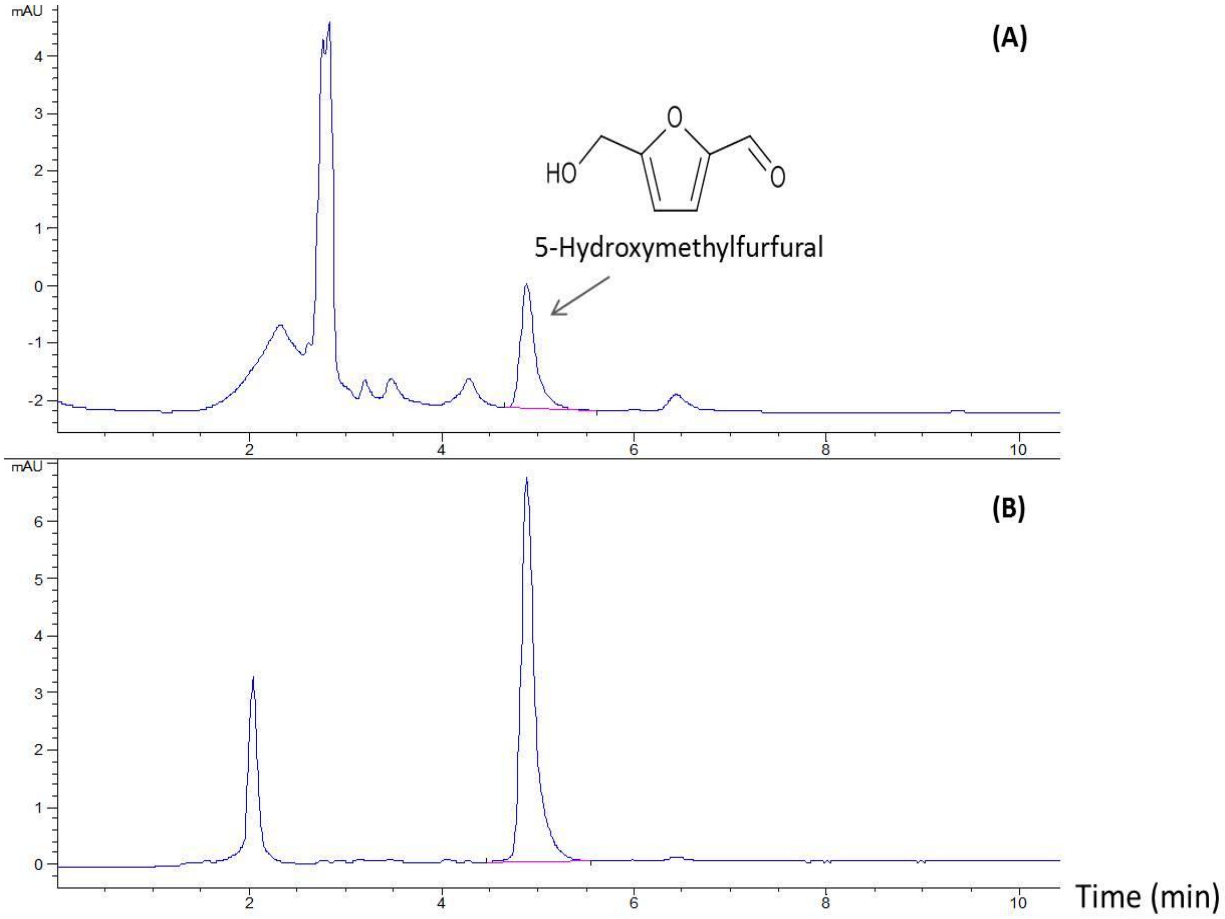

Fig.1. HPLC-DAD chromatograms of extracts obtained from (**A**) apple and yogurt-based baby food naturally contaminated with HMF at level of 3.5 mg kg^{-1} and (**B**) plum-based baby food naturally contaminated with HMF at level of 203 mg kg^{-1}.

Table 2. Performance characteristics of developed method for HMF analysis in baby food.

Parameters	HMF
LOD (mg kg^{-1})	0.02
LOQ (mg kg^{-1})	0.04
Linearity (0.04–4 mg kg^{-1}, R^2)	
Solvent	0.9979
Matrix	0.9981
Recovery (%, *n = 10*)	
0.04 mg kg^{-1}	102
2 mg kg^{-1}	98
4 mg kg^{-1}	101
Precision (RSD %)	*Intra-day, n = 5 (Inter-day, n = 10)*
0.04 mg kg^{-1}	4 (4)
2 mg kg^{-1}	3 (2)
4 mg kg^{-1}	2 (1)

LOD: limit of detection; LOQ: limit of quantification; R^2: coefficient of determination; RSD: relative standard deviation.

Linearity was evaluated in solvent and matrix-matched calibrations curves, which included seven points each with concentration levels ranging from 0.04 (LOQ) to 4 mg kg^{-1}. Adequate linearity was verified for solvent and matrix-based calibrations with coefficients of determination (R^2) exceeding 0.997 in all cases (Table 2). In addition, analysis of variance (ANOVA) demonstrated high significance of the regression ($p < 0.01$) and no significance of the lack of fit ($p > 0.999$) in the studied concentration range for solvent and matrix-matched calibration curves (Table 3). The slopes obtained for calibration curves of same concentration in solvent (deionized water) and matrix extracts (blank homemade baby food composed by apple, banana, orange, papaya and milk, did not submit to thermal treatment) were employed to estimate the matrix-induced effects using the equation: Matrix Effect (%) = [(matrix slope – solvent slope)/ solvent slope] x100. A low signal suppression (ME = –2%) was observed, indicating a negligible effect of the matrix on LC-DAD analysis. However, matrix-matched calibrations were employed for the HMF quantification in baby food samples.

Table 3. Analysis of variance (ANOVA) for solvent and matrix-matched calibration curves.

	Sum of squares	Degree of freedom	Mean square	*F* test [a]	*p* value
Solvent calibration					
Regression	42607.78	1	42607.78	6842.728	0.000000
Lack of fit	0.22	4	0.005	0.009	0.999829
Pure error	87.17	14	6.23		
Total	42695.32	20			
Matrix-matched calibration					
Regression	40819.07	1	40819.07	7982.816	0.000000
Lack of fit	0.4	4	0.1	0.02	0.999145
Pure error	71.59	14	5.11		
Total	40891.48	20			

[a] $F_{0.05;\ 1;14}$ (*F* tabulated) = 4.6

The efficiency of extraction of the developed method was verified by recovery experiments. For this, a representative blank baby food was spiked with working standard solution at levels of 0.04 (LOQ), 2 and 4 mg kg^{-1}. Before the extraction procedure, this mixture was allowed to stand for 1 h for proper interaction between the analyte and matrix. A total of ten independent replicates were analyzed at each spiked level on two different days. As a great result, mean recoveries between 98 and 102% were obtained for HMF (Table 2). According to requirements for quantitative methods of analysis stated by the Commission Decision 2002/657/EC, recoveries within the range 80–110% are acceptable for samples spiked at levels ≥ 10 μg kg^{-1} [27]. The precision, expressed in terms of relative standard deviations (RSD %), was evaluated under repeatability and within-laboratory reproducibility conditions. A total of five independent replicates at each spiked level was analyzed on the same day by the same analyst under the same chromatographic conditions, with RSD values ranging from 2 to 4% (Table 2). Under within-laboratory reproducibility conditions, a total of five independent replicates was analyzed on two different days by the same analyst under the same chromatographic conditions, totaling ten replicates for each spiked level, whose RSD values varied between 1 and 4% (Table 2), which did not distinguish those obtained under repeatability conditions, demonstrating the reliability of the proposed method.

3.2. Occurrence of HMF in commercial baby foods

The proposed sample preparation method was applied to twenty-five commercial samples of the most popular brands of baby food available in Brazilian market; HMF was detected in all analyzed samples, demonstrating its suitability for routine analysis. A wide variation in the mean content of HMF between 2.3 to 195.4 mg kg^{-1} was observed, with highest levels detected in plum-based baby foods (Table 4). In a study with dried fruits, the highest HMF levels were detected in plum (1600–2200 mg kg^{-1}) when compared to apple (80 mg kg^{-1}), pear (100 mg kg^{-1}) and pineapple (280 mg kg^{-1}) [28]. Also, high HMF level has been reported in plum-based jams (1200 mg kg^{-1}) [28].

HMF has also been reported in fruit-based baby foods commercialized in Spain. Levels between 0.3 and 8 mg kg^{-1} were observed in samples collected in Madrid [29]; additionally, higher levels, between 1 and 65 mg kg^{-1}, were related in other study with fruit-based baby food also commercialized in Madrid [30]. In addition, Gökmen and Senyuva (2006) described contents varying from < 1 to 57 mg kg^{-1} in samples of milk- and cereal-based baby foods [19]. Levels between 0.26 and 9.62 mg kg^{-1} were also reported by Bignardi, Cavazza and Corradini in cereal-based baby foods [14]. Regarding the occurrence of HMF in other foods intended for infant consumption, contents between 0.017 and 15.7 mg kg^{-1} have been detected in infant formulae [16, 34-37] (Table 1).

Table 4. HMF content in fruit-based baby foods commercialized in the Brazil.

Baby Food [a]	Mean (mg kg^{-1}) [b]
Apple *Brand A*	7.2 ± 0.2
Apple *Brand B*	4.2 ± 0.1
Apple *Brand C*	6.5 ± 0.1
Apple *Brand D*	13.6 ± 0.2
Apple, guava and banana	3.5 ± 0.01
Apple, papaya and orange	7.7 ± 0.2
Apple, pear and peach	12.8 ± 0.1
Apple and yogurt	3.6 ± 0.1
Banana *Brand A*	2.8 ± 0.2
Banana *Brand B*	9.6 ± 0.3
Banana and apple	5.5 ± 0.1
Banana and oat	2.4 ± 0.1
Banana and yogurt	2.8 ± 0.04
Banana, apple, mango and orange	6.3 ± 0.02
Banana, mango, orange and pineapple	3.8 ± 0.1
Banana, pineaple and orange *Brand A*	2.5 ± 0.2
Banana, pineaple and orange *Brand B*	2.3 ± 0.1
Orange and papaya	5.9 ± 0.2
Pear and mango	17.1 ± 0.3
Pear and yogurt	16.4 ± 0.3
Plum *Brand A*	37.4 ± 3.6
Plum *Brand B*	195.4 ± 11
Yogurt and apple	2.5 ± 0.04
Yogurt, banana, orange and pineapple	3.3 ± 0.1
Yogurt, apple, banana, papaya and strawberry	2.8 ± 0.2

[a] Principal ingredients; [b] $n = 3$.

3.3. Estimation of HMF dietary intake

In order to assess dietary exposure to HMF, the daily intake was estimated for infants aged 6–11 months taking into account only the contribution of fruit-based baby foods in their diet. Although data on the consumption of baby foods by infants and young children are limited in Brazil, in the present work an ingestion rate of 120 g per day was assumed on the basis of recommendations of the Brazilian Society of Pediatrics for fruit-based baby foods for infants aged 6–11 months [31]. A mean body weight (bw) of 8.4 kg was employed for dietary intake estimation [32].

Figure 2 shows the estimation of HMF daily intake by the point estimate approach at five different scenarios. Considering the minimum, mean and median experimental levels for HMF, intake ≤ 200 µg kg^{-1} bw day^{-1} was achieved through consumption of fruit-based baby foods. Nonetheless, maximum dietary exposures were estimated to be 2800 µg kg^{-1} bw day^{-1}, considering the most contaminated sample, and 2100 µg kg^{-1} bw day^{-1} considering 95th percentile, which represent the daily intake for high consumers taking into account the consumption of highly contaminated fruit-based baby foods. To our best knowledge, this is the first estimation of HMF dietary intake for infants. The European Food Safety Authority (EFSA) has established an acceptable daily intake (ADI) of 500 µg kg^{-1} bw for furfural, however, an ADI value was not stated for HMF. A threshold of concern of 540 µg per person per day for HMF was established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) from data on subchronic and chronic animal studies.On the other hand, a tolerable daily intake (TDI) of 132 mg per person per day was suggested by Zaitzev, Simonyan and Pozdnyakov by employing a 40-fold margin of safety [33].

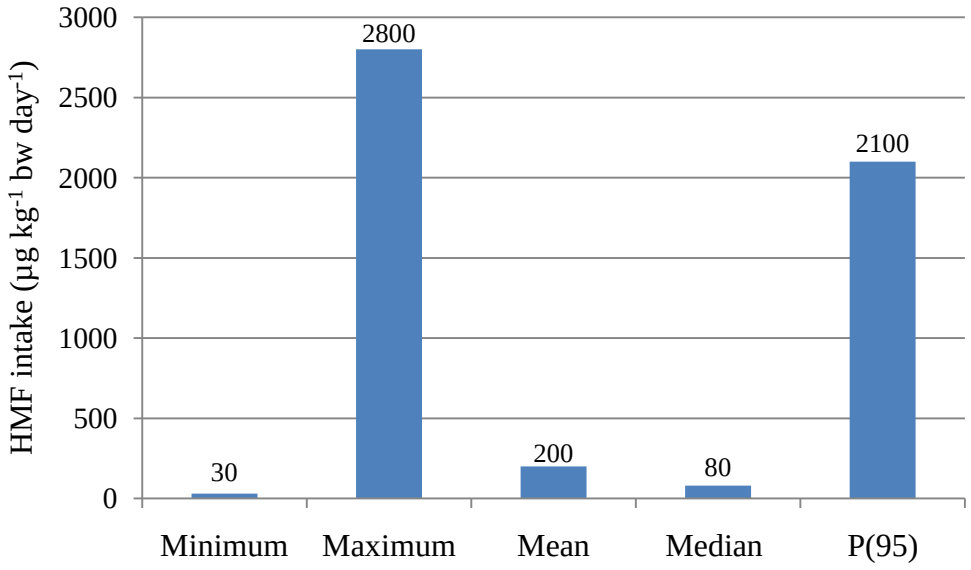

Fig. 2. Estimation of HMF dietary intake through fruit-based baby food consumption for infants aged 6–11 months.

Although HMF is commonly present in processed carbohydrate-rich foods and maximum limits have not been established for HMF in foodstuffs, except for honey as a quality indicator, the presence of HMF in baby foods raises great concern due to its conversion to 5-sulfooxymethylfurfural (SMF). The EFSA Scientific Panel on Food Additives, Flavourings, Processings Aids and Materials in contact with Foods (AFC) concluded that there is sufficient evidence about genotoxic potential of SMF, based on the mutagenic activity *in vitro* [33]. SMF was also detected in blood of mice after HMF intravenous administration [13], demonstrating HMF biotransformation to SMF *in vivo.* Additionally, children's metabolic pathways are not fully mature, especially in the first month after birth, therefore, detoxification and excretion process are less efficient than in adults; moreover, the high food consumption rate per kg of body weight contributes to higher susceptibility of this consumer group to food contaminants.

4. Conclusions

An environmental-friendly and simple sample preparation method has been proposed for the determination of HMF in baby food using LC-DAD. Analytical selectivity, high sensibility and adequate recoveries and precision, under repeatability and within-laboratory reproducibility conditions, were achieved for HMF analysis in fruit-based baby food. For the first time, HMF was investigated in baby foods available in Brazilian markets, whose mean levels varied between 2.3 and 195.4 mg kg^{-1}. Furthermore, dietary exposure to HMF through fruit-based baby food consumption was estimated for infants aged 6–11 months, with daily intake between 30 and 2800 µg of HMF per kg^{-1} of body weight, depending of the scenario evaluated. Due to the few data available about HMF in fruit-based baby foods, this report could subsidize further risk management as well as serve as basis for mitigation strategies to reduce HMF content in baby foods.

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CAPÍTULO 7 - Simplified determination of phthalates in baby food combining acetonitrile-based extraction with low-temperature cleanup and gas chromatography–mass spectrometry analysis

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Abstract

A simple, easy and cost-effective sample preparation method has been introduced for the determination of seven phthalates in baby food by gas chromatography–mass spectrometry (GC-MS). The developed method includes acetonitrile-based extraction followed by addition of salts sodium chloride and magnesium sulfate in order to induce phase separation, and then low-temperature cleanup at –18°C. As a great result, reduction of more of 50% in matrix co-extractives content was verified in the final extract by gravimetric measurements, when compared to crude baby food extract. Adequate performance characteristics were achieved including analytical selectivity, high sensibility with LOQs between 1 and 10 μg kg^{-1}, linearity in solvent and matrix-matched calibration curves, good recoveries (73–110%) and precision (RSD ≤ 16%), under repeatability and within-laboratory reproducibility conditions. The validated method was applied to twenty commercial fruit-based baby foods and the compounds BBP, DBP, DEHP, DEP and DnOP were detected in some samples with levels varying from 1.4 to 90.3 μg kg^{-1}, confirming the suitability of the proposed method for routine analysis.

Keywords: Phthalates; Baby Food; Low-temperature cleanup; Gas chromatography–mass spectrometry; Food contaminant.

1. Introduction

Phthalates, diesters of 1,2-benzenedicarboxylic acid, constitute a group of anthropogenic compounds, which are chemically inert and present high density, low to medium volatility, and high solubility in organic solvents. These compounds have been widely used as plasticizers for polymers to increase the flexibility and toughness of plastic materials such as polyvinyl chloride (PVC), being bis(2-ethylhexyl) phthalate (DEHP) the main representative for this application. Other phthalates, lower-molecular-weight diesters, including diethyl phthalate (DEP), dibuthyl phthalate (DBP) and benzylbuthyl phthalate (BBP), have been used as solvent to hold color and scent in several personal care products. Furthermore, phthalates have been employed in adhesives, glues and paints, as also in printing inks and lacquers to improve surface adhesion, flexibility and wrinkle resistance [1,2]. Nonetheless, the phthalates are structurally uncovalently bound in the products; consequently, these compounds continuously are released and leach out from the products to the environment [3].

Dietary intake has been the major human exposure route to phthalates; others such as inhalation, dermal and parental have also contributed to phthalates exposure [4]. In foods, the occurrence of phthalates is arising from processing, migration of packaging materials or environmental sources [2]. Adittionally, fatty foods have been predominantly contaminated with these compounds due to lipophilic characteristic of phthalates [2]. Several studies have investigated the presence of phthalates in different food matrices, and DEHP has been the most frequently compound analyzed, followed by DBP and BBP [2]. For instance, Fierens et al. (2012) reported the occurrence of phthalates in a wide variety of foodstuffs available on the Belgian market, including beverages, milk and dairies, sauces and condiments, fruits and vegetables, meat and fish-based products, oils and fats, cereals and bakery products; being the highest levels found for the compounds DEHP, DBP, BBP and diisobutyl phthalate (DiBP) [5]. Additionally, DEHP has been the most often phthalate detected in foods [2]. However, few works have been focused in the determination of phthalates in foods intended for infants [5,6].

According to International Agency for Research on Cancer (IARC), DEHP has been classified under group 2B as a possibly carcinogenic to humans [7]. Several adverse effects to human health have been associated with exposure to phthalates and its metabolites, especially in endocrine and male reproductive systems [8,9]. Particularly regarding children health, allergic diseases including asthma and eczema has been associated to phthalates exposure [10,11]. Additionally, the European Food Safety Authority (EFSA) has established a tolerably

daily intake (TDI) of 0.01 mg kg^{-1} of body weight (bw) for DBP [12], 0.05 mg kg^{-1} bw for DEHP [13], 0.5 mg kg^{-1} bw for BBP [14], and 0.15 mg kg^{-1} bw for diisodecyl phthalate (DIDP) and diisononyl phthalate (DINP) [15,16]. In addition, the European Commission has established specifications for materials intended to come into contact with food [17].

Gas chromatography (GC) coupled to mass spectrometry with electron ionization and single quadrupole mass analyzer has been the main analytical technique employed for the determination of phthalates in food matrices; being chromatographic columns of low polarity stationary phase such as 5% phenyl methylpolysiloxane commonly employed [2]. GC–tandem mass spectrometry (MS/MS) has also been described for phthalate analysis in fatty matrices [18] and bottled water [19]. However, flame ionization or electron capture detection has been less frequently used. On the other hand, high performance liquid chromatography, coupled to ultra-violet detector or MS/MS, has also been reported for phthalate analysis [2]. Regarding sample preparation methods applied to phthalates analysis, liquid-liquid partitioning with non-polar solvents such as chloroform, n-hexane, n-hepatne or isooctane has been used for the extraction of phthalates from non-fatty liquid matrices such as water, soft drinks and alcoholic beverages; while acetonitrile or mixtures of acetonitrile and water for non-fatty solid matrices. Particularly for fatty solid matrices, acetonitrile has achieved selective extraction of these compounds due to low solubility of fat in this organic solvent [2]. In addition, solid-phase extraction (SPE) and gel permeation chromatography (GPC) techniques have been employed for cleanup purpose [2].

In present work, the occurrence of seven phthalates including DEP, DBP, DiBP, DEHP, BBP, dimethyl phthalate (DMP) and di-n-octyl phthalate (DnOP) was investigated in baby foods available commercially in Brazilian markets. For this, a simple and cost-effective sample preparation method based on acetonitrile extraction and subsequent low-temperature cleanup at –18ºC was developed and in-house validated using gas chromatography–mass spectrometry.

2. Material and methods

2.1. Standards and chemicals

Analytical standards of phthalates and the internal standards (IS) DBP-d4 and DEHP-d4 were acquired from Sigma-Aldrich. Stock standard solutions were prepared in hexane at 5 µg mL^{-1}, while the IS solutions were prepared at 1 µg mL^{-1}. A multi-compound working solution was prepared in hexane at 0.5 µg mL^{-1} by combining appropriate aliquots of individual stock solutions, and all solutions were stored at –18°C. Acetonitrile HPLC grade

was purchased from Linchrosolv, and hexane from Sigma. Sodium chloride (NaCl) was supplied by Synth, and anhydrous magnesium sulfate ($MgSO_4$) was acquired from J.T. Baker.

2.2. Samples of baby foods

Commercial samples of fruit-based baby foods were purchased in the city of Campinas, SP, located in the South-eastern region of Brazil, between January and February 2016. A total of twenty samples was randomly collected from three supermarkets. All samples were kept in their original packaging, glass jar (120 g each) or plastic bag (113 g each), at room temperature until analysis.

2.3. Determination of phthalates

2.3.1. Sample preparation

Five grams of homogenized baby food sample and 10 mL of acetonitrile were added to a 50 mL glass centrifuge tube and the mixture was vortexed for 1 min. Then, 1 g of NaCl and 4 g of $MgSO_4$ were added to mixture and the tube was again vortexed for 1 min, and centrifuged at 3000 rpm for 15 min. After centrifugation step, the tube was maintained at –18°C for 3 h, and then the supernatant was collected for subsequent GC-MS analysis.

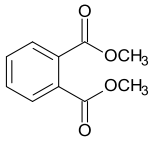
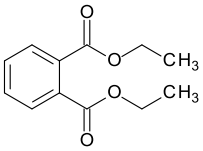
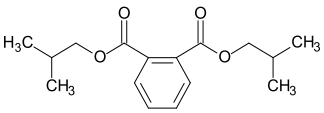
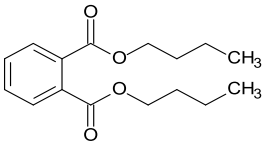
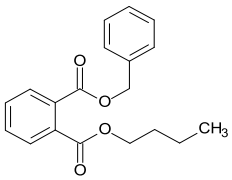
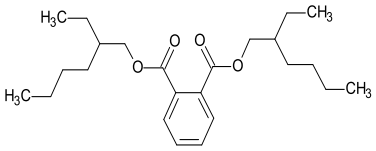
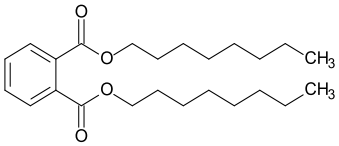
2.3.2. Gas chromatographic and mass spectrometric conditions

A 7890A gas chromatography system interfaced to a single quadrupole inert mass selective detector (5975C) with electron ionization (EI) source (Agilent Technologies) was employed for phthalates analysis. Agilent ChemStation platform was used for qualitative and quantitative data analysis. GC separation was achieved on a ZB-5HT Inferno capillary column (20 m x 0.18 mm x 0.18 µm; Zebron, Phenomenex), which was maintained initially at 50°C for 1 min, increased to 320°C at 15°C min^{-1}, then ramped at 5°C min^{-1} to 350°C and held for 5 min, resulting a total run of 30 min. A solvent delay of 3 min was applied. The injector was maintained at 320°C and 1 µL of extract was injected in splitless mode (purge flow of 100 mL min^{-1} at 1 min). Ultra-high purity helium (99.9999 %) was employed as carrier gas at constant flow of 1 mL min^{-1}. The electron energy was 70 eV, transfer line was maintained at 280°C, EI source at 230°C, while quadrupole mass analyzer at 150°C.

2.3.3. Identification and quantification

Individual phthalate standard solutions, all prepared in hexane at 5 μg mL^{-1}, were analyzed in full-scan mode from *m/z* 50 to 500 in order to establish the retention time and characteristic spectrum of each analyte. One target ion and another qualifying ion were selected for each phthalate, based on their abundance and specificity (Table 1). Data acquisition was divided in 6 different ion groups in selective ion monitoring (SIM) mode, and the dwell times (50–100 ms) were set depending on the number of ions per group. The identification of the compounds was established by the retention time of target ion equal to that obtained in calibration standards, as well as by the qualifier to target ion ratios. The internal standards (IS) DBP-d4 (*m/z* 153, t_R = 12.5 min) and DEHP-d4 (*m/z* 153, t_R = 15.9 min) were added to sample at level of 5 μg kg^{-1} prior to the extraction procedure, and the phthalates were quantified employing six-point matrix-matched calibration curves constructed by plotting analyte/IS peak area ratio against concentration levels.

Table 1. Phthalates evaluated in the present study, their molecular weight (MW), retention time (t_R) and ions monitored for quantification and confirmation purposes.

Analyte (abbreviation)	Chemical structure	MW (g mol^{-1})	t_R (min)	Target ion (*m/z*)	Qualifying ion (*m/z*)
Dimethyl phthalate (DMP)		194.18	8.8	163	194
Diethyl phthalate (DEP)		222.24	9.9	149	177
Diisobutyl phthalate (DiBP)		278.34	11.9	149	223
Dibutyl phthalate (DBP)		278.34	12.5	149	223
Benzylbutyl phthalate (BBP)		312.36	14.9	149	206
Bis(2-ethylhexyl) phthalate (DEHP)		390.56	15.9	149	167
Di-n-octyl phthalate (DnOP)		390.56	16.8	149	279

3. Results and discussion

3.1. Sample preparation

The ubiquity of phthalates represents the main difficulty to the development of methods for quantitative analysis of these compounds in food matrices. Their occurrence in laboratory environment can lead to contamination of the materials as well as of the investigated samples. Reagents, plastic materials and equipment (particularly injector parts of GC system such as syringe, septum and insert) are potential sources of phthalates. Therefore, blank values (obtained from unspiked representative baby food sample plus reagents) were monitored daily for phthalate contamination at the beginning of GC-MS analysis, whose

levels were constant during our method development and below the concentration of the lowest calibration curve level (Table 2). Blank correction was applied for quantitative analysis.

In order to maintain minimal phthalate contamination during analytical procedure, acetonitrile acquired from three manufacturing companies (J.T. Baker, Panreac and Linchrosolv) was checked regarding phthalates level before use as extracting solvent. In addition, the salts NaCl and $MgSO_4$ used in the extraction step were muffled at 500°C for at least 5 h and stored in glass flasks. Plastic materials such as polypropylene centrifuge tubes were avoided, thus entire sample preparation procedure was carried out using glass flasks, which were rinsed out with hexane followed by heating at 500°C for at least 5 h. Moreover, the plastic screw cap of glass flasks and chromatography vials were wrapped with aluminum foil in order to prevent contact of reagents and sample extract with polypropylene material. Also, before use, all disposable points of micropipette were immersed in a glass flask containing 50% ethanol solution and submitted to 45°C for at least 12 h, followed by drying at 45°C, focusing remove free phthalates.

In our method development the main goal was to obtain a simple and easy sample preparation method for accurate quantification of target phthalates with minimal generation of residues. Taking into account the lipophilic character of phthalates and the complexity of baby food matrices, acetonitrile was chosen as extracting solvent. Acetonitrile has extracted less matrix components as proteins and lipids [20] when compared to ethyl acetate, acetone and other non-polar solvents. Due to high mean moisture content of the analyzed baby food samples (84 ± 2%, n = 10), the salts NaCl and $MgSO_4$ were employed in order to induce phase separation and force the phthalates into acetonitrile phase, on the basis of QuEChERS procedure [21]. After, shaking and centrifugation steps, the tubes were maintained at –18°C for 3 h in order to freeze fats.

The baby foods analyzed presented in their composition milk or other dairy ingredients, cereal flours, starch, besides fruits purée, potential sources of analytical interferences for GC-MS analysis. Low-temperature cleanup (LTC) has been reported as an attractive cleanup procedure for analysis of different food matrices [22-24]. Although this procedure is time-consuming, several benefits have been associated with LTC such as simplicity of execution and high sample throughput. Furthermore, this cleanup procedure avoids additional organic solvents and solid phase extraction (SPE) cartridges or dispersive-SPE sorbents, which makes it less costly and environmental-friendly. Particularly for phthalates analysis, this cleanup procedure reduces the chances of contamination by

phthalates of the extracts due to little manipulation of sample without addition of extra solvents and reagents; as well as it contributes to easy collection of extract for subsequent GC-MS analysis.

The efficiency of the cleanup procedure for removal of matrix co-extractives was assessed by gravimetric measurements. Therefore, a representative baby food, composed by a mixture of fruit purées (apple, banana, papaya and strawberry), rice flour, starch and heavy cream, was extracted according to proposed sample preparation procedure and then, the obtained extracts (duplicate) were submitted to LTC. After 3h at –18°C, 7 mL of acetonitrile extracts were transferred to pre-weighed glass tubes, which were previously heated at 110°C for at least 1 h to eliminate the moisture, and these extracts were evaporated to dryness under nitrogen stream. Then, the glass tubes containing the dried residues were again heated at 110° for at least 1 h and weighed (Fig. 1). Matrix co-extractives removal efficiency was estimated as difference in weight of co-extractives before and after LTC employing the equation: [(co-extractives weight before LTC – co-extractives weight after LTC)/ co-extractives weight before LTC] x 100 [25]. As a good result, this cleanup procedure provided a removal of 52% in the co-extractives content compared to crude acetonitrile extract without cleanup (Fig. 1), contributing to lower insertion of matrix components into GC-MS system which could affect the long-term system performance during analysis and the ruggedness of the method.

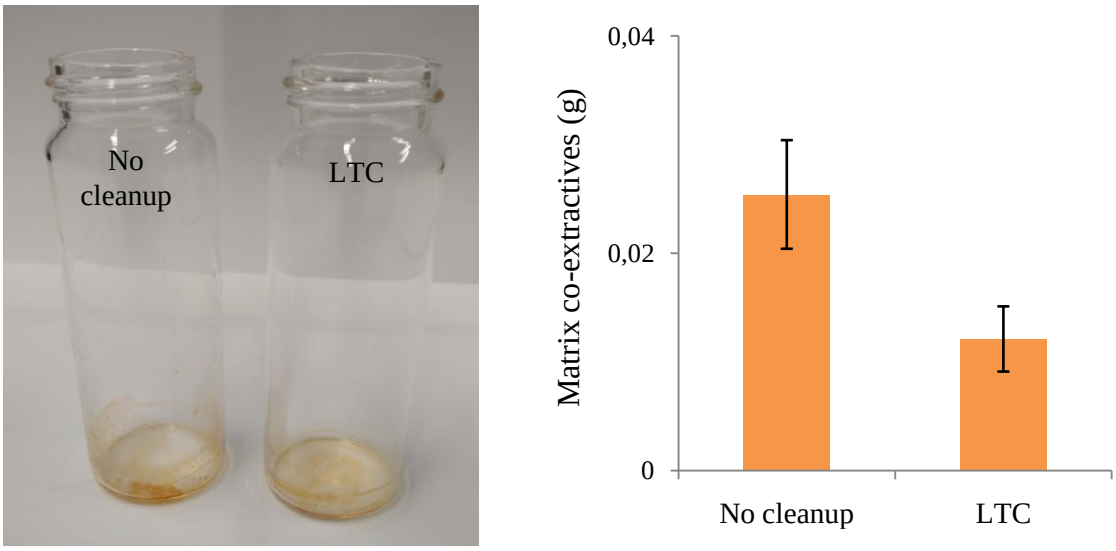


Fig. 1. Evaluation of matrix co-extractives removal efficiency using low-temperature cleanup (LTC) (*n* = 2).

3.2. Method validation

The performance characteristics of the developed method were established by in-house validation procedure including analytical selectivity, sensibility (limits of detection and quantification), linearity, recovery and precision, under repeatability and within-laboratory reproducibility conditions. A representative baby food – composed by a mixture of fruit purées (apple, banana, papaya and strawberry), rice flour, starch and heavy cream, was used for recovery and precision experiments as also to obtain the matrix-matched calibration curves for quantitative analysis.

Analytical selectivity was verified on the basis of total ion chromatograms (TIC) obtained in SIM mode and no interfering peaks were observed in the retention time of each phthalate, demonstrating the ability of proposed method to accurately quantify the target compounds in the presence of other matrix components [26] (Fig. 2).

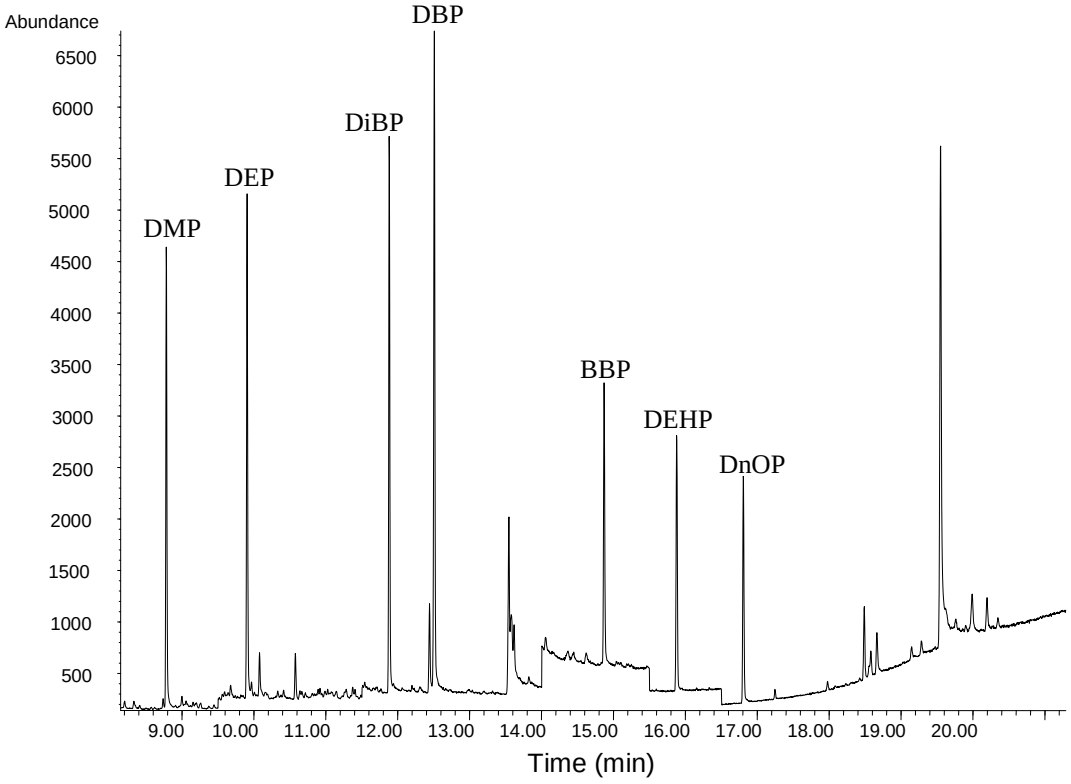


Fig. 2. Total ion chromatogram obtained in selected ion monitoring (SIM) mode of a fruit-based baby food composed by a mixture of fruit purées (apple, banana, papaya and strawberry), rice flour, starch and heavy cream spiked at 50 µg kg^{-1}.

High analytical sensibility was achieved wich the proposed method. The LODs and LOQs were estimated on the basis of mean blank values from analysis of 10 independent replicates of unspiked baby food sample plus three and ten-fold the standard deviation obtained, respectively. Low LODs were obtained using this sample preparation approach, with values ranging between 0.5 and 5 µg kg^{-1}, while the LOQs varied from 1 (DMP and DnOP) to 10µg kg^{-1} (DiBP) (Table 2).

Table 2. Blank values obtained for investigated phthalates and respective limits of detection (LOD) and quantification (LOQ).

	DMP	DEP	DiBP	DBP	BBP	DEHP	DnOP
Mean blank values* (μg kg^{-1})	0.2	1	3	0.7	0.4	2	0.2
LOD[a] (μg kg^{-1})	0.5	1.5	5	1	1	3	0.5
LOQ[b] (μg kg^{-1})	1	3	10	2	2	6	1

*Blank values obtained from 10 independent replicates using an unspiked baby food sample. [a] LOD = mean blank value + 3 x *SD*; [b] LOQ = mean blank value + 10 x *SD*; *SD*: standard deviation.

Linearity was verified in solvent and matrix-matched calibration curves, which included 6 concentration levels each. To obtain matrix-matched calibrations, appropriate volumes of working standard solutions were added to 5 mL of baby food extract (obtained from a representative baby food composed by a mixture of fruit purées – apple, banana, papaya and strawberry, rice flour, starch and heavy cream) to obtain 1–100 μg kg^{-1} of standard equivalent in the sample for DMP and DnOP, 3–100 μg kg^{-1} for BBP, DBP and DEP, and 10–100 μg kg^{-1} for DEHP and DiBP. For solvent standard calibrations, the same concentration levels were prepared in 5 mL of acetonitrile. Adequate linearity was achieved for all analytes with coefficients of determination (R^2) exceeding 0.991 for solvent and matrix-matched calibrations (Table 3). Therefore, matrix-matched calibration curves were employed for quantification of the analytes in the samples as also for recovery and precision estimation, which were constructed by plotting the analyte/IS peak area ratio against the concentration levels.

Efficiency of extraction of the method was evaluated by recovery experiments. A representative baby food was spiked with working standard solution at levels of 10, 25 and 50 μg kg^{-1}. Before the extraction procedure, these fortified samples were allowed to stand for 1 h for better interaction between the analytes and matrix. A total of fifteen independent replicates at each level were analyzed on three days (five replicates per day) resulting in mean recoveries between 79 and 110%, 75 and 110%, and 73 and 108% at 10, 25 and 50 μg kg^{-1}, respectively (Table 3). The precision was expressed in terms of relative standard deviation (RSD). Under repeatability conditions, the precision was estimated from five independents replicates at each level of spiked samples analyzed on the same day by the same analyst under the same chromatographic conditions, with RSD values ranging from 2 to 16%, 1 to 16%, and from 2 to 12% at 10, 25 and 50 μg kg^{-1}, respectively (Table 3). Under within-laboratory reproducibility conditions, the precision was calculated from five replicates of spiked samples

analyzed on three days by the same analyst under the same chromatographic conditions, totaling fifteen independent replicates for each level, with RSD values between 5 and 15%, 10 and 16%, and 5 and 12% at 10, 25 and 50 µg kg^{-1}, respectively (Fig. 3).

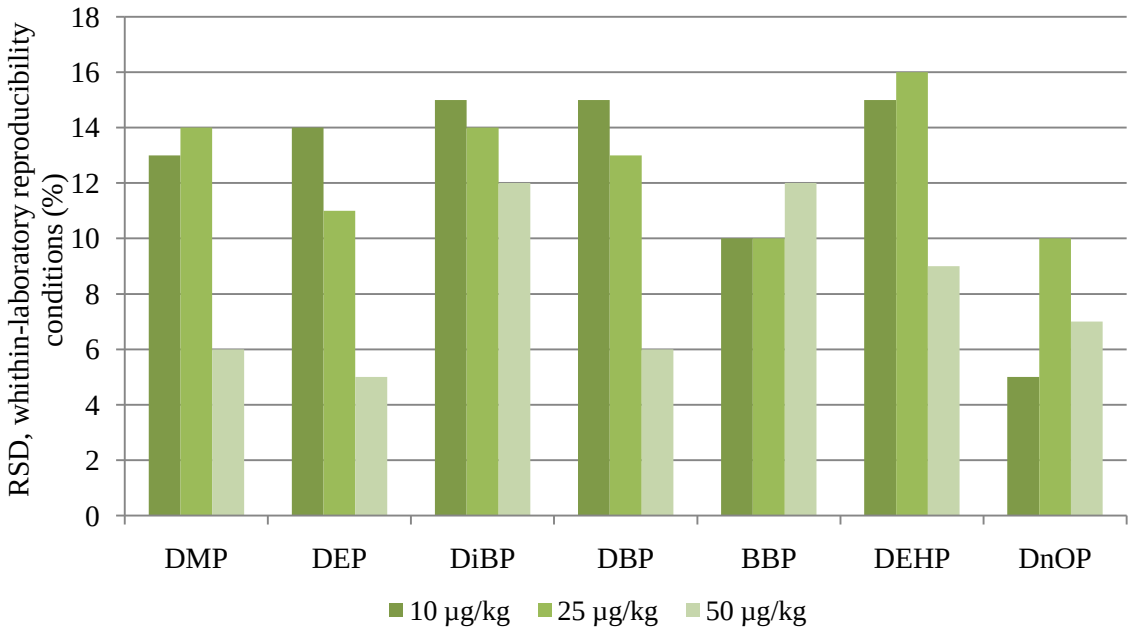


Fig. 3. Relative standard deviation (RSD) estimated from spiked baby food samples analyzed on three days (n = 15).

Table 3. Performance characteristics of developed method for the determination of phthalates in baby food, obtained on three days.

Analyte	Linearity * (R^2)	1st day			2nd day			3th day		
		10 µg kg^{-1}	25 µg kg^{-1}	50 µg kg^{-1}	10 µg kg^{-1}	25 µg kg^{-1}	50 µg kg^{-1}	10 µg kg^{-1}	25 µg kg^{-1}	50 µg kg^{-1}
		%R (RSD$_r$)	%R (RSD$_r$)	%R (RSD$_r$)	%R (RSD$_r$)	%R (RSD$_r$)	%R (RSD$_r$)	%R (RSD$_r$)	%R (RSD$_r$)	%R (RSD$_r$)
DMP	0.9915	86 (11)	98 (7)	105 (3)	88 (13)	90 (4)	104 (5)	105 (4)	110 (3)	102 (6)
DEP	0.9911	82 (12)	90 (2)	104 (3)	79 (16)	91 (5)	99 (5)	98 (8)	109 (1)	106 (5)
DiBP	0.9916	105 (5)	109 (2)	104 (7)	110 (14)	105 (7)	104 (8)	99 (15)	102 (9)	108 (12)
DBP	0.9973	109 (6)	75 (3)	99 (3)	90 (8)	94 (10)	81 (6)	92 (5)	86 (3)	82 (2)
BBP	0.9991	107 (4)	99 (2)	96 (3)	90 (8)	88 (4)	79 (5)	91 (3)	80 (2)	73 (2)
DEHP	0.9946	93 (10)	94 (16)	92 (3)	95 (7)	82 (13)	83 (2)	93 (15)	79 (14)	83 (5)
DnOP	0.9995	101 (5)	96 (15)	101 (4)	103 (7)	92 (8)	89 (3)	107 (2)	101 (5)	101 (3)

*Matrix-matched calibration curves; R^2: coefficient of determination; %R: percentage of recovery; RSD$_r$: relative standard deviation under repeatibility conditions (n = 5).

3.3. Analysis of commercial baby food samples

The occurrence of target phthalates was investigated in twenty ready-to-eat samples of the most popular brands of baby food available in the Brazilian markets. The compounds BBP, DBP, DEHP, DEP and DnOP were detected in some fruit-based baby foods, demonstrating the suitability of the proposed method for routine analysis. Blank-value-corrected-results are reported in the Table 4. DnOP was detected in four baby food samples with levels between 1.4 and 2.5 μg kg^{-1}, while the highest levels detected were of DEHP (48.5 and 90.3 μg kg^{-1}). Additionally, two samples presented more than one compound, namely, DEP and BBP (total of 7.7 μg kg^{-1}), and DEHP and DnOP (total of 92.8 μg kg^{-1}).

Phthalates have also been reported in foods intended for infant consumption (milk powder, soups, vegetable purées and fruit purées) available in Belgium markets during 2009 and 2010, including DMP (not detected – 0.2 μg kg^{-1}), DEP (n.d. – 1.6 μg kg^{-1}), DiBP (0.1 – 16 μg kg^{-1}), BBP (n.d. – 16 μg kg^{-1}), DEHP (n.d. – 67 μg kg^{-1}), DnOP (n.d. – 3 μg kg^{-1}), DBP (0.1 – 32 μg kg^{-1}) and dicyclohexyl phthalate (n.d. – 1.8 μg kg^{-1}), whose highest level was observed for the compound DEHP [5]. Additionally, DBP, BBP and DEHP were quantified in one (40 μg kg^{-1}), one (5 μg kg^{-1}) and two (360 μg kg^{-1}; 630 μg kg^{-1}) baby food samples collect in Danish markets, respectively; while BBP was related in two infant formulae (4 μg kg^{-1}; 10 μg kg^{-1}) and DEHP in other two infant formulae samples (40 μg kg^{-1}; 60 μg kg^{-1}) [6]. Furthermore, a total daily intake of 4.42 μg kg^{-1} bw per day (median value) was estimated from DEHP metabolite levels detected in the urine of Danish children between 3 and 6 years old [27].

Table 4. Occurrence of phthalates in fruit-based baby foods commercialized in the Brazil.

Baby food [a]	Mean value, $n = 3$ (μg kg^{-1})							Σ (μg kg^{-1})
	DMP	DEP	DiBP	DBP	BBP	DEHP	DnOP	
Apple *Brand A*	n.d.	n.d.	n.d.	< 2	n.d.	n.d.	n.d.	---
Apple *Brand B*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Apple *Brand C*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Apple, guava and banana	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Apple, papaya and orange	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Banana	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Banana and apple	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Banana and oat	n.d.	4.4 ± 0.3	n.d.	n.d.	3.3 ± 0.6	n.d.	n.d.	7.7
Banana and yogurt	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5 ± 0.2	1.5
Banana, apple and mango	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.4 ± 0.1	1.4
Banana, mango, orange and pineapple	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Banana, pineaple and orange *Brand A*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Banana, pineaple and orange *Brand B*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Orange and papaya	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Pear and mango	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Plum *Brand A*	n.d.	n.d.	n.d.	n.d.	n.d.	48.5 ± 7.3	n.d.	48.5
Plum *Brand B*	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---
Yogurt and apple	n.d.	n.d.	n.d.	n.d.	< 2	90.3 ± 13.2	2.5 ± 0.3	92.8
Yogurt, banana, orange and pineapple	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.4 ± 0.1	1.4
Yogurt, apple, banana, papaya and strawberry	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	---

[a] Principal ingredients; n.d.: not detected

4. Conclusions

This work reports the successful development of a simple and cost-effective sample preparation method for the determination of seven phthalates in fruit-based baby food. QuEChERS-based extraction combined with low-temperature cleanup provides accurate quantification of target compounds using GC-MS, with acceptable recoveries (73–110%) and precision (RSD ≤ 16%), under repeatability and within-laboratory reproducibility conditions, as well as high analytical sensibility (LOQs between 1 and 10 μg kg^{-1}). Moreover, efficient cleanup of baby food extracts was achieved using this environmental-friendly sample preparation approach, with a reduction of more of 50% in the total matrix co-extractives content. The application of the method to commercial baby food samples and detection of the compounds BBP, DBP, DEP, DEHP and DnOP demonstrate the feasibility of developed method for routine analysis.

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DISCUSSÃO

Atualmente, na análise de alimentos, há um grande interesse por técnicas de preparo de amostra que se destaquem pela simplicidade e rapidez na execução, o consumo mínimo de reagentes e solventes orgânicos, principalmente os de baixa toxicidade, e consequentemente baixo custo e geração de resíduos químicos. Paralelamente, os alimentos infantis representam complexas matrizes para as análises cromatográficas devido aos seus principais ingredientes, os quais são fontes de potenciais interferentes como açúcares, ácidos orgânicos, lipídeos, pigmentos e proteínas. Assim, a escolha da técnica de preparo de amostra representa uma das etapas mais críticas na análise de contaminantes em alimentos infantis, frente aos possíveis interferentes analíticos e os baixos níveis em que estes contaminantes podem estar presentes nesses produtos.

A combinação das técnicas de preparo de amostra QuEChERS (*quick, easy, cheap, effective, rugged and safe*) e microextração líquido-líquido dispersiva (DLLME – *dispersive liquid-liquid microextraction*) mostrou-se promissora para a determinação de resíduos de 24 agrotóxicos pertencentes a 12 classes químicas em alimentos infantis, usando cromatografia a gás acoplada a um analisador de massas quadrupolo. A aplicação da DLLME no extrato obtido com o procedimento QuEChERS contribuiu para alta sensibilidade analítica do método assim permitindo o monitoramento dos analitos no limite máximo de resíduo (LMR) de 10 μg kg^{-1} estabelecido pela Comunidade Europeia para alimentos infantis. Por outro lado, uma eficiente limpeza dos extratos foi obtida com a técnica de extração em fase sólida dispersiva (d-SPE – *dispersive solid-phase extraction*) usando os sorventes amina primária e secundária (PSA – *primary secondary amine*) e C_{18} resultando em um menor efeito de matriz durante as análises cromatográficas.

Até o momento, cromatografia a gás tem sido a principal técnica analítica utilizada para a determinação de resíduos de piretróides e piretrinas em alimentos. Como uma alternativa a esta técnica, um método empregando cromatografia a líquido acoplada ao analisador de massas triplo quadrupolo foi desenvolvido para a análise simultânea das piretrinas naturais e seus análogos sintéticos os piretróides em alimentos infantis. As técnicas de preparo de amostra extração líquido-líquido assistida por *salting out* (SALLE – *salting-out assisted liquid-liquid extraction*) e microextração líquido-líquido dispersiva assistida por ultrassom (UA-DLLME – *ultrasound-assisted dispersive liquid-liquid microextraction*) foram otimizadas resultando métodos de simples execução e pequeno consumo de solventes orgânicos. SALLE envolveu apenas 2 mL de solvente extrator e uma rápida extração sob

agitação e centrifugação, porém alguns analitos apresentaram limites de quantificação maior que o LMR de 10 μg kg^{-1} para resíduos de agrotóxicos em alimentos infantis. Em contrapartida, a aplicação de ultrassom no procedimento DLLME contribuiu para um preparo de amostra mais rápido resultando na dispersão do solvente extrator clorofórmio na fase aquosa contendo os analitos de interesse sem a necessidade da injeção de solvente de dispersão, comumente usado na técnica DLLME. O pequeno volume de solvente extrator utilizado promoveu um enriquecimento dos analitos no extrato final, permitindo a quantificação de piretrinas e piretróides em níveis abaixo do LMR estabelecido para alimentos infantis.

Frente à alta toxicidade dos hidrocarbonetos policíclicos aromáticos (HPAs), um limite máximo de 1 μg kg^{-1} foi estabelecido pela Comunidade Europeia para o composto benzo[a]pireno em alimentos infantis, individualmente ou combinado com criseno, benzo[a]antraceno e benzo[b]fluoranteno. Assim, uma estratégia de preparo de amostra, envolvendo extração com QuEChERS, seguida de limpeza do extrato por d-SPE com os sorventes PSA e C_{18}, e finalmente concentração dos analitos no extrato final usando DLLME, foi proposta visando adequadas recuperações, mínimo efeito de matriz e alta sensibilidade analítica. O uso do delineamento de seleção de variáveis Plackett-Burman permitiu a identificação de potenciais variáveis do método proposto que afetam a recuperação dos HPAs, tais como a quantidade do sorvente C_{18} na etapa de limpeza e o volume do solvente extrator tolueno no procedimento DLLME. Uma promissora inovação neste preparo de amostra foi a combinação do procedimento DLLME com ultra-baixa temperatura (–80°C), a qual contribuiu para uma fácil coleta dos poucos microlitros do solvente extrator utilizado, e a purificação por precipitação de proteínas e congelamento de gorduras presentes no extrato final. Como um bom resultado, baixos limites de detecção e quantificação foram obtidos usando cromatografia a gás acoplada a um analisador de massas quadrupolo.

Purificação do extrato usando baixa temperatura foi também uma eficiente estratégia durante as análises de ftalatos nos alimentos infantis. Uma das maiores dificuldades na determinação destes compostos é a presença de alguns deles no ambiente do laboratório, além disso, reagentes, materiais plásticos e equipamentos são potencias fontes de ftalatos. Após extração com acetonitrila, o extrato foi mantido –18°C por 3 h, resultando em uma significativa redução na quantidade de co-extrativos provenientes da matriz no extrato final, quando comparado ao extrato que não foi submetido à purificação com baixa temperatura, a qual foi determinada gravimetricamente. Assim, evitando o uso de cartuchos de extração em fase sólida (SPE – *solid phase extraction*), sorventes de limpeza e outros solventes orgânicos,

reduzindo as chances de contaminação do extrato final. Usando cromatografia a gás acoplada a espectrometria de massas, adequadas características de desempenho foram obtidas para o método proposto incluindo alta seletividade e sensibilidade analítica.

Outra inovação na determinação de contaminantes em alimentos foi a análise simultânea de acrilamida e 4-hidroxi-2,5-dimetil-3(2H)-furanona. Cromatografia a líquido acoplada ao analisador de massas triplo quadrupolo permitiu adequada quantificação dos compostos a baixos níveis nas amostras de alimentos infantis, entretanto, esta técnica analítica não evitou um intenso interferente analítico próximo ao tempo de retenção da acrilamida, uma vez que este interferente apresentou a mesma transição *m/z* 72 > 55 monitorada para acrilamida. SPE em cartuchos de troca catiônica foi a técnica mais adequada para a remoção deste co-extrativo, a qual não afetou a sensibilidade e recuperação da acrilamida e 4-hidroxi-2,5-dimetil-3(2H)-furanona, e evitou a possibilidade de falsos positivos.

Um método de preparo de amostra bastante simples, econômico e rápido foi desenvolvido para a determinação de 5-hidroximetilfurfural. Alta sensibilidade analítica e seletividade foram obtidas usando cromatografia a líquido com detector de arranjo de diodos, após uma simples extração com acetonitrila e então diluição do extrato com água deionizada para grandes volumes visando reduzir possível efeito de matriz. Outro avanço, foi a otimização de uma metodologia para a determinação das poliaminas espermina e espermidina usando extração por par iônico com o reagente bis(2-etilhexil)fosfato e cromatografia gasosa acoplada a espectrometria de massas. Através de um planejamento fatorial, o tempo de extração e o tempo da reação de derivatização com anidrido heptafluorobutírico foram otimizados para amostras de alimentos infantis, resultando em um método mais rápido comparado ao original. O uso de um forno de micro-ondas convencional reduziu o tempo da reação de derivatização de 60 para 5 minutos, indicando ser uma atrativa alternativa para métodos morosos.

A análise de amostras comerciais de alimentos infantis e a detecção de alguns dos compostos investigados indicam a eficiência dos métodos analíticos desenvolvidos. Ademais, o presente estudo fornece os primeiros dados nacionais sobre acrilamida, ftalatos, 5-hidroximetilfurfural e resíduos de agrotóxicos em amostras de alimentos infantis a base de frutas comercializadas no Brasil. Também, é o primeiro estudo na literatura a relatar os níveis das poliaminas espermidina e espermina, e de 4-hidroxi-2,5-dimetil-3(2H)-furanona em alimentos infantis, tendo uma grande contribuição na área de análise de alimentos.

CONCLUSÃO GERAL

Novos métodos analíticos foram introduzidos para a análise de resíduos e contaminantes em alimentos infantis empregando cromatografia a líquido, cromatografia a gás e espectrometria de massas. Os métodos propostos apresentaram adequadas características de desempenho, incluindo seletividade analítica, linearidade, exatidão e precisão, para a determinação de resíduos de agrotóxicos, piretrinas naturais, hidrocarbonetos policíclicos aromáticos, ftalatos, acrilamida, 4-hidroxi-2,5-dimetil-3(2H)-furanona, 5-hidroximetilfurfural e as poliaminas espermidina e espermina em amostras de alimentos infantis a base de frutas. Alta sensibilidade analítica foi obtida com os métodos desenvolvidos permitindo assim o monitoramento destes contaminantes nos níveis máximos estabelecidos pela Comunidade Europeia para alimentos infantis. Além disso, os métodos de preparo de amostra desenvolvidos apresentam importantes características para análises de rotina, incluindo fácil execução, pequena quantidade de amostra, baixo consumo de solventes e reagentes, e consequentemente menor geração de resíduosquímicos. A aplicação dos métodos em amostras comercias de alimentos a base de frutas destinadas ao público infantil a partir do 6° mês de vida, disponíveis em mercados da cidade de Campinas, SP, Brasil, demonstrou a adequabilidade deles como também revelou a presença de alguns dos compostos investigados. A ocorrência do fungicida procimidona em um nível acima do limite máximo de resíduo demonstra a necessidade de um constante monitoramento de resíduos de agrotóxicos em alimentos infantis, como também de estudos abrangendo um número maior de amostras para a avaliação da exposição dos lactentes e crianças de primeira infância a esses contaminantes.

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ANEXO I

Safren, Susan, Springer US <Susan.Safren@springer.com> 14:04 (Há 21 horas)

para mim

inglês › português Traduzir mensagem Desativar para: inglês

Dear Mateus,

Yes, you have the right to do this.

Sincerely,
Susan

-----Original Message-----
From: SpringerAlerts@springeronline.com [mailto:SpringerAlerts@springeronline.com]
Sent: Monday, May 08, 2017 1:04 PM
To: Safren, Susan, Springer US
Subject: Customer question from website

Dear Susan Safren,

Hope you are doing great!

I am writing to request permission to include my article "Determination of polyamines in baby food by gas chromatography-mass spectrometry: Optimization of extraction and microwave-assisted derivatization using surface response methodology" in full in my thesis for non-commercial purposes.

Kind regards,
Mateus H Petrarca

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Subject: Customer question from website
Sender name: Mateus Henrique Petrarca
Sender email: petrarcamh@gmail.com
Page name: Food Analytical Methods - incl. option to publish open access Page url: http://www.springer.com/food+science/journal/12161