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Contamination of Dipeptide by Polymer Stabilizers Leached from Gloves and Packaging during Scale-up

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Abstract:

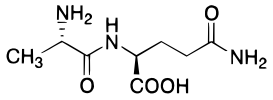
In dipeptide production, it was observed that a water solution of a peptide became turbid. The origin of turbidity was monitored and identified by LC-MS analysis. The impurities, derived from natural rubber gloves, and polyethylene bags caused the turbidity when the wet cake of the peptide contacted them.

Introduction

Scale-up is a minefield! Impurities from somewhere contaminated the product. In GMP philosophy, impurities on an industrial scale must be lower than at the laboratory level. The reason is that raw materials, solvents, and reagent qualities are checked, and reactor vessels, pipelines and centrifuges are cleaned with great care prior to the GMP production. The contamination from the facilities, consequently, might be slight. However, we found unexpected impurities in the case of scale-up. They could not be completely anticipated in the laboratory.

Dipeptide Production Process

The production process of L-alanyl-L-glutamine (AlaGln, **1**), modified for an industrial scale from D-chloropropionyl



AlaGln (1)

chloride and L-glutamine, has already been published.[1] The purification method for the synthesized crude AlaGln was as follows: a solution of the crude AlaGln cake in H_2O was passed through an anionic resin column in order to remove impurities.[2] The eluent was then concentrated and decolorized with activated carbon. The eluent was heated to 50 °C, and EtOH was then added for crystallization. The crystals of AlaGln cake were filtered off and dried in vacuo.

Scale-up Problem

The AlaGln product obtained on a plant scale had a problem with turbidity (runs 2, 3 in Table 1). A 10% water solution of AlaGln visually showed pale white turbidity. Such turbidity was not observed in laboratory products. The transparency measured with UV at 430 nm also showed lower values. The total contents of the byproducts in the product,[2] mainly the diastereomer, D-alanyl-L-glutamine, were almost the same as in the pilot sample (Table 1). Thus, it seemed that the byproducts did not cause the turbidity.

Table 1. **AlaGln on scale-up production; turbidity and byproducts**

run	facility	scale, kg	transparency (T %)[a]	total contents of byproducts[b], %
1	pilot	43	99.5	0.13
2	plant	160	93.9	0.18
3	plant	170	95.4	0.17
4	pilot	63 (recrystallization of run 2)	88.1 (wet cake)	0.05

[a] 10% water solution (C = 10), 430 nm. [b] HPLC area.

Identification of the Turbidity Compounds

At first, the turbidity seemingly originated from the plant equipment contamination such as vessel packing-seal grease (silicon oil) or stirring-shaft lubricating oil, because they seemed to soak into the reactor while not in use. The former was used in vessel packing when the upper (cover) and lower (vessel) parts were installed. The latter was used for the lubricant stirring-shaft and the cover. However, during a challenging test using them, turbidity was not observed.

Next, isolation of the impurities was attempted as follows: the turbid water solution of the AlaGln was filtered through a 0.2 μm mesh filter, and no turbidity was observed in the filtrate (transparency was the same as blank water, 100%). Therefore, all of the turbidity compounds were filtered off. The filter was washed with MeOH, and then the MeOH was analyzed by HPLC.

Four peaks were recognized mainly (HPLC retention time: 3 min (peak A), 5 min (peak B), 13 min (peak C), and 24 min (peak D), Figure 1). LC-MS analysis was conducted on each peak, and the structure was determined from the mass library[3] (Table 2). All of these compounds are widely used as polymer stabilizers (antioxidants, stabilizing agents to prevent discoloration). Peak C was thought to be an oxidized compound of tris (2,4-di-*tert*-butylphenyl) phosphite. Although the compound was not detected in Figure 1, it is commonly used as an antioxidant. Peaks A

(1) Sano, T.; Sugaya, T.; Inoue; K., Mizutaki, S.; Ono, Y.; Kasai, M. *Org. Process Res. Dev.* **2000**, *4*, 147.

(2) Major impurities are; *N*-(2*S*)-2-hydroxypropionyl-L-glutamic acid diamide, (2*S*,5*S*)-2-(2-carbamoylethyl)-5-methylpiperazine-3,6-dione, L-alanyl-L-glutamic acid diamide, (2*S*,4*S*)-2-aza-2,4-dimethyl-1,5-pentanedioyl-di-L-glutamine, D-alanyl-L-glutamine.

(3) The NIST (the National Institute of Standards and Technology) Mass Spectral Library Database, which was attached to the LC-MS, was used.

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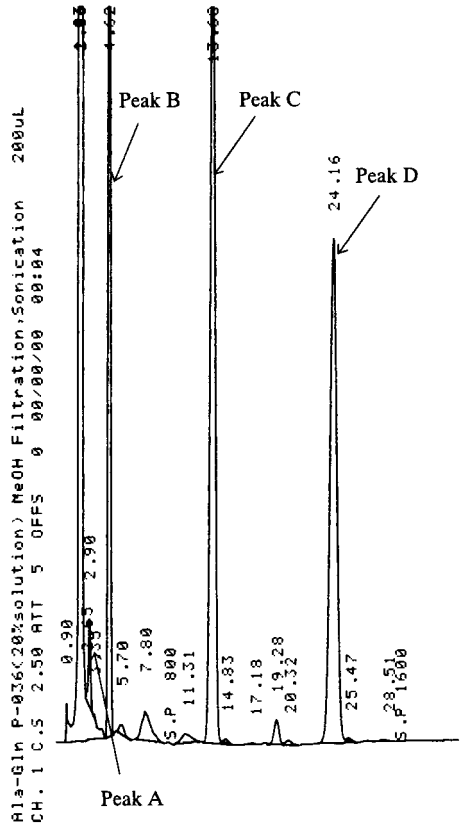


Figure 1. **HPLC chart of MeOH. Washing the filter is described in the text; run 2 is listed in Tables 1 and 3.**

and B (BHT and DOP) were supposedly from the filter itself because the blank test (see Experimental Section) also showed peaks A and B. Peak B was from a widely used polymer plasticizer, and it was easily detected from vinyl tape, the electric wire peel, a neutral filter, and so on.[4] The concentrations of these compounds were measured by HPLC analysis (Table 3). Fundamentally, peak A (BHT) and peak B (DOP) were found in MeOH treated with a blank filter. The measured value was revised with the blank filter concentration.

Study on the Turbidity Compounds

Prior to the purification, the crude AlaGln cake showed no turbidity In this purification, during the treatment with the resin column followed by activated carbon, no turbidity was found in the water.[5]

From these results (the wet cake showed turbidity), the product did not become contaminated with turbidity compounds before the activated carbon treatment or after drying. Therefore, all of the equipment, crystallizer, feed pipe, pressure filter, pumps, clothes, gloves, and storage bags were checked. That is, those components were rinsed with MeOH, and the MeOH was analyzed by HPLC. After that, no equipment, except for the natural rubber gloves, contained the peak B material, and the polyethylene bags used in this process contained peak C and peak D material!

The gloves were used to prevent contamination from human skin, and the material of the gloves was polyethylene in runs 1−3 and natural rubber in run 4 (Tables 1 and 3). The wet cake contacted the natural rubber gloves during transfer to the dryer pans. Thus, the wet cake of AlaGln during run 4 (Tables 1 and 3) contained peak B material at high concentration (runs 1 and 3 were treated with polyethylene gloves). However, the concentration of peak B changed with the location of the crystal of the wet cake. Peak B content on the surface of the lump crystal was 32 ppm, but only 5 ppm in the central part. Such distribution was evidence of the fact that peak B represented contamination from the surface of the crystal. It was found that a pair of natural rubber gloves released more than 2 g of the peak B material with EtOH[6] (about 10 ppm in 200 kg production approximately).

Peak C and peak D were found in polyethylene bags. The manufacturer of the polyethylene bags used 5 ppm of these two reagents as stabilizers. Polyethylene bags were used as containers for the wet cake storage. The polyethylene bag was changed in plant production. The bag size was 200 L on the plant scale but 90 L in the pilot plant.

Each polyethylene was a low-density type, and the bags and the "stabilizers" were from different manufacturers. During the wet cake storage, these stabilizer compounds transferred from the polyethylene to the cake. The experiments on storage of the wet cake using miniature bags, prepared from the same polyethylenes of real bags, were carried out, and the results are shown in Table 4. 200 g of AlaGln wet cake, prepared from 100 g of AlaGln dry cake and 100 g of EtOH, was packed into a polyethylene bag revised to 30-cm^2 size. It was stored in an isothermal box at 50°C for 5 h, followed by drying the cake and analyzing the concentration of impurities. The transfer of the polymer stabilizer to the product by the organic solvent media is a widely known phenomenon in the food area.[7] In this case, the temperature of the filtered cake was high,[8] and EtOH was used in the crystallization. The compounds causing turbidity were transferred very easily from the gloves and bags to the cake.

During runs 2 and 3 in Table 1, the turbidity was caused by the 200-L polyethylene bag. It was caused by natural rubber gloves during run 4 (Tables 1 and 3). To prevent the turbidity, the process has been changed as follows: the material of the gloves was returned to polyethylene. In this case, the contact time with the wet cake is very short; therefore, there is no worry about turbidity. The containers

(4) The operation rooms for this process have HEPA filters. DOP was used for the tracer for the HEPA filter ability ("DOP filter test"). However, the tested HEPA filter never spreads DOP.; Irie, T.; Mitui, Y.; Saiki, A.; Suzuki, M.; Shionozuka, M.; Otake, N. *Evaluation of organic components in clean room air using atmospheric pressure ionization mass spectrometer, The 10th Symposium of air cleaning and contamination control Japan*, 1991.

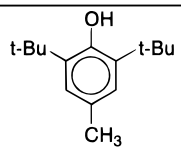
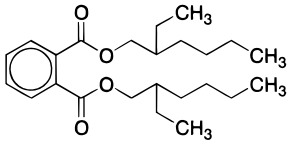
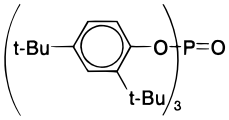
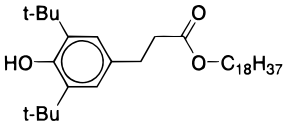
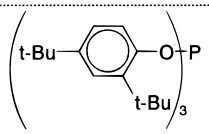
(5) Before the resin treatment, the water solution (about 30 wt %) of the crude cake did not show turbidity in an optical view check.

(6) A pair of natural rubber gloves (20 g) was soaked in 300 mL of EtOH for 10 min under reflux conditions, and the EtOH was evaporated. The residual was peak B material, and the weight was 2.2 g.

(7) For example: Bourges, F.; Bureau, G.; Pascat, B. *Food Addit. Contam.* **1993**, *10*, 443. Sarbach, C.; Yagoubi, N.; Sauzieres, J.; Renaux, D.; Ferrir, D.; Postaire, E. *Int. J. Parm.* **1996**, *140*, 169. We searched the database "CA File" on *CAS on line* (April, 1998, updated) for the words "polyethylene" and "transfer"; all of the literature sources were within the food area.

(8) The temperature of the filtration cake was maintained over 40 °C because of polymorphism.

Table 2. **Materials caused turbidity**

Peak	Name [CAS Registry No.]	Structure	Molecular Weight
A	2,6-Di-tert-butyl-4-methylphenol (BHT) [128-37-0]		$C_{15}H_{24}O$ 220.36
B	Dioctylphthalate (DOP) [117-81-7]		$C_{24}H_{38}O_4$ 390.56
C	Tris (2,4-di-tert-butylphenyl) phosphate [95906-11-9]		$C_{42}H_{63}O_4P$ 662.92
D	Stearyl 3- (4'-hydroxy-3',5' -di-tert-butylphenyl) propionate [2082-79-3]		$C_{35}H_{62}O_3$ 530.86
*	Tris (2,4-di-tert-butylphenyl) phosphite [31570-04-4]		$C_{42}H_{63}O_3P$ 646.92

*Precursor of peak C.

Table 3. **Concentration of the contaminants**

run	facility	peak B[a]	peak C	peak D
1	pilot	1.5 ppm	0.7 ppm	N.D.[b]
2	plant	4.5 ppm	1.3 ppm	2.2 ppm
3	plant	3.5 ppm	3.1 ppm	3.5 ppm
4	pilot	41.0 ppm	0.6 ppm	N.D.[b]

[a] Values were revised with blank filter (see Experimental Section). [b] Not detected.

Table 4. **Results of wet cake storage; turbidity and HPLC analysis**

container	*T*%[a] after 50 °C/5 h storage	peak B[b]	peak C	peak D
glassware	99.8	1.1 ppm	N.D.	N.D.
90 L polyethylene bag	97.0	1.3 ppm	9.6 ppm	N.D.
200 L polyethylene bag	92.2	2.0 ppm	8.6 ppm	8.4 ppm

[a] Before storage, *T*% was 99.9%. [b] Values were revised with blank filter.

for the wet cake storage were changed from polyethylene bags to stainless steel buckets. After these modifications, no turbidity was observed on the plant scale (Table 5).

Conclusions

During the production process of the dipeptide, we found some contaminants from gloves and bags. These contaminated compounds were not expected on a laboratory scale.

Table 5. **Modified production; turbidity and HPLC analysis**

run	scale	*T*%	peak B	peak C	peak D
1	160 kg, plant	100	1.4 ppm	0.04 ppm	N.D.
2	155 kg, plant	99.8	2.4 ppm	0.02 ppm	N.D.

[a] Values were revised with blank filter

So far, the transfer of the polymer stabilizer has been reported only with organic solvents. However, the storage of the wet cake, containing an organic solvent in a polyethylene bag, carries a risk of such contamination.

Experimental Section

All of the materials were commercially available. The analysis of the contaminated materials was carried out using HPLC as described below. LC-MS spectra were measured on a Micromass Quattro mass spectrometer. Transparency was recorded on a Hitachi Spectrometer U-1100.

Transparency Analysis. 1 (1.0 g cake) was dissolved in water to 10 mL, and a glass cell (1 cm) was used. The wavelength: 430 nm. The water transparency was set to 100% as the blank value.

Filtration Procedure. 1 (60 g cake) was dissolved in water to 300 mL (20 w/w%). The solution was filtered through a 0.2 mm cellulose nitrate filter (PALL Co., POSIDYNE N66, 47 mm) under pressure (0.8 kgf/cm^2). The filtering equipment: Advantec BT-700S.

HPLC Analysis. The column was Tohso ODS-120T, 250 × 4.6 mm and the precolumn was Advantec Dismic-13HP

(0.45 mm). The column temperature was 30 °C. The mobile phase was CH_3CN. The flow rate was 1.5 mL/min. Detection was UV 210 nm.

After the filtration mentioned above, the filter was washed with 25 mL of MeOH, and the material was analyzed with HPLC (charge volume: 200 μL).

The concentrations of peaks A−D were analyzed by the absolute calibration method with commercial reagents. The quantities were revised with the blank value of the filter. The blank test was operated as follows: a filter that passed 300 mL of water was washed with 25 mL of MeOH, and the MeOH solution was analyzed with HPLC. Peak A (BHT) was present at 13 ppm, and peak B (DOP) at 11 ppm appropriately.

LC-MS Measurements. HPLC conditions were as follows: column, Tohso ODS-120T, 250 × 4.6 mm; column temperature, 30 °C; mobile phase, CH_3CN; flow rate, 1.5 mL/min; detection, UV 210 nm. MS conditions were as follow: ion mode, ESI positive; scan range, *m*/*z* 50−800, source temperature, 150 °C. Found (*m*/*z*) 2,6-di-*tert*-butyl-4-methylphenol (BHT, peak A) $C_{15}H_{24}O$ *m*/*z* 221 $(M + H)^+$, Tris(2,4-di-*tert*-butylphenyl) phosphate (peak C[9]) $C_{42}H_{63}O_4P$ *m*/*z* 663 $(M + H)^+$, stearyl 3-(4′-hydroxy-3′,5′-di-*tert*-butylphenyl) propionate (peak D) $C_{35}H_{62}O_3$ *m*/*z* 531 $(M + H)^+$. The retention time and fragment patterns of all compounds were the same as those of the commercial reagents and the synthesized reagent (peak C[9]).

(9) Tris(2,4-di-*tert*-butylphenyl) phosphate (peak C) was synthesized as follows: 6.47 g (10 mmol) of tris (2,4-di-*tert*-butylphenyl) phosphite (Aldrich Co.) was added to THF (100 mL) and H_2O (10 mL) solution at room temperature. Iodine (7.61 g, 30 mmol) in THF (30 mL) was then added dropwise over a few minutes. The reaction mixture was stirred for 1 h at the same temperature followed by condensation and extraction with $CHCl_3$. The organic layer was then concentrated. The residue was treated with MeOH (100 mL) at room temperature, and the white crystal of peak C material was precipitated. The yield was 65% (4.3 g), and the structure was confirmed by NMR spectroscopy and LC-MS. Such iodine oxidation from phosphite to phosphate is referred to in Letsinger, R.; Lunsford, W. B. *J. Am. Chem. Soc.* **1976**, *98*, 3655. Compound C was synthesized by γ radiation by the following literature: Allen, D. W.; Leathard, D. A.; Smith, C. *Chem. Ind.* **1987**, *12*, 854.

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