RJC

RASĀYAN *J. Chem.*
Vol. 9 | No. 2 |160 - 168 | April - June | 2016
ISSN: 0974-1496 | e-ISSN: 0976-0083 | CODEN: RJCABP
http://www.rasayanjournal.com
http://www.rasayanjournal.co.in

EVALUATION OF PHTHALATES IN JORDAN DIARY PRODUCTS BY HPLC/MS-MS

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ABSTRACT
Higher-molecular-weight phthalates, such as di-2-ethylhexyl phthalate, are primarily used as plasticizers to soften polyvinyl chloride products, while the lower-molecular-weight phthalates, such as diethyl phthalate, di-n-butyl phthalate, and butyl benzyl phthalate, are widely used as solvents to hold colour and scent in various consumer and personal care products. The average calculated concentration ranges of di-n-butyl phthalate, Di(2-ethylhexyl) phthalates and Di-n-octyl phthalates in all the samples were 1.43-15.8µg/L, 0.0893-58.3µg/L and 0.107-51.5µg/L respectively. Among the analysed samples, Shaneinah Hamoodeh (yougort syrup) has the highest concentration of di-n-butyl phthalate and local cow milk has the lowest concentration. This may be due to nature of the packaging system during manufacturing in the industry. However, local Camel milk has the highest concentration of Di-n-octyl phthalates compared to Cow milk which has the lowest concentration, Values obtained are within acceptable limits.
Keywords: Phthalate, Dairy products, HPLC/MS-MS, Plasticisers, Milk.

INTRODUCTION

Phthalates are a group of di-esters of ortho-phthalic acid (di-alkyl or alkyl aryl esters of 1, 2-benzenedicarboxylic acid). They are a group of chemical compounds widely used in cosmetic products, food packaging materials (e.g. Plastic containers), and polyvinyl chloride plastics. They are used as plasticizers to improve flexibility, pliability and elasticity in a broad range of plastic products. Since these phthalates are not chemically bound in the plastics, so they can be released into the environment or may be exposed to the human via ingestion, inhalation, or dermal routes[1].

Sources of phthalates in milk

PVC- tubing is commonly used in the milking process and in the bulk transfer of milk between tankers and storage tanks in dairy farms and dairy processing plants. Like many other PVC products, plasticizers such as phthalates are used in PVC tubing to make it more flexible, and among which DEHP is the most frequently used with as much as 40% in the tubing[2]. Since they are not chemically bonded to the polymer, plasticizers can migrate from the PVC tubing into milk, especially at relatively higher temperature during the milking process.

Food-packaging films

Thin packaging films, also known as the cling films, are widely used for wrapping a variety of foods. Several types of films are available, such as PVC film, poly vinylidene chloride (PVDC) film, polyethylene (PE) film, regenerated cellulose film (RCF), cellulose acetate film etc. The most commonly used PVC film is plasticized mainly with DEHA, although DEHP is also used in some other countries[3]. Like plasticizers in PVC products, plasticizers in packaging films are also not bonded chemically to the polymer and can migrate when they are in contact with foods, especially fatty foods.
Printing inks plasticizers, such as DBP and DEHP, are part of printing ink formulations (2% to 8%) to improve its adhesion on surfaces and thus its flexibility and wrinkle resistance.[4]

Since ink is printed on the outer surfaces of food packaging materials (film, cardboard, and so on), it will be another source of phthalates in foods in addition to the packaging materials. Other sources include PVC gloves, aluminium foil-paper laminates, and Polyethylene terephthalate (PET) etc.

General structure of phthalates

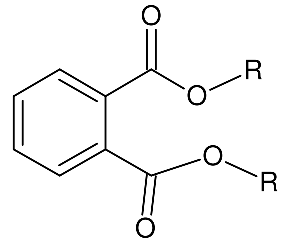
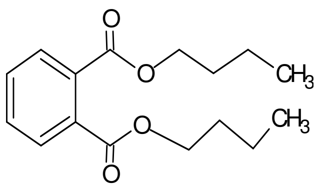
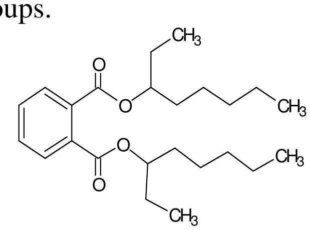


Fig.-1: General structure of PAE.

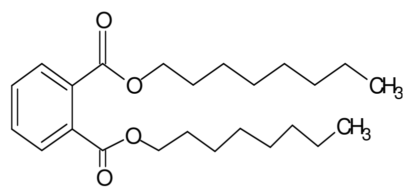
Where, R and R¨ are the same or different alkyl or aryl groups.



(a.) DBP



(b.) DEHP



(c.) DNOP

Fig.-2: Structures of plasticizers.

Due to their lipophilic nature phthalates may accumulate in animal tissues, muscle, fat, and also may pass from the digestive tract to the milk, which leads to another potential threat chain [5].

Although the acute toxicity PAE is relatively low, continuously running production exposes the population to chronic exposure. Chronic exposure of phthalates, especially of di-2-ethylhexyl phthalate, may also have teratogenic and carcinogenic effects (liver cancer cell) and affect the reproductive ability of the body (decreased weight of the testes, ovaries, sperm count)[6]. Phthalates potentially disrupt the human hormonal system, sexual development, reproduction, potentially encourage asthma and skin diseases of young children. Some phthalates are considered developmentally toxic substances harmful to re production[7].

EXPERIMENTAL

Materials and Methods

De-ionized water, Methanol HPLC grade, Ammonium formate, Formic acid, Di-butyl phthalate (DBP), Di(2-ethylhexyl) phthalates (DEHP), Di-n-octyl phthalates (DNOP), 10ml glass centrifuge tube, Sonicator, Vortex mixer, Centrifuge machine, H Volumetric flasks of different volumes, Micropipettes of different sizes (Socorex, Switzerland) were used for solution preparation.

Preparation of standard solutions

10mg of DBP, DEHP and DNOP reference standard were accurately weighed in 100ml volumetric flask, dissolved and diluted to volume with methanol as mixed stock solution. The mixed stock solution was diluted with methanol and the following working standard solutions were obtained: 0.5, 1, 5, 10, 15, 25µg/L.

Sample preparations

A total of seven (7) different Jordanian dairy products were collected randomly from different local shops and supermarket. One fresh milk sample was obtained from two different local shops .The samples of Jordanian dairy products were obtained from Carrefour supermarket located in Irbid City Jordan. These include; raw cow milk, Shaneinah (Teeba), shaneinah (hammoudeh), Yoghurt (Teeba), Juice Milk, Almarai milk and fresh camel milk. The milk samples were stored in freezer until analysis. Milk Serum Extraction, 1g of each milk sample was weighed accurately and transferred into a 50ml volumetric flask, 45ml of methanol was added and sonicated for 30mins and cooled to room temperature. It was then diluted with methanol to volume and mixed with vortex mixer and allowed to stand for several minutes. Two layers were formed; about 5ml of the upper solution was transferred into a 10ml centrifuge tube, and centrifuge at 3500 rpm for 10mins. The supernatant was used as sample solution for LC-MS analysis.

Instrumentation

Phthalic Acid Esters were determined using LC/MS/MS API 3200 model, using multiple reactions monitoring (MRM) method. ACQUITY BEH C18, 1.7µm. 2.1mm i.d. × 100mm, or equivalent product is used as the column, with an injection volume of 10µL. Evaluation was performed using mobile phase of the following compositions: Ammonium formate: water: formic acid (5mM: 1L: 0.1%) and 100% methanol with flow rate of 0.35mL/min.

RESULTS AND DISSCUSSION

LC/MS-MS Results

Plasticizers in dairy products were analyzed using LC/MS/MS method. Mobile phase solution comprises of 5mM Ammonium formate, 0.1% formic acid, 1L H_2O and 100% methanol.

Calibration curves

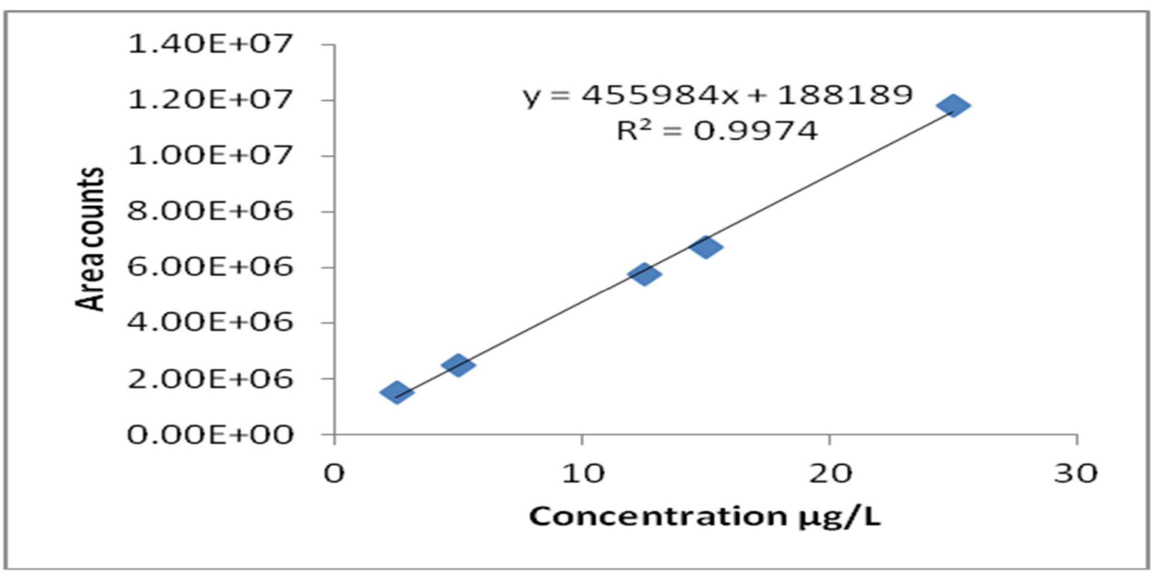


Fig.-3: Calibration curve for DBP

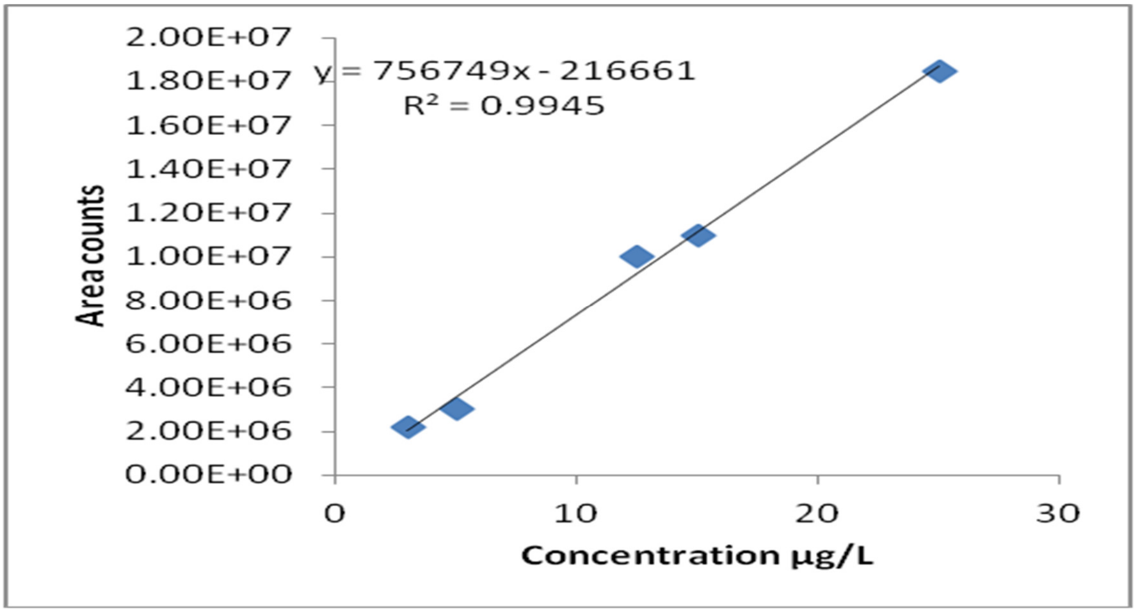


Fig.-4: Calibration curve for DNOP

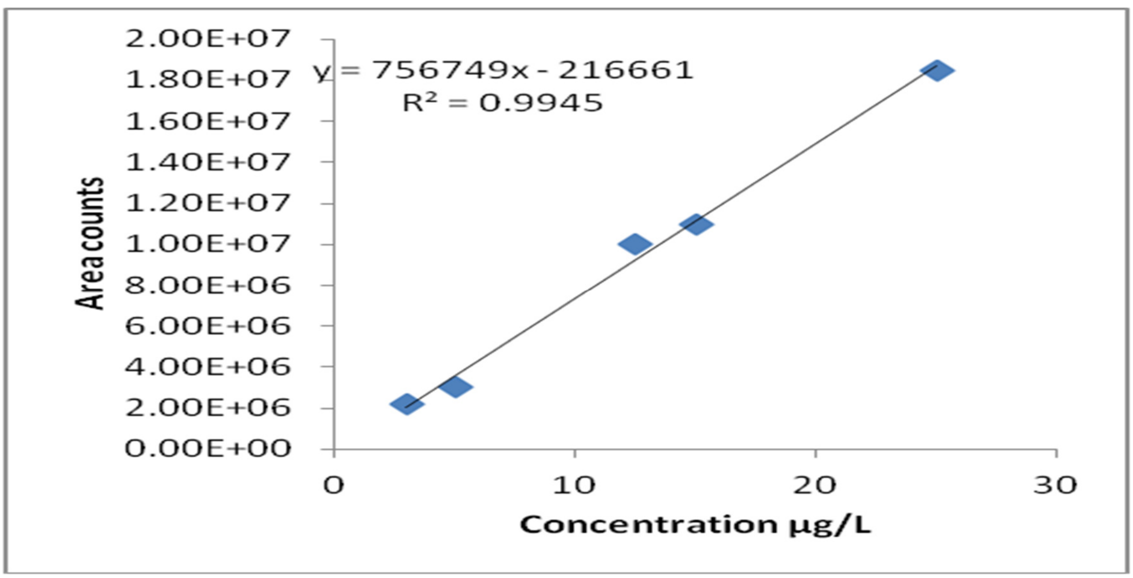


Fig.-5: Calibration curve for DEHP

Method Validation

The reliability of the method used was validated by studying the assessed performance in terms of recovery, linearity, precision, and accuracy, limit of detection and limit of quantification.

Linearity, LOD and LOQ

A working standard solution series ranging in concentration from 0.5 to 25µg/L was prepared by diluting the stock solutions in methanol. The standard solutions were then directly injected into the LC/MS/MS instrument. Peak areas were plotted versus the amount of the analyte injected, and linear regression equations were applied. The linear ranges were 0.5–25µg/L, and the range of correlation coefficients varied from 0.994 to 0.997, as shown in Table-1.

The results obtained for LODs and LOQs are also demonstrated.

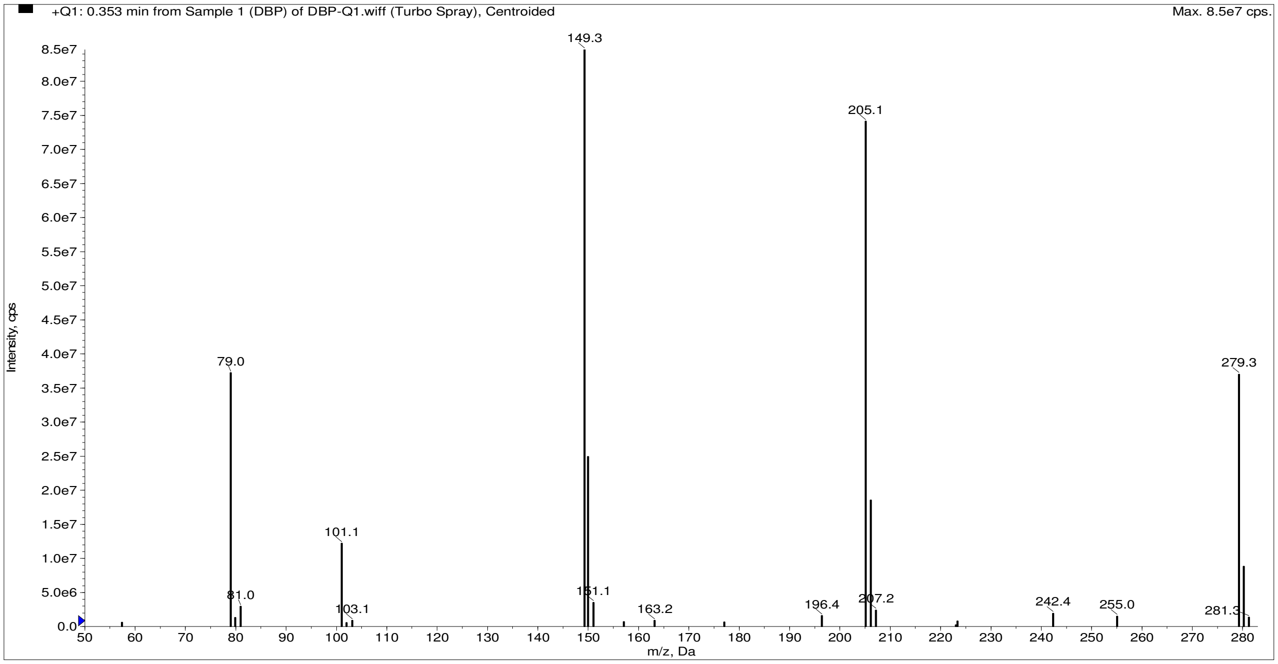


Fig.-6: Mass spectra of Plasticizer

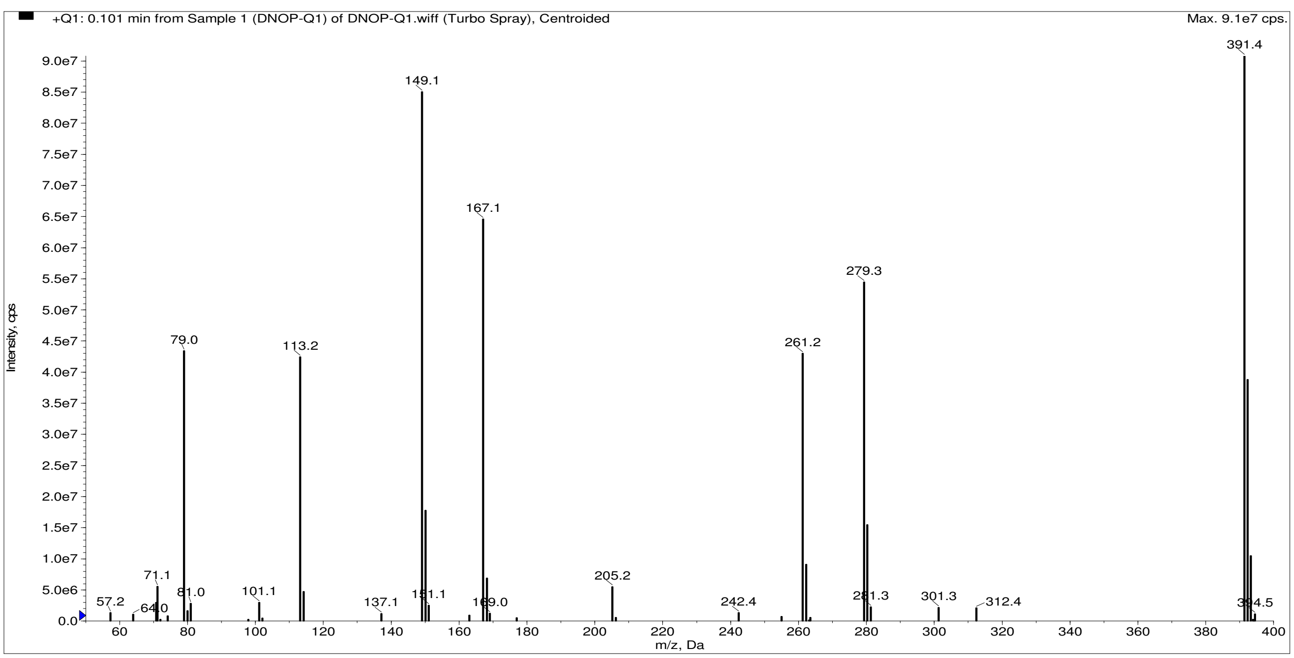


Fig.-7: Parent ion and fragments of DBP plasticizer

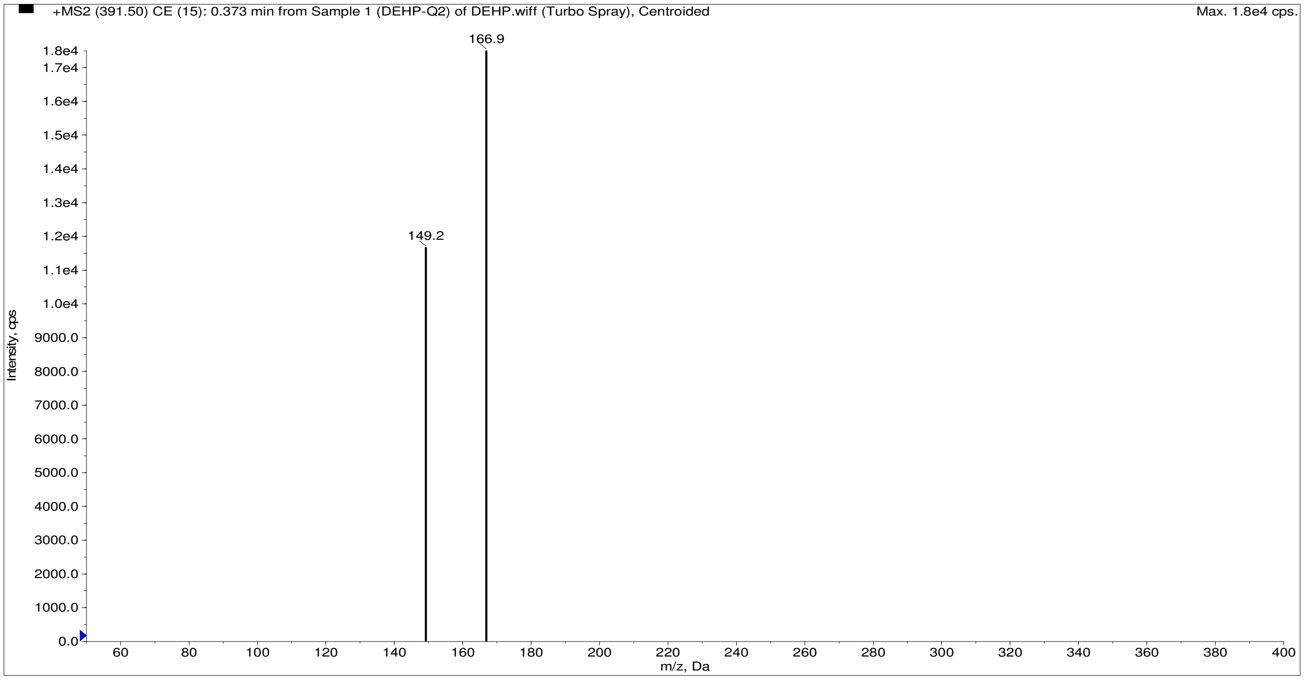


Fig.-8: Parent ion and fragment ions of DNOP plasticizer

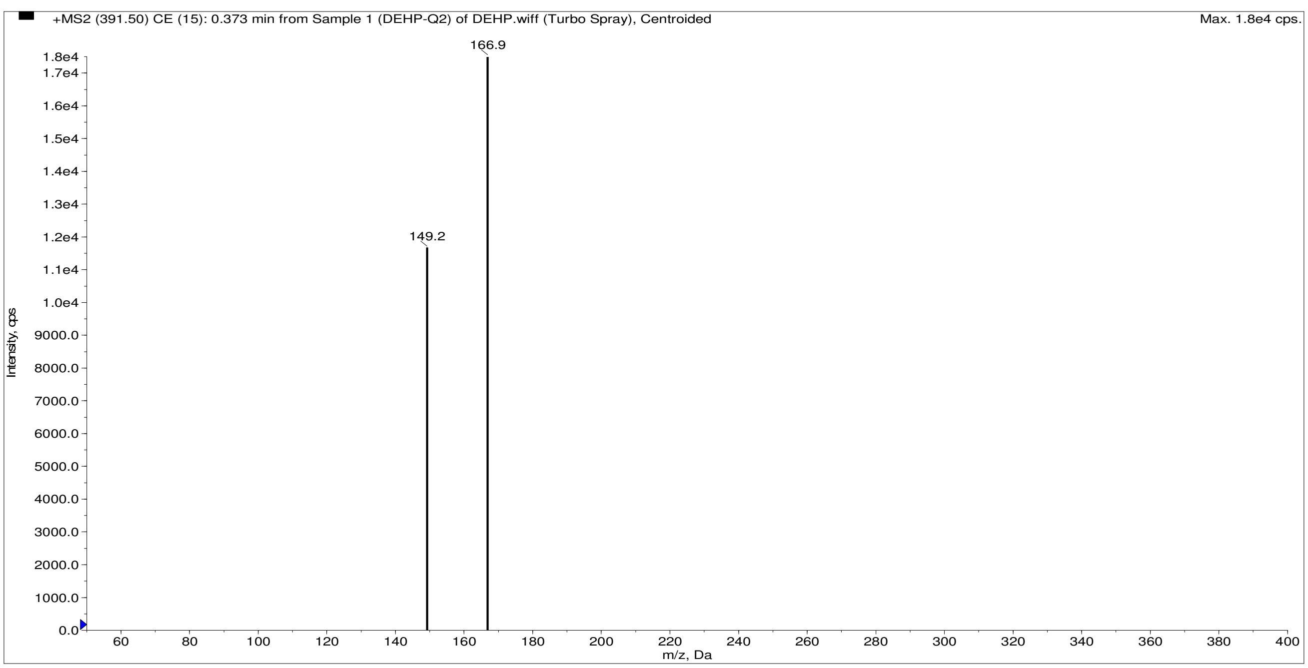


Fig.-9: Fragments of DEHP plasticizer

Cow Milk

Exp. RT (8.60) min

Calculated
Conc. 1.45 μg/L
Area: 6.70e+004

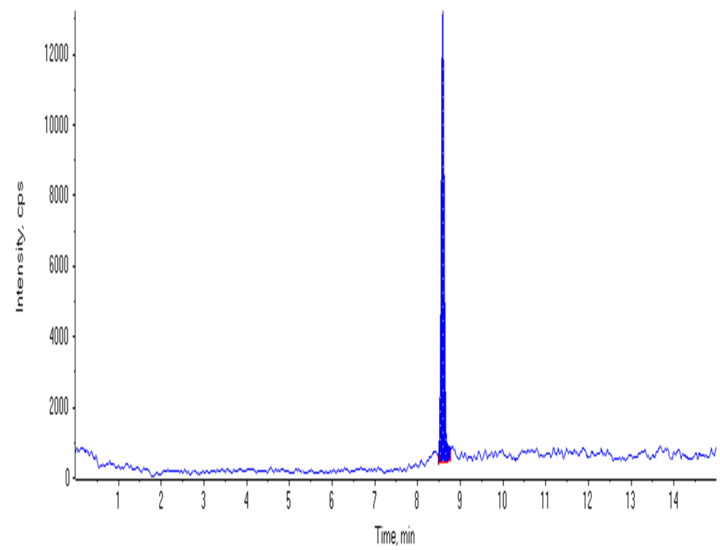


Fig.-10 : Typical representative chromatograms, retention time, areas and calculated concentrations of DBP in one of the samples

Table-1: Shows Linear ranges, correlation coefficients, Mean Recoveries, LODs and LOQs

Phthalate	Linear range(μg/L)	Correlation Co-efficient(R^2)	LOD	LOQ	Mean Recoveries
DBP	0.5-25	0.994	0.43	8.9	94.2
DNOP	2.5-25	0.997	2.43	8.1	99.4
DEHP	3-25	0.994	2.40	7.9	96.4

Table-2: Shows Mean Calculated Concentrations of plasticizers in μg/L in the Samples

S.No.	Sample Type	DBP	DNOP	DEHP
1.	Local (Cow)	1.45	0.0893	0.774
2.	Local (Cow)	1.43	0.108	0.809
3.	Local(CML)	14.3	58.3	51.5
4.	Local(CML)	13.0	52.0	47.4
5.	Y. Teeba	ND	ND	0.107
6.	Y. Teeba	ND	ND	0.184
7.	Almarai	ND	ND	0.275
8.	Almarai	ND	ND	0.228
9.	S. Teeba	2.22	36.6	34.9
10.	S. Teeba	1.81	38.7	33.5
11.	S. Hamodeh	15.8	55.0	49.2
12.	S. Hamodeh	14.7	54.2	50.6

ND: Not Detected

Recovery and accuracy

The recovery rate of the proposed method was evaluated by spiking milk samples with different concentrations (5, 10, 15, and 25μg/L) of phthalate standard solutions. The sample preparation process was carried out using the previously mentioned pre-treatment method. The reconstituted samples were analyzed and the results shown in Table-1 demonstrated that the average recoveries of phthalates ranged from 94.2 to 99.4. The reproducibility of the analytical method was reliable.

The purpose of this study is to ascertain the presence or absence of food contamination due to plasticizers by conducting a wide survey using LC/MS/MS method. Analyst software version 1.4 (AB Sciex) was used for data acquisition, statistical calculations and quantitation. Linear regression analysis using the least-square method was employed to evaluate the calibration curve of each analyte as a function of its concentration in the dairy products.

Liquid–liquid extraction was applied to sample preparation in this study and the method was more reliable and rapid than those reported for milk samples[8]. Due to the complex matrix effects of milk products, proteins must be removed from the samples to increase the extraction efficiency. The final samples were kept in freezer before injection and the sonication step help to reduce solvent consumption and extraction time, giving an improvement of the whole recoveries The extract could not be filtered, because the plastic syringes were easy to leach phthalates, especially the DIOP, DOP, and DMP. The membrane also contains phthalates, though it contributed less on the back ground. The mobile phase of 100% methanol maintaining from 3.5 to 11 min can maximize the removal of fat. The ion source can be cleaned after sampling for many times. After all, the preprocessing procedure was much reliable, and helps to minimize the chances of external contamination. The ammonium formate and formic acid were used as buffer in the mobile phase to improve the chromatographic separation. The mass of the analyzer was operated in MS-MS mode. Parent ions were isolated and specific daughter ions were monitored. The mass spectrum for the phthalates was very similar. DBP and DNOP shows base peak at m/z 149 and DEHP at m/z 166.

The concentration of each sample is measured in duplicate. The average concentration was reported as the calculated concentration of each plasticizer in the sample as shown in table . The average calculated concentration ranges of DBP, DNOP and DEHP in all the samples were 1.43-15.8µg/L, 0.0893-58.3µg/L and 0.107-51.5µg/L respectively shown in table 2. Among the analysed samples, Shaneinah Hamoodeh has the highest concentration of DBP while local milk (cow) has the lowest concentration. This may be due to nature of the packaging system during manufacturing in the industry[6]. However, local milk (Camel) has the highest concentration of DNOP compared to Cow milk which has the lowest concentration. The variation in the concentration might be due to milking method either by machine or manually by hand (which depends on the nature by which the plastic material is being made)[7]. Also local milk (Camel) has the highest concentration of DEHP and Yoghurt teeba has the lowest concentration. However, differences were also observed between local fresh milk samples and other dairy products analyzed. Results showed lower values than the Tolerable daily intakes (TDI) which have been specified by the European Food Safety Authority (EFSA) for several phthalates, and they are 0.01 and 0.15 mg/kg body weight/day for DBP and DEHP respectively.

Based on comparison with previous work, a case study was carried out on 9 phthalate residues in milk products by simultaneous determination using HPLC-ESI-MS-MS. Liquid-liquid extraction was also employed for sample preparation and the mobile phase was acetonitrile, methanol, ammonium acetate and 0.1% formic acid. The results obtained were similar to our own values with excellent recoveries and correlation coefficient (R^2) 0.9913 to 0.9986[8]. These 9 phthalates are DMP, DEP, DBP, DPP, BBP, DCHP, DIOP, DOP, DEHP which are also termed as the major plasticizers. The plasticizers DBP, DNOP and DEHP were also analyzed in this study and the remaining six might also be quantified if standards are available.

This LC/MS/MS method provides both quantitative measurement and a high degree of specificity[10,11]. It also affords excellent sensitivity and is linear over a broad and physiologically relevant concentration range[9].

CONCLUSION

The modern developed method of liquid chromatography tandem mass spectrophotometry (LC-MS/MS) has proved to be a sensitive, selective and reliable approach for the evaluation of plasticizers in fresh milk samples as well as the dairy products in general.

The LC/MS/MS method employed in this study provides shorter analysis time and simpler clean-up procedure with efficient selectivity and sensitivity over GC/MS method.

Promotion of the substitution of toxic phthalates by other health-harmless substances such as citrates, phenol alkyl sulfonates, benzoates, especially in the production of materials used in agriculture, food industry, and health care in order to reduce the risks of phthalates. Levels of phthalates in Jordanian dayari products were within acceptable values[1].

AKNOWLEDGMENT

Authors aknowledge the support of Jordan University of Science and Technology.

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[RJC-1409/2016]