絞り酸エステルで汚染された大豆もやし（発芽大豆, Glycine Max.）と油脂に含まれるエステルの Sephadex LH-20 カラムクロマトグラフィーによる分析の可能性

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Soybean Moyashi (Germinated Soybean, *Glycine Max.*) contaminated with Phthalates and Sephadex LH-20 column Chromatography as a Possible Means for Their Analysis in Oils.

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Introduction

The contamination of fish and other food items with PCB has called much attention of the general public in recent years. However, much less attention has been paid to the contamination of foods with the esters of phthalic acid, which are used as plasticizers in various forms of plastics, via already contaminated river waters in this country1). This situation may be attributed firstly to their large LD₅₀ values (for example, 4.0g/Kg body weight for dibutyl phthalate and 14.19g/Kg. body weight of dioctyl phthalate2) and secondly to the fact that some of large food manufacturers took a quick step a few years ago to eliminate their use in plastic food containers that come directly in contact with foods. A FAO/WHO special committee has set a permissible dose of the esters for human daily intake to be 1~2mg/kg body weight3). Their acute and chronic toxicities are, however, under debate and remain to be studied although their toxicity had been observed in 1970 in patients who received the transfusion of blood contaminated with these esters4). A recent study on their metabolism in animals showed that 55% of the orally administered diethylhexyl phthalate was eliminated in the urine and 35% in the feces in mice and (ω-1)oxidized forms5).

The presence of phthalates in soybean moyashi was observed in our laboratory a few years ago during the course of a study on low molecular weight components in the TG (triglyceride) fraction from moyashi6), and reproducible results were obtained by different experimenters for the three consecutive years. Furthermore, they were also found to be present in moyashi sold on a local market in the spring of 1973.

Another point of interest in this study is that the method described here is simple enough to find an application to the analysis of phthalates in oil samples. There have been two methods available for the analysis of their trace amounts in oils: One is an indirect method involving the analysis of phthalic acid and alcohol components after saponification7); and the other is the one developed by Williams8), in which they were extracted with acetonitrile, followed by epoxidation, silicic acid column chromatography and gas chromatography. The present method requires no such elaborate techniques and the analysis can be performed simply by fractionation on a Sephadex LH-20 column.

Two unknown compounds were detected in a low molecular weight TG fraction from the axis and their mass spectrometric analysis indicated that they are oxidized forms of the esters of phthalic acid.

Material and Methods

1 Solvents

The precautions pointed out by Katase2) were exercised in handling solvents. Thus, all the solvents used for extraction and fractionation of lipids were carefully distilled and stored in bottles without plastic inner caps, from which the diesters used as plasticizers are said to dissolve into solvents and samples treated with such contaminated solvents will give rise to their artefact peaks on gas chromatograms. Special attention was also paid to the distilled water used for the analysis of moyashi procured from a local market since this sample was subjected to a
semiquantitative analysis.

II The esters of phthalic acid

The following standard samples were purchased from Wako Chemical Co.; DEP (diethyl phthalate), DiBP (diisobutyl phthalate), DBP (dibutyl phthalate), and MEP (monoethyl phthalate). The following monosteros were synthesized essentially according to the method described by Gogans and Copenhagen, using sublimed phthalic anhydride and redistilled anhydrous alcohols: MiBP (monoisobutyl phthalate), MBP (monobutyl phthalate), MHxP (monohexyl phthalate), MHpP (monooctyl phthalate), and MnOP (monooctyl phthalate). The monoesters were purified several times by acid precipitation, followed by recrystallization at low temperatures; the m.p. of MiBP and MBP were 64-65°C and 71-73°C, respectively.

The purity of the diesters were checked by gas chromatography and that of the monoesters by thin layer chromatography, using chloroform-methanol-water (65:25:4) as a solvent system. Their Rf values were 0.74 for MEP, 0.60 for MiBP, 0.68 for MBP, 0.76 for MHxP, 0.80 for MHpP, and 0.84 for MnOP.

III Germination

Soybeans used for germination were of two varieties. Sample (a) was “Tamanishiki”, which were harvested in the autumn of 1971 on the farm of Osaka Furiitsu Daigaku. During their cultivation, plastic tubes and films were used for water spray and weed control. Sample (b) was an unknown variety imported from a foreign country, and sample (c) soybean moyashi procured from a local market and used for the estimation of the ester content in their cotyledons.

A lot of 3-5kg. of sample (a) and (b) was germinated for 4 days in a dark room provided with automatic control systems of moisture, temperature, and intermittent warm water spray, where no plastic tubes or tanks were used. The 4th day moyashi was used since earlier observations showed that low molecular weight components in the TG fraction from the axis were most abundant at this stage.

IV Extraction of lipids and the isolation of TG fraction

In early experiments with sample (a) and (b) fresh samples were used but in later study with sample (c) moyashi was freeze-dried prior to extraction of lipids.

The axes separated from cotyledons were treated with hot water, followed by air-drying prior to the extraction. The minced axes were extracted a number of times with petroleum ether-diethyl ether (1:1) and the cotyledons were homogenized in the same solvent system and their lipids extracted, in the same manner as in the case of the axis. The TG fraction from the cotyledons was obtained by silicic acid column chromatography by running chloroform as an eluting solvent system and that from the axis by thin layer chromatography by developing with petroleum ether-diethyl ether-acetic acid (100:15:1). The thin layer chromatoplates used were prewashed with chloroform or with petroleum ether-diethyl ether (70:30) prior to the application of a sample. The TG fraction was removed from the plate and extracted with petroleum ether-diethyl ether (1:1). A number of blank tests were performed to see if any artifact peaks suspected to be phthalates could be detected on gas chromatograms by scraping off silica gel in an amount equal to that of the TG spot and injecting its extract into a gas chromatograph. No peaks were detected.

V Sephadex LH-20 column chromatography

The procedure described by Calderon et al. was followed.

A semi-quantitative study made with the cotyledons of sample (c) will be described as a typical example. The weight ratio of the cotyledons to the axes was 7:3 and the weight of the fresh cotyledons decreased by 50% on freeze-drying. A total amount of the crude fat extracted from the freeze-dried sample was 12.3% and that of the TG fraction from it was 63% when 800 mg. of the crude fat was subjected to chromatography by a 20-g. silicic acid column. The TG fraction amounting to 252.15mg. was fractionated on a column packed with 150g. of Sephadex LH-20 with chloroform as an eluting solvent. Two 10ml. fractions collected after running 200 ml. of the solvent contained a single substance when monitored by gas chromatography, eluting at 146-147°C (Fig. 2). The following 10 ml. fraction (6 of Fig. 2) also contained a small amount of this compound mixed with other components. The former two fractions (4 and 5 in Fig. 2) were combined and the solvent was removed; the residue weighed 3.3mg. An amount of this compound, being identified to be DiBP by mass spectrometry as will be discussed later, in 100g.
of freeze-dried and fresh cotyledons was estimated to be 101.7 mg. and 50.4 mg., respectively. Some DiBP was detected in the axes but its contribution to the above estimated amount would be negligible since the ratio of the crude fat content in the cotyledon to that in the axis was found to be approximately 100:1.

VI Gas Chromatography (Column I) (GC)

Yanagimoto GCG-550 FP, provided with a FID detector, was used for monitoring the fractions obtained by Sephadex LH-20 column chromatography. The conditions used were as follows: A stainless steel column (3 mm x 450 mm) packed with 2% Silicone OV-17 coated on Chromosorb W (AW 80-100 mesh); column temperature 120-320 °C (programmed at a rate of 6 °C/min.); nitrogen and hydrogen gas flow rate 60 ml./min. and 40 ml./min., respectively; and injection and detector temperature 310 °C and 320 °C, respectively.

The same conditions were used for testing the response of the monoesters, except that a longer column, 3 mm x 2 m, was used.

VII Gas Chromatography (Column II)—Mass spectrometry (GC-MS)

The instrument used was Shimazu LKB-9000 installed at the Osaka Furitsu Hoshasen Kenkyusho, Sakai. The conditions used were as follows: A glass column (3 mm x 2 m) packed with 0.5% Silicone OV-17 coated on Chromosorb W 60-80 mesh; He gas flow rate 30 ml./min.; column temperature 120-270 °C (programmed at a rate of 6 °C/min.); flash heater and separator temperature 290 °C and 300 °C, respectively; and ion potentials 15 and 70 e.v.

The mass spectra of the monoesters were obtained by their direct introduction into the mass spectrophotograph under the ion potentials of both 15 and 70 e.v.

Results and Discussion

I Sephadex LH-20 column chromatography-GC

The efficiency in the separation of a diester of phthalic acid by Sephadex LH-20 column chromatography is demonstrated in Fig. 1, where the TG fraction from the axis of germinated soybean (a) has been subjected to the analysis and similar results have been obtained for three consecutive years of study with germinated soybean (b) as well as sample (c). The low molecular weight fractions of the TG from the

![Fig. 1. The reproduction of the gas chromatograms obtained on Column I. A The TG fraction from the axes of sample (a) or (b) before purification by Sephadex LH-20 column chromatography. B A TG fraction obtained from a Sephadex LH-20 column](image)

![Fig. 2. The reproduction of the gas chromatogram obtained on Column I. The TG fraction from the cotyledons of Sample (c) fractionated on a Sephadex LH-20 column. 4 represents a 10 ml. fraction after running 200 ml. of chloroform. 5 and 6 are the following 10 ml. fractions](image)
Fig. 3. The reproduction of a part of the gas chromatogram obtained on column II to show the relative Rt and response of compound B, C, D, and E in a fraction collected from a Sephadex LH-20 column (The Axis TG from sample (a)).

cotyledons of sample (c) also show a distinct single peak eluting at $146 \sim 147^\circ C$ on column I as depicted in Fig. 2. In addition to these DOP and two unknown compounds, B and D (Fig. 3) were detected from the axes of germinated soybean (a). The chemical structure of these unknown compounds will be discussed later.

Thus, the use of Sephadex LH-20 column chromatography will simplify the analysis of the esters of phthalic acid, which are readily soluble in oils and difficult to separate by solvent extraction. More efficient packing materials, which are being developed, than the LH-20 in conjunction with a high speed liquid chromatographic instrument will achieve the analysis of high molecular weight esters such as DOP.

II Contamination of moyashi with the esters of phthalic acid

The amount of DiBP in moyashi sample (c) purchased from a local market is estimated roughly to be 0.51 ppm on the wet basis. This amount is considered to be minimum since other esters and metabolites are apt to be present and their amounts would contribute to a certain extent. In connection with the contamination of foods, Mayer et al.12) reported that channel catfish from various localities were contaminated with $0.4 \sim 0.8$ ppm of DOP in average and in some cases with as high as 3.2 ppm. The review by Katase1) also points out the contamination of field picked as well as cultivated “mitsuba” with some of these esters. Thus, the present study indicates that more thorough examination of other food items should be conducted for the sake of the health control of the general public.

III Identification of the known phthalate esters by mass spectrometry

The mass spectral patterns of compound A (Fig. 4), C (Fig. 5), and E (Fig. 6) are identical with those taken with the standard samples of DiBP, DBP, and the spectrum reported for these and DOP in the literature13, 14, 15) respectively. Compound A, however, may be suspected to be DEP since the maximum fragment ion observed is at m/e corresponding to its molecular weight. The mass spectrum of DEP shows quite intense fragment ions at m/e 177 (M-45) and m/e 163 (M-59) when its spectrum is taken under the identical conditions and these fragment ions are reported to be the characteristic ions derived from DEP. Since the spectrum shown in Fig. 4 A does not show such fragment ions, compound A must be DiBP.

Furthermore, its elution temperature on GC-column I corresponds to that of the standard DiBP, 146°C. Compound C (Fig. 5) is identified to be DBP since its elution temperature at 157°C on GC-column I corresponds to that of the standard sample and its mass spectrum is similar to that of the standard DBP, showing its molecular ion at m/e 278. The mass spectrum of compound E (Fig. 6) is in agreement with that reported for DOP13, 14, 15) its maximum fragment ion being generally observed at m/e 279 (M-113+2).

IV The mass spectra of the synthetic monoesters of phthalic acid

Compound B and D were at first suspected to be monoesters of phthalic acid since Hayashi et al.16) reported the detection of MiBP, eluting between DiBP and DBP on an NGS packed column, in “mitsuba” (Cryptotaenia canadensis DC var. Japonica Makino) and mentioned the mass spectrum of MiBP (without showing its spectrum, however) gives rise to the fragment ions at m/e 167 and m/e 149 that are the characteristic fragment ions of the diesters. In order to check this point, a series of the monoesters prepared synthetically were subjected to mass spectrometry at
ion potentials of 15 and 70 e.v. Since the patterns obtained at both ion potentials are quite similar, those obtained at 15 e.v. are shown in Table 1. The mass spectra of these monoesters have not been reported previously as far as we are aware of.

The relative intensities of the molecular ions of all the compounds examined, except MEP, are rather weak. On the other hand, the relative intensities of the fragment ion at m/e 167 \( [C_6H_4(COOH)_2]^+ \) is from a medium to large in all except MEP. The fragment ion at m/e 149 \( [C_6H_4(COO^-)OH]^+ \) is not necessarily the base peak for all the monoesters— it is so for MBP, MHxP, and MHpP. The base peak of MEP is a fragment ion at m/e 150 (149+1) and the relative intensity of the fragment ion at m/e 149 is only 49%. On the other hand, the base peak of MiBP and MnOP is a fragment ion at m/e 104 \( [C_6H_4CO]^+ \). The relative intensities of the latter fragment ion in the cases of MBP, MHxP, and

### Table 1: The mass spectra of the monoesters of phthalic acid

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* metastable ions, m/e 132.8 or 132.9
The values underscored indicate the relative intensities of the molecular ions.
MHpP are rather large, indicating that the losses of the RO and -COOH groups would readily occur in the monoesters under electron impact. This may account for the observed small intensities or absence of the fragment ion at m/e 132 [C₆H₄(CO)₂]⁺ and at m/e 121 [C₆H₅CO₂]⁺. The former fragment ion is generally observed as a metastable ion at m/e 132.8 or 132.9 in all the cases, except MEP. MEP shows the

Fig. 4. The mass spectra of compound A (DiBP) and B (an oxidized product, see the text)

Fig. 5. The mass spectrum of compound C (DBP)

Fig. 6. The mass spectra of compound D (an oxidized product, see the text) and E (DOP)
fragment ion at m/e 122 with a relative intensity as large as 39%. The spectra of MEP, MiBP (also MBP), MHP, MHP, and MnOP show rather small intensities of RO and R fragment ions at m/e 45, 73, 101, 115 and 129 and 29, 57, 85, 99, and 113, respectively. The relative intensities of the latter series are smaller that those of the (R-1) fragment ions, except MEP. Furthermore, these monoesters do not give rise to the fragment ions at m/e 28 and 18 with relative intensities as large as those observed with compound B and D. Thus, these two unknown compounds do not correspond to any of the simple monoesters examined here.

V Proposed chemical structure for compound B and D

While this manuscript was under preparation, Albro et al. (17) published the papers on the metabolism of the phthalate ester, di-2-ethylhexyl phthalate, and reported that lipases of animal tissues hydrolyze the diester to the stage of the monoester and also that the metabolites isolated from the urine of rats after its oral administration consist of the following four monoesters in addition to a small amount of phthalic acid:

(1) \( C_6H_4COOH \)

(2) \( C_6H_4COOCH_2CH-(CH_2)_2CH_3 \)

(3) \( C_6H_4COOCH_2CH-(CH_2)_3-COOH \)

(4) \( C_6H_4COOCH_2CH_2CH_2-COOH \)

The mass spectra of these esters (20 e.v.) show no characteristic fragmentation of their side chains and the relative intensities of the fragment ion at m/e 132 are very small, 1.1-1.8%, those at m/e 121 or 122 either absent or less than 1%, and only a fragment ion at m/e 120 is observed at a 1.1% level in compound (3).

On the other hand, the examination of the lower mass region of the spectrum of compound B shows the following fragment ions not being observed in the spectra of the synthetic monoesters and the metabolites (1)-(4): m/e 47 [HC\( \text{COOH} \)] derived from an acetal or [CHO\( \text{H} \)] derived from an orthoester, m/e 44 [CO\( \text{2} \)] or [CH\( \text{2} = \text{CHOH} \)] derived from an aldehyde; and m/e 39 an ion of a diene with three carbon atoms. Furthermore, the spectrum of compound B shows considerably larger intensities of the ions at m/e 71, 95, and 97 in comparison with the ions observed in the spectra of MiBP, MBP, MHP and the above metabolites. Thus, B must have an oxygenated side chain or chains, being different from those of the metabolites, and the following structures may be proposed:

\[
\begin{align*}
\text{C}_6\text{H}_4\text{COO-CH}_2\text{CH}_2\text{CH}_2\text{CH} & \quad \text{COO-CH}_2\text{CH}_2\text{O} \\
\text{B-1} & \quad \text{M} = 266 \\
m/e 222 = \text{M} - 44 \\
m/e 205 = 222 - 17 \\
m/e 167 = \text{M} - 99 (\text{C}_4\text{H}_9\text{COO}-2)
\end{align*}
\]

\[
\begin{align*}
\text{B-2} & \quad \text{M} = 268 \\
\end{align*}
\]

Both structures would satisfy the large intensities of the fragment ions at m/e 28 and 18 and also the presence of ions at m/e 47, 44, and 39. However, the structure represented by B-2 should show an ion at m/e 45, which is absent in the spectrum of B. Furthermore, a compound with such a structure with the highly polar groups should be eluted following DBP on GC, if not decomposed by a high temperature. With this respect, MEP, MiBP, and MBP (in the form of the free acid) were injected into the gas chromatograph under the conditions described earlier, except that a column used was 3mm x 2m; no responses were observed, indicating their decomposition. Thus, a hemiacetal structure represented by B-1 is preferred.

Analogously, compound D (Fig. 6) may also have a side chain or chains with an oxygen atom or atoms, being indicated by the presence of the fragment ions at m/e 58 (24%), 44 (23%), 28, and 18. However, in the absence of the fragment ion at m/e 47, compound D has no acetal or a hemiacetal moiety. The
pattern of the lower mass region (m/e 41-58) (Fig. 6) is more like that of hexanal than that of 2-hexanone or a lactone. The large intensity of the ion at m/e 28 would eliminate the ketone structure. A fragment ion at m/e 155 corresponds to the RCO ion with C_{10} or a C_{11} hydrocarbon ion and considered to be a secondary ion formed under electron impact since no smaller fragment ions following this are detected. Thus, the following structures are proposed for compound D.

The structure represented by D-3 is not likely because the elution temperature of a compound with such a structure is expected to be close to that of OBP. Therefore, the most appropriate structure for compound D may be represented by D-1, as far as its mass spectrum is concerned.

The proposed structures for compound B and D suggest that \( \omega \) or (\( \omega-1 \)) oxidation also occurs in the plant system. Since the amounts or these compounds detected in the axis of germinated soybeans are very small (see Fig. 3), no further work will be conducted. However, the study of this nature will be of considerable interest in view of the present interest in the contamination of various foods with diesters of phthalic acid.

The esters of phthalic acid detected in soybean moyashi in the present study are summarized in Table 2. Among these esters, DiBP and DBP are most readily detected from the TG fraction of the axis and cotyledon and determined semiquantitatively by Sephadex LH-20 column chromatography.

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### References

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浦上他：大豆もやしのフタール酸エステル


要約
大豆もやしの胚軸のトリグリセリド区分より次のものを検出した。ジイソブチルフタレート（DiBP）、ジブチルフタレート（DBP）、ジオクチルフタレート（DOP）および2コの未知物質。この未知物質の化学構造式を知るため、次のようなモノエステルを合成しそれらのマススペクトルと比較した。モノイソブチルフタレート（MiBP）、モノブチルフタレート（MBP）、モノヘプチルフタレート（MHP）、モノヘプチルフタレート（MHPP）およびモノ−n−オクチルフタレート（MnOP）。その結果、2コの未知物質はこれらモノエステルのいずれとも相当しなかった。未知物質は次の化学構造式を有すると推定した。

ここで用いたSephadex LH-20カラムクロマトグラフィは油脂に微量含まれるフタール酸エステルの分析に適しており、従来から用いられている方法に比べ簡便である。この方法を用いて市販大豆もやしを分析した結果、最少限0.51PPmのDiBPが含まれていることがわかった。