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화장품에 함유된 미량의 프탈레이트 함량을 정확히 분석하기 위한 가스크로마토그래피-질량분석 시험법 및 그 시험법의 유효성

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Accurate Analysis of Trace Phthalates and Method Validation in Cosmetics using Gas Chromatography with Mass Spectrometric Detection

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요 약: 네일락카와 헤어스프레이 같은 화장품에 존재하는 미량의 프탈레이트를 정량분석하기 위하여, 가스크로마토그래피와 질량분석기를 사용한 효과적이면서 환경친화적인 분석방법을 개발하였다. 이들 화장품들은 다량의 유기용매를 함유되어 프탈레이트를 분석하기 위하여 널리 사용되는 시료의 클린업 방법이 적합하지 않았다. 더군다나 미량의 프탈레이트 분석시에는 실험과정 중에서의 오염으로 인해 실제보다 높은 분석값을 산출하게 되는 경우가 매우 많다. 이에 정확한 함량분석 및 이차오염을 방지하기 위해 유기용매를 사용하여 시료를 직접 희석하는 시료 전처리를 적용하였다. 이 분석방법은 높은 정확성, 분석감도, 그리고 시료전처리를 간략히 할 수 있는 이점을 가진다. 화장품에서의 검출되는 빈도가 높고, 사람과 동물에 영향을 미치는 환경호르몬으로 보고되는 dibutyl phthalate (DBP)와 di (2-ethylhexyl) phthalate (DEHP) 두 종의 프탈레이트를 분석대상으로 선정하였다. 정량시 그 정확도 향상을 위해서 내부표준물질로 두 물질의 중수소치환체인 DBP-d_4,와 DEHP-d_4를 사용하였다. 시험법의 유효화를 시행한 결과 본 시험법이 ppm 농도의 프탈레이트 정량분석에 적합함을 확인하였으며, 네일락카와 헤어스프레이 제품에 약 25 μg/g의 농도로 표준물질을 첨가하여 분석한 회수율은 95 ~ 106.1 % 범위였고, % 상대표준편차 값은 3.9 % 이하였다.

Abstract: An effective, environmentally friendly analytic methods using gas chromatography with mass spectrometric detector (GC-MSD) have been developed for the quantitative analysis of trace phthalate levels in cosmetics such as nail lacquer and hair spray. Since such cosmetics are largely comprised of organic solvents, conventional clean-up methods that have been widely used for phthalate analyses are in adequate. In addition, analysis of trace phthalate levels is notorious for its sensitivity to contamination, which causes high analytical values. A direct sample dilution method using an organic solvent was adopted to the sample preparation process to determine the exact amounts of phthalates and simultaneously avoid the high risk of secondary contamination. The method has many advantages including high accuracy, sensitivity, and simplicity in sample preparation. Dibutyl phthalate (DBP) and di (2-ethylhexyl) phthalate (DEHP) were selected for analysis because they have been frequently detected in cosmetics and consistently reported as endocrine disruptors in humans and animals. Internal standard method using two deuterium substitutes (DBP-d_4, DEHP-d_4) as the internal standard was also used. The results of 'Method validation' showed the capabilities of this method for the routine analysis of phthalates at the ppm level. The recovery ranges were between 95 % and 106.1 %, and relative standards deviations (RSD) were less than 3.9 % in fortified nail lacquer and hair spray samples at the concentration of 25 μg/g.

Keywords: *cosmetics, dibutyl phthalate, di (2-ethylhexyl) phthalate, gas chromatography with mass spectrometric detector, method validation*

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1. Introduction

The widespread production and use of phthalates in commercial goods caused many issues to the present environment. Since these sorts of persistent and refractory organic pollutants have a propensity to accumulate in living tissues, they give high risk to human health. Phthalates have been reported as cancer-causing and endocrine-disrupting materials[1,2], and some phthalates and their metabolites showed reproductive effects[3,4]. Such factors are very important for trace analysis. Some research on phthalate (and/or their metabolites) levels in human urine, blood, and breast milk[5-7] revealed that phthalates extensively migrate into the human body from various sources, including cosmetics. Accordingly, the use of dibutyl phthalate (DBP), benzylbutyl phthalate (BBP), and di (2-ethylhexyl) phthalate (DEHP) as cosmetic ingredients is prohibited by law in many countries because of their well-known genotoxicities. Additionally, the use of dibutyl phthalate (DEP) is of concern, although it is rarely reported to be genotoxic.

While phthalates are not used as cosmetic ingredients, some cosmetics such as nail lacquer, hair spray, and perfume contain phthalates as impurities. According to the European Cosmetic Toiletry and Perfumery Association (COLIPA), trace amounts of phthalates in the product itself can be caused from contamination and/or carryover from plastic and raw materials used in production or storage. COLIPA has asked the European Commission on Health & Consumer Protection Directorate-General to evaluate whether the presence of such trace levels would constitute a risk to consumers. In 2007, the Scientific Committee on Consumer Products (SCCP) of the European Commission agreed that "traces of DBP, BBP, and DEHP up to 100 $\mu g/g$ (or $\mu g/mL$) total or per substance don't indicate a risk to the health of the consumer"[8]. Nevertheless, non-governmental organizations (NGO) have consistently sought to eliminate these chemicals from cosmetics, contending that even trace amounts of phthalates will be very harmful to humans. As a result, the cosmetic industry needs an reliable, accurate, and convenient analytical method to detect low levels of phthalates and thus control the quality of their products.

Previous researchers have focused solely on analysis of phthalates in environmental samples such as water-based matrices and soil samples[9-15], and in food stuffs such as milk, vegetable oil, and wine[16-18]. However studies on phthalates in cosmetics are rare. Two papers using gas chromatography with a flame ionization detector (GC-FID) and high performance liquid chromatography (HPLC) exist[19,20]. But their quantitative concentration levels were disappointingly above 0.1 % (1000 $\mu g/g$). The most common techniques used for sample preparation were solvent extraction[12,15], liquid-liquid extraction (LLE), adsorption column chromatography, and solid phase extraction (SPE)[5,6,12,13,16,21]. However these treatments are expensive, time-consuming, laborious, and harmful to both analyst and environment because they use organic solvent extraction and pre-concentration steps to analyze trace levels. Moreover, analysis of phthalates at low concentrations is notorious for being contaminated by the apparatus and solvent, which results in high analytical results[22]. Accordingly, phthalate analysis requires extra steps to remove possible contamination from the sample. To overcome the drawbacks of the sample preparation method mentioned above, solid-phase microextraction (SPME)[17,18] and liquid-phase microextraction (LPME)[9,11,14] have been developed and used in environmental, food, biological, and pharmaceutical analyses. These procedures simplify the sampling, extraction, and concentration steps, and greatly reduce secondary contamination of phthalates. In addition, they use a minimal amount of solvent and achieve good concentration efficiency.

Nail cosmetics, hair sprays, and perfumes are the main cosmetic products in which phthalates (particularly DBP, and DEHP) are detected. These cosmetics share the feature of containing plenty of organic solvents, which causes ready phthalate contamination during production or storage and makes sample treatment for trace analysis difficult. According to our experimental observations, the sample preparation meth-

ods discussed above are ineffective for phthalate analysis of these cosmetics. In this study, we adopted a direct sample dilution method with an organic solvent, and quantified DBP, and DEHP at low levels in nail cosmetics and hair sprays with GC-MSD. Two phthalates (DBP and DEHP) were selected for analyses because they have been identified in these cosmetics and are possible endocrine disruptors in humans. We confirmed the capabilities and validity of our described method through the 'method validation' procedure referred to in EPA methods 8000C[23] and 8270D[24].

2. Experimental

2.1. Chemicals and Reagents

The grade of methanol, acetone, and n-hexane were 'B&J GC_2 capillary GC/GC-MS solvent. For trace analysis at or below the ppb level' (Honeywell. USA). Dibutyl phthalate (DBP), and di (2-ethylhexyl) phthalate (DEHP) were obtained from Sigma-Aldrich (USA). Di-n-butylphthalate-3,4,5,6-d_4 (DBP-d_4) and di (2-ethylhexyl)phthalate-3,4,5,6-d_4 (DEHP-d_4), which were used as the internal standards, were purchased from CDN Isotopes Inc. (USA).

2.2. Instruments and Apparatus

The GC-MSD system used was the 6890GC (Agilent, USA), which included capillary column split/splitless EPC inlet and liquid autosampler, 5975B inert XL MSD (Agilent, USA), and Mass Chemstation data system. The analytical column was HP-5MS (30 m × 0.25 mm, 0.25 μm).

All glassware was ultrasonicated in acetone for at least 30 min, then washed with n-hexane and dried in a phthalate-free desiccator at least 1 day to avoid phthalates contamination.

2.3. Preparation of Sample and Standard Solutions

An internal standard (IS) solution was made by dissolving two deuterium substitutes in methanol and diluting the solution to a concentration of about 10 μg/mL. A standard stock solution was prepared by dissolving DBP and DEHP together into a 100 mL volumetric flask with methanol. This stock solution was used to prepare the seven levels of standard solutions ranging 0.01 μg/mL to 2.5 μg/mL. Adequate portions of standard stock solution with the addition of 0.5 mL of IS solution were diluted to 10 mL with methanol to prepare the working standard solutions.

About 0.5 g of sample was transferred into 10 mL flask with 0.5 mL of IS solution. This sample solution dissolved with acetone, then was ultrasonicated for at least 30 min, and was filled up with acetone to given volumn. When insolubles existed, they were removed by centrifugation. The upper clear layer was moved to the GC vial and then injected in the GC.

Table 1. Operation Condition for Analysis of Phthalates

Inlet	260 ℃, Splitless (purge time 1 min, purge split 40:1)
Column	HP-5MS, 0.25 mm × 30 m × 0.25 mm
Flow	He, 0.8 mL/min, constant flow
Oven	70 ℃, 2 min → 10 ℃/min → 300 ℃, 5 min
Injection volume	1 μL
Detector	MSD interface Temp. : 280 ℃ MS source Temp. : 230 ℃ MSD Quadrupole Temp.: 150 ℃ Ionization mode : EI Ionization voltage : 70 eV Dwell time : 50 ms
Quantification	Internal standard method using SIM mode

Group	Analyte	Quantitative ion (m/z)	Confirmative ion (m/z)
1	DBP	149	150, 104
	DBP-d_4	153	
2	DEHP	149	167, 279
	DEHP-d_4	153	

2.4. Identification and Quantification

The standard solution, sample solution, and solvent blank solution were injected into the split/splitless inlet in splitless mode by the autosampler. Selected ion monitoring (SIM) mode was used for quantification (m/z

149 comes from phthalates and *m/z* 153 comes from IS) and analyte identification (*m/z* 104 and *m/z* 150 for DBP, *m/z* 167 and *m/z* 279 for DEHP). DBP-d_4 was used as an IS for DBP and DEHP-d_4 was used for DEHP. The operation conditions are summarized in Table 1.

2.5. Method Validation

We also performed 'Method Validation' which refers to EPA method 8000C 'Determinative chromatographic separations', and 8270D 'Semivolatile organic compounds by Gas Chromatography/Mass Spectrometry'. The specificity (peak identification) was checked by relative retention time (RRT), which was expressed as retention time (RT) of the *m/z* 149 ion over RT of *m/z* 153 ion derived from IS and ion ratios (two % abundances of confirmative ions compared with quantitative ion).

Linearity was evaluated by calibration curves of first-order and second-order least squares regression. The correlation coefficient derived from standard solutions in the range of 0.01 ~ 2.5 μg/mL. % differences were calculated to confirm the representativeness of the data. This checks fitness of the calibration data back to the model of the calculated amount of each of the standards against the expected amount of the standard, which was determined by using the following equation:

$$\% \text{ difference} = (C_c - C_e)/C_e \times 100$$

where C_c is the calculated amount of standard in concentration units

C_e is the expected amount of standard in concentration units.

The % recovery and precision were calculated to check the accuracy of these methods by analyzing spiked hair spray and nail lacquer samples that contained a certain amount of standard solution. Hair spray and nail lacquer samples, which were prepared in concentrations of 25.0 μg/g of each DBP and DEHP, were analyzed. Fortified samples were created from well-cleaned nail cosmetics and hair spray samples by adding a certain amount of analytes.

Table 2. % Abundances[a] of Confirming Mass Relative to Quantitative Mass

	DBP			DEHP		
	% Abundance			% Abundance		
	m/z 104	*m/z* 150	*m/z* 149	*m/z* 167	*m/z* 279	m/z 149
STD solution1	5.6	9.4	100	36.9	16.8	100
STD solution2	5.7	9.1	100	34.9	15.7	100
STD solution3	5.5	9.2	100	33.4	11.8	100
STD solution4	5.6	9.0	100	31.9	10.9	100
STD solution5	5.5	9.0	100	32.7	11.0	100
STD solution6	5.5	9.1	100	29.6	9.5	100
STD solution7	5.7	9.1	100	30.3	10.2	100
Nail lacquer (Fortified)	5.6	9.3	100	30.6	10.1	100
Hair spray (Fortified)	5.5	9.0	100	31.2	10.4	100

[a][Abundance of confirming mass/abundance of quantitative mass (*m/z* 149)] × 100

3. Results

Analyte level in standard solution lower than 0.01 μg/mL was difficult to quantify by this method. We confirmed the reliability of the quantification range of 0.2 ~ 50 μg/g in sample for GC-MSD. Obviously, this method minimizes sample handlings and the amounts of solvent, which show their potentials for analyzing trace level of phthalates. Acceptable criteria for validation were based on those described in EPA methods.

3.1. Specificity (Peak Identification)

GC-MS chromatograms and mass spectra of each peak provide some information for peak identification,

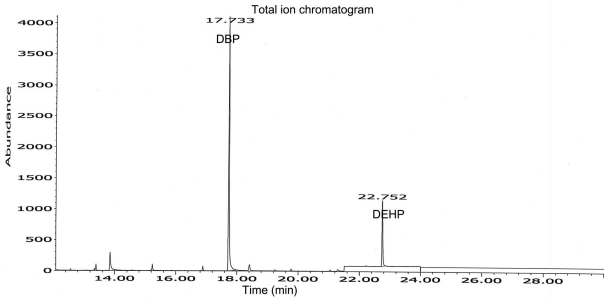

Figure 1. Total ion chromatogram of standard solution.

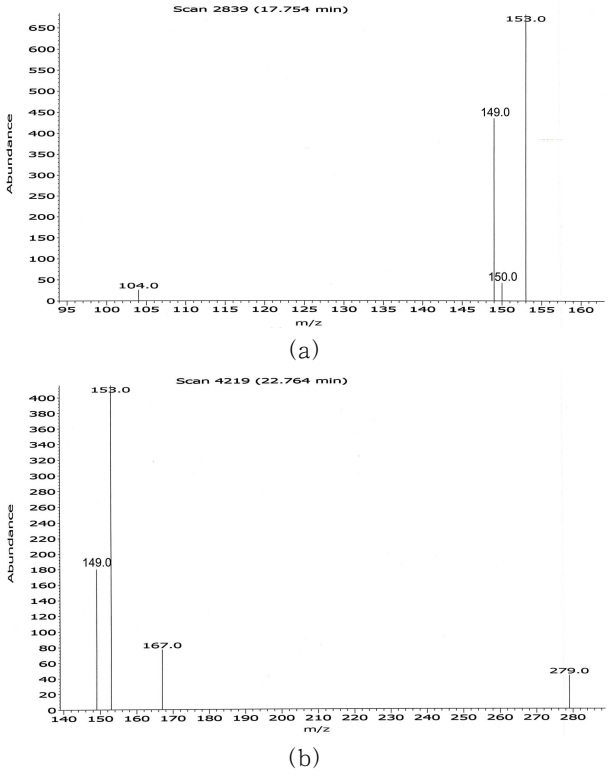

Figure 2. Mass spectra of DBP peak (a) and DEHP peak (b).

including RT and relative intensity of confirming masses. Deviations of RRT windows (criterion : ≤ 0.06 min) were within 0.001 min, deviations of absolute RT windows (criterion : ≤ 0.03 min) were within 0.02 min, and deviation of % abundance of confirmative ions (criterion : within ± 20 %) were lower than 5 % (Table 2). A peak suspected to be phthalate was not considered when it did not satisfy any one of these identification criteria.

3.2. Linearity / % difference

Table 3. Parameters of Calibrations

	First order		Second order	
	DBP	DEHP	DBP	DEHP
Equation	y = 0.9658 x + 0.0069	y = 0.9212 x + 0.007	y = 0.0267 x^2 + 0.8761 x + 0.0421	y = 0.0014 x^2 + 0.9158 x + 0.0095
r^2	0.9992	0.9986	0.9998	0.9986
LOD (μg/g)	0.06	0.09	0.06	0.09
LOQ (μg/g)	0.2	0.3	0.2	0.3

0.01 ~ 2.5 μg/mL, 7 levels (n = 3)

Table 4. % Difference of Standard Solutions (n=3)

Standard solutions	First order		Second order	
	DEP	DEHP	DEP	DEHP
1	57.3	- 2.6	16.4	- 5.3
	48.9	43.9	7.3	41.3
	54.7	46.1	13.5	43.6
2	14.4	4.4	- 2.7	3.3
	14.1	- 1.0	- 2.8	- 2.1
	11.5	- 8.9	- 5.7	- 10.1
3	- 4.7	- 0.4	- 2.7	- 0.3
	- 3.4	- 2.0	- 1.3	- 1.8
	- 4.3	- 3.7	- 2.3	- 3.6
4	- 2.1	- 3.2	0.8	- 3.0
	- 1.8	- 2.8	1.1	- 2.6
	- 1.3	0.1	1.6	0.2
5	- 1.8	1.5	0.0	1.6
	- 2.4	0.3	- 0.6	0.4
	- 0.7	0.1	1.1	0.2
6	- 0.7	0.4	1.1	0.7
	0.4	- 0.9	3.3	- 0.5
	- 0.9	- 2.0	- 1.6	- 1.6
7	- 2.0	1.3	- 1.2	0.0
	1.3	2.0	3.0	0.6
	2.0	1.3	- 3.1	- 0.1

The calibration curve was obtained using first-order and second-order least squares regression. The correlation coefficients (r^2, criterion : 〉 0.99) were greater than 0.997 (Table 3). All results of % differences in

Table 5. % Recovery and % RSD of Fortified Sample Solutions (n=6)

	DBP		DEHP	
	% Recovery	RSD (%)	% Recovery	RSD (%)
Hair spray (Fortified)	95.1	0.8	95.0	0.6
Nail lacquer (Fortified)	97.3	0.9	104.2	0.8

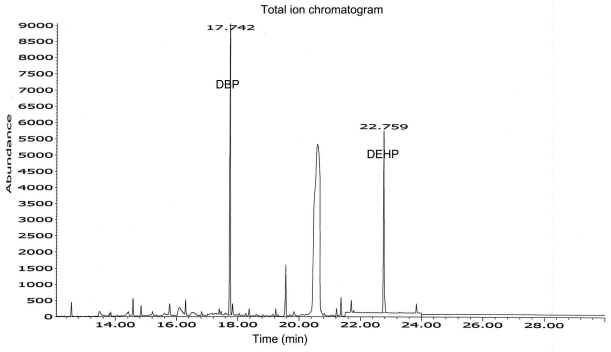

Figure 3. GC-MSD total ion chromatogram of a fortified nail lacquer sample.

standard solutions were suitable for the criteria (% difference ≤ 20 %) except the lowest concentrations (Table 4). As shown in Table 4, there is a tendency for % difference values higher than 20 % in low-level standards, especially DEHP in spite of the efforts to make the apparatus and chemicals phthalate-free, It means that a number of variations exist in this region. Minimal contamination caused high deviation at even very low concentration levels. This arises, we think, from slight contamination of the autosampler syringe, washing solution, GC inlet, and other solutions used during the analysis. Both first-order and second-order calibration in GC-MSD method can be used for quantification, second-order calibration was more suitable and accurate for quantification of low concentrations.

3.3. Precision (Repeatability) & Accuracy (% Recovery)

The % recovery and % RSD data summarized in Table 5 reveals the applicability of these methods. Good recoveries ranged from 95 % to 106.1 %, and precision was observed with % RSD less than 3.9 %. A Total ion chromatogram of a fortified nail lacquer sample in Figure 3.

Table 6. Measured Concentrations in Nail Lacquer Cosmetics (n=3, μg/g)

	DBP	DEHP
Sample1	Not detected	0.4
Sample2	17.1	0.7
Sample3	Not detected	16.7
Sample4	0.7	52.9

3.4. Limit of Detection (LOD) and Limit of Quantification (LOQ)

LOD and LOQ were estimated as the analyte concentration in the vicinity of the lowest concentration that gives the ratio of signal to noise (S/N) = 3 and limit of quantification (LOQ), where S/N = 10 (Table 3).

3.5. Analysis of Real Samples

The methods of quantification and sample preparation were applied to the analysis of four nail lacquer cosmetic products which are purchased at market. The measured concentrations are displayed in Table 6.

4. Conclusion

We have devised the method that uses simple sample treatment and GC techniques to analyze solvent-based cosmetics for phthalates. This method is faster, cheaper, and more environmentally friendly than currently used methods. Further, it is highly accurate because they eliminate phthalate contamination during the analytical process and use deuterium derivatives as internal standards. Method validation showed that these methods are capable of routinely detecting DBP and DEHP in nail cosmetics and hair sprays at the ppm level analysis.

5. Discussion

This study was an outgrowth of long-term unsuccessful attempts to test various preparation methods such as SPE, LLE, and adsorption column chromatog-

raphy to establish an adequate analytical method that would enable analysis of organic solvent-based samples.

We have reported GC-FID method for the analysis of phthalates in same matrices previously[25]. We mentioned several important cautions in GC experiment and limitation of GC-FID method at this article. The highest concentration of standard solution was set at 2.5 μg/mL because the injection of significant amounts of phthalate causes serious contamination of the GC inlet. Once serious contamination took place, extensive cleaning and time was essential to eliminate it. Such contamination frequently causes positive blank values [22]. A clean analytical system and an extensive effort to eliminate phthalates are continuously needed for trace level analysis, and in order to minimize contamination from phthalates, pesticide analysis-grade solvents or their equivalents must be used. Glass apparatus are preferred and all apparatus should be precleaned with phthalate-free organic solvent and then stored in a clean container. Analytical instruments should also be kept from excessive exposure to phthalate. According to our experimental results over several years, even an injection of standard solution over the 10 μg/mL level of phthalate can seriously pollute the GC system, especially the GC-MSD. Once the GC is contaminated, cleaning of the injection port, washing solution, and syringe are inevitable[22].

Analyzing traces of phthalates in solvent-based cosmetics is tedious, and sample preparation is difficult. These kinds of cosmetics are also easily contaminated by phthalates during the experimental process such as sample extraction and pre-concentration steps; these steps are time-consuming and labor-intensive, and they require large amounts of organic solvent. We adopted a direct-dilution method to overcome these shortcomings and to make sample preparation of solvent-based samples better. This method simplifies the sample can be diluted with a small amount of organic solvent and 'splitless injection' technique, which sends sufficient analytes to the GC column to lower the detection limit of phthalates. In this way, we can lower the detection limit, experimental time, cost, and required amount of solvent.

The mass selective detector generally tends to show the phenomenon of response increase or decrease by ion suppression or enhancement depending on the concentration of analytes and their matrices. Large enhancement of peak intensities of diluted sample solutions (even more than 10 fold) were always observed in our experiment even in standard solutions, which resulted in high positive results of phthalates when internal standards were not used for quantification. We can compensate that deviation by using internal standard. The use of internal standards was vital to the accuracy and reliability of results obtained with our GC-MSD method.

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