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Contaminants in biotechnologically manufactured L-tryptophan

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Abstract

The epidemic outbreak of a new disease, the eosinophilia–myalgia syndrome (EMS), was traced back to the intake of L-tryptophan (Trp) of certain lots from a single manufacturer. Since some trace contaminants were related to EMS, it appeared to be necessary to identify and find the origin of most trace contaminants in the EMS related Trp in order to apply this knowledge to a prospective manufacturing practice. Seventeen contaminants were determined in an implicated Trp lot by a single reversed-phase high-performance liquid chromatography run using UV and fluorescence detection. Most of these contaminants were classified as Trp metabolites, non-physiological oxidation or carbonyl condensation compounds of Trp. The amount and the pattern of contaminants were compared with recently manufactured Trp and Trp-containing preparations.

Keywords: Tryptophan; Eosinophilia–myalgia syndrome (EMS); Biotechnology

1. Introduction

A new multisystemic disease, the eosinophilia–myalgia syndrome (EMS), has been linked to the intake of L-tryptophan (Trp) as a dietary supplement and as a “natural” drug prescribed for insomnia and depression [1]. World-wide more than 1600 people have been affected by this illness, including 38 deaths [2]. The epidemic outbreak of EMS was traced back to Trp from a single Japanese manufacturer, Showa Denko KK (SD). These findings led to the conclusion that one or more contaminants in the implicated Trp might cause EMS [1]. Radiochemical and microbiological studies have detected no significant contamination nor has analysis for inorganic elements done so [3]. Although SD-Trp was generally more than 98.5% pure (which fulfilled the de-

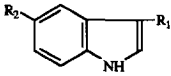
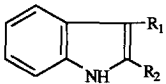
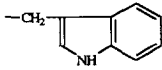
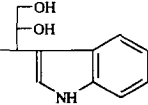
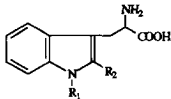
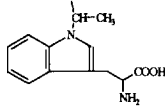
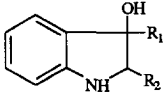
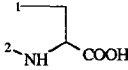
mands of US and German pharmacopoeia at that time) more than 60 contaminant peaks could be detected by reversed-phase high-performance liquid chromatography (RP-HPLC) and UV detection [4].

To date, several trace contaminants (<0.1%) have been identified, either by assignment to standard substances or by isolation from the implicated Trp-material and subsequent structural analysis (Table 1). 1,1'-Ethylidenebis(L-tryptophan) (peak E) (1,1'-EBT) was structurally elucidated by Mayeno et al. [5] and Smith et al. [6] contemporaneously. Toyo'oka et al. [7] identified 5-hydroxytryptophan (5-OH-Trp), indolealdehyde and indole (Ind). Müller et al. [8] characterized the four major contaminant peaks that are typical for the by-product pattern of SD-Trp: *cis*- and *trans*-1-(3-indolylmethyl)-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (IMTHCC); 2-(3-indolylmethyl)-L-tryptophan (IMT) and 2-[2,3-dihydroxy-1-(3-indolyl)propyl]-L-

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Table 1
Contaminants identified in EMS related Showa-Denko-L-tryptophan (for chromatogram see Fig. 3)

1 a) Indole derivatives

	R ₁	R ₂	Name, peak number, abbreviation, first citation
	-H -CHO -CH ₂ -COOH $\begin{matrix} \text{NH}_2 \\ \\ -\text{CH}_2-\text{CH}-\text{COOH} \end{matrix}$	-H -H -H -OH	Indole (Ind) [7] Indolealdehyde [7] Indole-3-acetic acid (16) (IAA) 5-Hydroxytryptophan (5) (5-OH-Trp) [7]
	$\begin{matrix} \text{NH}_2 \\ \\ -\text{CH}_2-\text{CH}-\text{COOH} \end{matrix}$ $\begin{matrix} \text{NH}_2 \\ \\ -\text{CH}_2-\text{CH}-\text{COOH} \end{matrix}$ $\begin{matrix} \text{NH}_2 \\ \\ -\text{CH}_2-\text{CH}-\text{COOH} \end{matrix}$	  -OH	2-(3-Indolylmethyl)-L-tryptophan (21) (IMT) [8] 2-[2,3-Dihydroxy-1-(3-indolyl)propyl]-L-tryptophan (20) (dhPIT) [8] 2-Hydroxytryptophan (Ox-indolylalanine) (8) (Oia) [10]
		-H	1,1'-Ethylidenebis(L-tryptophan) (18) (1,1'-EBT) [5, 6]
	$\begin{matrix} \text{NH}_2 \\ \\ -\text{CH}_2-\text{CH}-\text{COOH} \end{matrix}$	=O 	Dioxindolylalanine (2) (DiOia) [10] 3a-Hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-indole-2-carboxylic acid (1) (PIC)

1 b) Phenyl derivatives

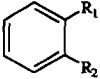
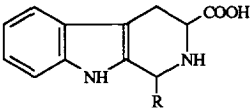
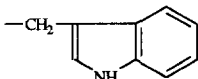
	R ₁	R ₂	Name, peak number, abbreviation, first citation
	-COOH $\begin{matrix} \text{O} & \text{NH}_2 \\ & \\ -\text{C}-\text{CH}_2-\text{CH}-\text{COOH} \end{matrix}$ $\begin{matrix} \text{O} & \text{NH}_2 \\ & \\ -\text{C}-\text{CH}_2-\text{CH}-\text{COOH} \end{matrix}$ $\begin{matrix} \text{NH}_2 \\ \\ -\text{NH}-\text{CH}_2-\text{CH}-\text{COOH} \end{matrix}$	-NH ₂ -NH ₂ -NHCHO -H	Anthranilic acid (9) (AA) Kynurenine (4) (Kyn) [10] N-Formylkynurenine (6) (NFK) 3-Phenylamino-L-alanine (7) (3-PAA) [9]

Table 1. Continued

1 c) 1,2,3,4-Tetrahydro- β -carboline-3-carboxylic acid derivatives

	R	Name, peak number, abbreviation, first citation
	-H	1,2,3,4-Tetrahydro- β -carboline-3-carboxylic acid (12) (THCC) [8]
	-CH ₃	1-Methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (cis) (13) (MTHCC) [34]
		

tryptophan (dhPIT). In addition, they identified 1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (THCC) and 1-methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid (MTHCC) as minor contaminants. Goda et al. [9] elucidated the structure of a contaminant peak as 3-phenylamino-L-alanine (3-PAA). Oxindolylalanine (Oia), dioxindolylalanine (DiOia) and kynurenine (Kyn), three tryptophan oxidation compounds, were determined in SD-Trp by the authors [10].

SD-Trp was manufactured by fermentation with genetically modified strains of *Bacillus amyloliquefaciens*. Prior to the epidemic outbreak of EMS, the manufacturing process had undergone several modifications in the fermentation (genetic modification of the bacillus strain used) and purification processes. Belongia et al. [1] determined the use of *B. amyloliquefaciens* strain V and the reduction of the amount of powdered activated carbon to be significantly correlated with EMS.

Hill et al. [4] summarized the attempts to associate single contaminants with the occurrence of EMS by the comparison of case and non-case lots of SD-Trp. They found 6 of 60 examined contaminant peaks to be associated with EMS. Three of these are structurally characterized as 1,1'-EBT, 3-PAA and IMT (Table 1).

Some features of EMS could be triggered with 1,1'-EBT in mice [11] or Lewis rats [12]. After administration to Wistar rats, very small amounts of 1,1'-EBT and its decomposition products could be

determined in blood and urine [13]. 1,1'-EBT was shown to become incorporated in vitro into proteins [14]. Under acidic conditions it decomposes to MTHCC and Trp [15,16].

3-PAA was detected in blood, liver, kidney and brain after administration to Lewis rats, which indicated passage of this substance through the blood-brain barrier [17]. The detection of four metabolites of 3-PAA led to the conclusion that it is metabolized enzymatically, analogous to the phenylalanine pathway [18]. Sidransky et al. [19] assume that 3-PAA is incorporated into proteins. As yet nothing is known about the formation, toxicology and metabolism of IMT.

However there is still no animal model that establishes all the features of EMS and there is still a lack of statistical significance for the association of EMS with the three above mentioned substances [4,20]. This raises the possibility that the etiologic agent of EMS is still unknown [21]. For that reason, the licence for the use of Trp containing drugs for oral application has been withdrawn by the German authorities [22]. Since the use of Trp and 5-OH-Trp is obligatory for certain diets and parenteral nutrition, new requirements for the purity of Trp raw materials have been introduced into the monograph for Trp in the German pharmacopoeia DAB 10 [23]. In this paper, we report a simple and sensitive method for the simultaneous determination of EMS related compounds, Trp breakdown products and compounds of the Trp pathway. We discuss the

results of this application to Trp raw material from three different sources and to Trp-containing infusion solutions.

2. Experimental

2.1. Apparatus

The HPLC apparatus consisted of a Merck L-6200 pump, L-6000 UV detector, F-1080 programmable fluorescence detector (Darmstadt, Germany) and a Waters 994 programmable photodiode array detector (Milford, MA, USA). Signals were acquired and calculated by Bruker Chromstar software (Bremen, Germany). UV maxima were determined with a Kontron Uvikon 930 spectrophotometer (Neufahrn, Germany). Excitation and emission spectra of fluorescent substances were acquired by the fluorescence detector mentioned above.

2.2. Chromatographic conditions

The best results were obtained with the chromatographic conditions described in Table 2. UV and fluorescence detection was performed simultaneously by connecting the monitors in series. Photodiode array detection was only done for confirmation of the identity of detected substances. For the determination of Ind in SD-Trp, the conditions as described in [10] had to be chosen because coelution with dhPIT occurred when using the method given in Table 2. Methanol (gradient grade), acetonitrile (gradient grade) and trifluoroacetic acid (TFA) (Uvasol) were purchased from Merck. Deionized water was purified by Heraeus-Destamat Bi18E (bidestillator) (Kleinstheim, Germany).

2.3. Materials

N-Acetyl-D,L-tryptophan (AcTrp), Ind, indole-3-acetic acid (IAA), anthranilic acid (AA) and 5-OH-Trp were purchased from Merck. D,L-Kynurenine (Kyn) and D,L-indole-3-lactic acid (ILA) were obtained from Sigma (Deisenhofen, Germany) and serotonin creatininesulfate from Fluka (Neu-Ulm,

Table 2
HPLC conditions

Stationary phase	Nucleosil 120 3-C ₁₈ , 250×4 mm, precolumn 20×2 mm			
Mobile phase	0.1% TFA	Methanol	Acetonitrile	
	(%)	(%)	(%)	
Gradient	0 min	90	10	0
	0–5 min	86	14	0
	5–10 min	79	14	7
	10–40 min	41	14	45
	40–45 min	41	14	45
	45–46 min	90	10	0
	46–55 min	90	10	0
Temperature	37°C			
Flow-rate	1.0 ml/min			
Injection volume	20 µl			
Detection	UV, 260 nm			
	Fluorescence program:			
		Ex (nm)	Em (nm)	
	0–11 min	230	342	
	11–13 min	325	425	
	13–40 min	280	335	
	Photodiode array detection UV-Vis spectra, 200–400 nm			

Germany). Pharmaceutical grade Trp raw material was made available by two different manufacturers. EMS associated Trp (Showa Denko lot 67 236 201) was granted by Centers of Disease Control (CDC, Atlanta, GA, USA).

Oia, DiOia, N-formylkynurenine (NFK), THCC and MTHCC were synthesized as had been published previously [10]. By fractionated crystallisation of the diastereomeric mixture of (1*S*,3*S*)- and (1*R*,3*S*)-MTHCC (14:1) from water, we obtained the 1*S*,3*S*-diastereomer in good purity (>98%, RP-HPLC-UV_{280 nm}) (absolute configuration was determined by [24]). 3a-Hydroxy-1,2,3,3a,8,8a-hexahydropyrrolo-indole-2-carboxylic acid (PIC) was synthesized according to Savige [25] by treating 5 g of Trp dissolved in 500 ml ice cooled water with 17 ml peracetic acid solution (prepared by stirring 24.5 g acetic anhydride and 5 g 30% hydrogen peroxide for 4 h at 40°C and 18 h at room temperature). After 40 h stirring at 0°C, 100 µl dimethylsulfide was added

to arrest the reaction. After removal of the solvents, the residue was suspended in 40 ml water and filtered. For separation of the product, cation-exchange clean-up of the filtrate was performed as follows: column, Dowex 50×2 (H^+ -counter ion) 100–200 mesh, 120×26 mm; eluent A, water (200 ml); eluent B, 6 M formic acid (200 ml); linear gradient 0–100% B; flow-rate 2 ml/min. The remaining Trp was subsequently eluted with 2 M NH_3 . Only the earlier eluting product of 2 diastereomers (the *trans*-isomer according to [26]) was isolated in good purity (>98% RP-HPLC-UV_{240 nm}).

Synthesis of 3-PAA, 1,1'-EBT and 2,2'-ethylidenebis[L-tryptophan] was performed by Eulitz et al. (publication in preparation).

2.4. Sample pretreatment

Infusion solutions were directly injected onto the HPLC system, while Trp materials were dissolved in 0.1% aqueous TFA (10 mg/ml) and ultrasonic-treated prior to injection.

3. Results and discussion

3.1. Analytical features

Trp and eighteen metabolites and degradation compounds were separated in a single run (Fig. 1). An acid pH of the eluent at about 2.0 provides protonation of the carboxyl group and by this improves retention on RP-phases. For this application, the ion pairing effect of TFA proved to give better selectivity than that of phosphoric acid. The use of a volatile buffer system simplifies handling of the chromatographic system. Oia diastereomers (no. 8 in Table 3) are interconverted by enol-tautomerism during chromatography. A ternary gradient was therefore chosen, to achieve similar retention times for both isomers.

Table 3 lists spectroscopic properties of the examined substances and the sensitivity of UV detection at a signal-to-noise ratio of 3:1 and the HPLC conditions described in Table 2. Above the detection limit, the UV-detector response proved to be linear for all the examined standard substances.

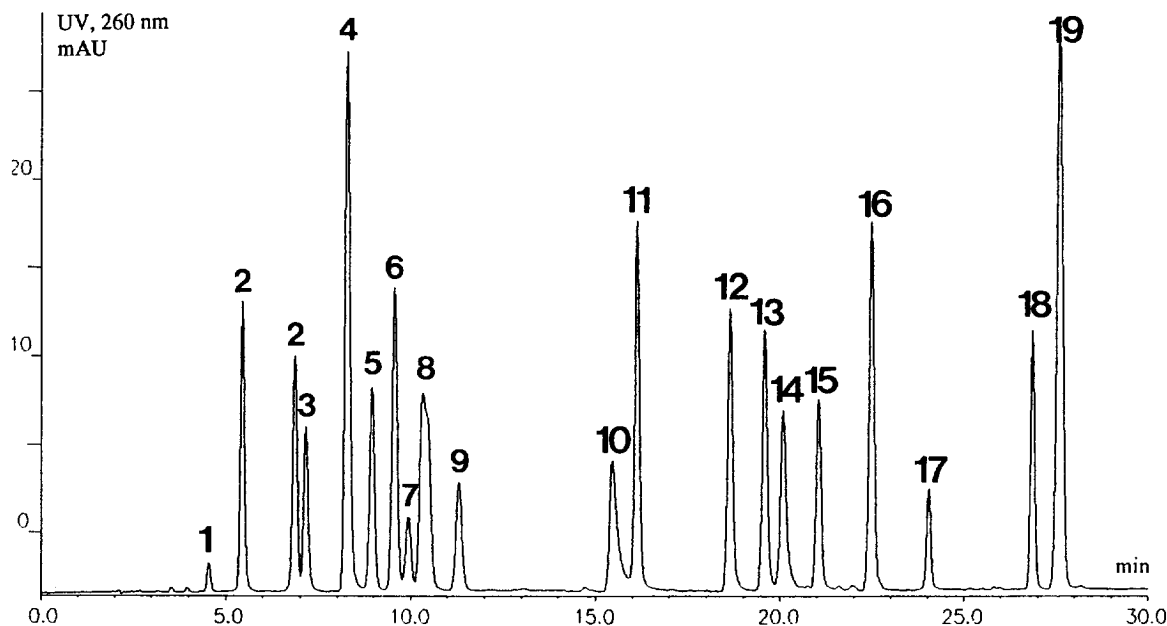


Fig. 1. Chromatogram (UV_{260 nm}) obtained from a standard mixture of 19 compounds (10 mg/l) (for conditions see Table 2, for peak identification Table 3).

Table 3

Abbreviations, spectral properties and UV_{260 nm} detection limit of standards (for chromatogram see Fig. 1)

Peak no.	Substance	Abbreviation	Fluorescence ($\lambda_{ex}/\lambda_{em}$, max) (nm)	UV (λ_{max}) (nm)	Detection limit (UV _{260 nm}) ($\mu\text{g/l}$)
1	3a-Hydroxy-1,2,3,3a,8,8a-hexahydro-pyrroloindole-2-carboxylic acid	PIC A	230, 285/340	233, 291	400
2	Dioxindolylalanine	DiOia A,B	none	207, 252, 287	70
3	Serotonine	5-HT	215, 270/335	218, 273	70
4	Kynurenine	Kyn	none	224, 255, 360	20
5	5-Hydroxytryptophan	5-OH-Trp	220, 270/330	215, 274	50
6	N-Formylkynurenine	NFK	225,255,315/435	224, 255, 326	30
7	3-Phenylamino-L-alanine	3-PAA	238, 285/340	240, 288	150
8	Oxindolylalanine	Oia	none	204, 248	60
9	Anthranilic acid in water in 0.1% TFA (pH 2)	AA	215, 305/395 218, 275, 335/420	210, 240, 311 218, 272, 328	150
10	Tryptamine	Tra	270/350	215, 277	100
11	Tryptophan	Trp	215, 270/350	216, 277	30
12	1,2,3,4-Tetrahydro- β -carboline-3-carboxylic acid	THCC	215, 265/340	220, 276	40
13	1-Methyl-1,2,3,4-tetrahydro- β -carboline-3-carboxylic acid	MTHCC, <i>cis</i>	215, 265/345	217, 269, 275	40
14	Indole-3-lactic acid	ILA	215, 270/360	218, 278	50
15	N-Acetyltryptophan	N-AcTrp	215, 270/355	218, 278	60
16	Indole-3-acetic acid	IAA	215, 270/360	216, 277	40
17	2,2'-Ethylidenbis(L-tryptophan)	2,2'-EBT	215, 275/355, 430	216, 280, 345	50
18	1,1'-Ethylidenbis(L-tryptophan)	1,1'-EBT	215, 270/350	216, 280	60
19	Indole	Ind	210, 265/350	212, 267, 283	30

This was not influenced by the matrix of infusion solutions or by overloading the column with Trp (10 mg/ml). The fluorescence detector response was found to be non-linear at concentrations below the UV_{260 nm} detection limit. Because of their importance as EMS related substances [4], a practical limit of detection for 3-PAA (Fl 230/340 nm) (no. 7 in Table 3) and 1,1'-EBT (Fl 280/335 nm) (no. 18 in Table 3) was determined by standard addition to Trp-material not contaminated by these substances. Both could be detected in Trp at the 1 ppm level (1 mg contaminant/kg Trp) using fluorescence detection (Fig. 2a,b) and at 6 ppm (1,1'-EBT, UV_{260 nm}) and 5 ppm levels (3-PAA, UV_{240 nm}) using UV detection. This corresponds with the results of Knight and Davidson [27] who determined the limit of detection for 1,1'-EBT in Trp at 3 ppm (Fl 280/342 nm) and 8 ppm (UV 220 nm), respectively. By using C₁₈ solid-phase extraction, 0.6 ppm 1,1'-EBT (UV 280 nm) are reported to have been detected in spiked Trp [28]. However, the clean-up procedure of this method is both time and cost consuming and most of the

other contaminants, such as 3-PAA, are either lost or coeluted with clean-up artefacts.

The fluorescence of 3-PAA proved to be dependent on protonation of aniline-N. If necessary, the sensitivity of 3-PAA detection might be enhanced by raising the pH of the eluent. The identification of peaks was done by retention time and their response in UV- and fluorescence detectors, both compared with standard runs. Further confirmation was obtained by comparison of UV spectra of standards with those obtained by HPLC with photodiode array detection and by spiking of the samples with standard substances. Peaks were assigned to IMTHCC (22,23), dhPIT (20) and IMT (21) by comparison of the RP-HPLC typical SD-Trp contaminant pattern with the characterization reported by Müller et al. [8] (Fig. 3).

The sum parameters of UV_{260 nm}-detectable contaminants prior to and after the Trp peak were calculated as Trp by the addition of peak areas (UV, 260 nm; least signal-to-noise ratio, 3:1) and by assigning them to an external Trp standard.

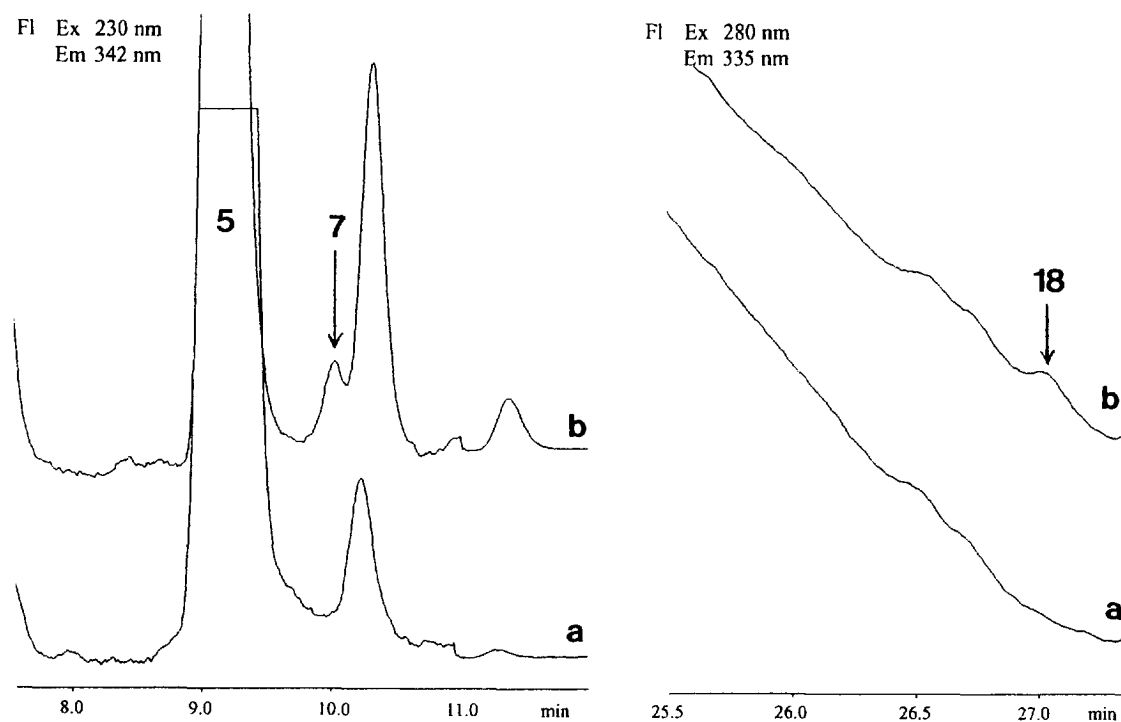


Fig. 2. Chromatograms obtained from (a) 1,1'-EBT and 3-PAA free Trp (10 mg/ml) and (b) the same Trp spiked with 1 ppm of 1,1'-EBT and 3-PAA (for conditions see Table 2). Peaks: 5=5-OH-Trp; 7=3-PAA; 18=1,1'-EBT.

3.2. Contaminants in an EMS related SD-Trp raw material

Fig. 3 shows a chromatogram of an EMS related SD-Trp lot (10 mg/ml). Most UV_{260 nm}-detectable substances with a high response could be identified and classified according to their formation into three groups (Table 4): (1) compounds derived from Trp by oxidative degradation; (2) compounds derived from Trp by carbonyl condensation reaction of Trp and aldehydes; (3) physiological metabolites of Trp.

3.2.1. Oxidation compounds

Non-physiological oxidation is known to occur during heat or acidic treatment of Trp in the presence of air [29]. The detection of PIC (1), DiOia (2) and Oia (8), which are all non-physiologic Trp oxidation compounds [30], indicates a rough treatment of SD-Trp during the manufacturing process. Peaks numbered 24 are supposed to be OH-Trp isomers because of their elution and fluorescent properties

(Fig. 3). Similar eluting peaks were derived from γ -irradiated Trp solutions. Their entire characterization is in progress. They may likewise be classified as non-specific oxidation compounds. Hill et al. [4] mentioned that the structure of case associated Peak C (named by Hill et al.) might be an isomer of 5-OH-Trp.

3.2.2. Carbonyl condensation compounds

Reaction of Trp and aldehydes is known to lead to the formation of the so called 1,2,3,4-tetrahydro- β -carboline-3-carboxylic acids via the Pictet-Spengler mechanism. Since acet- and formaldehyde are formed during fermentation processes, THCC (12) and MTHCC (13) are likely to be found in any biotechnologically derived Trp. Surprisingly the Trp-dimer 1,1'-EBT (18), which is likewise formed by carbonyl condensation reaction of Trp and acetaldehyde, was found in even higher amounts in EMS related SD-Trp than MTHCC (Table 3). The IMTHCC diastereomers (22, 23) are supposed to be

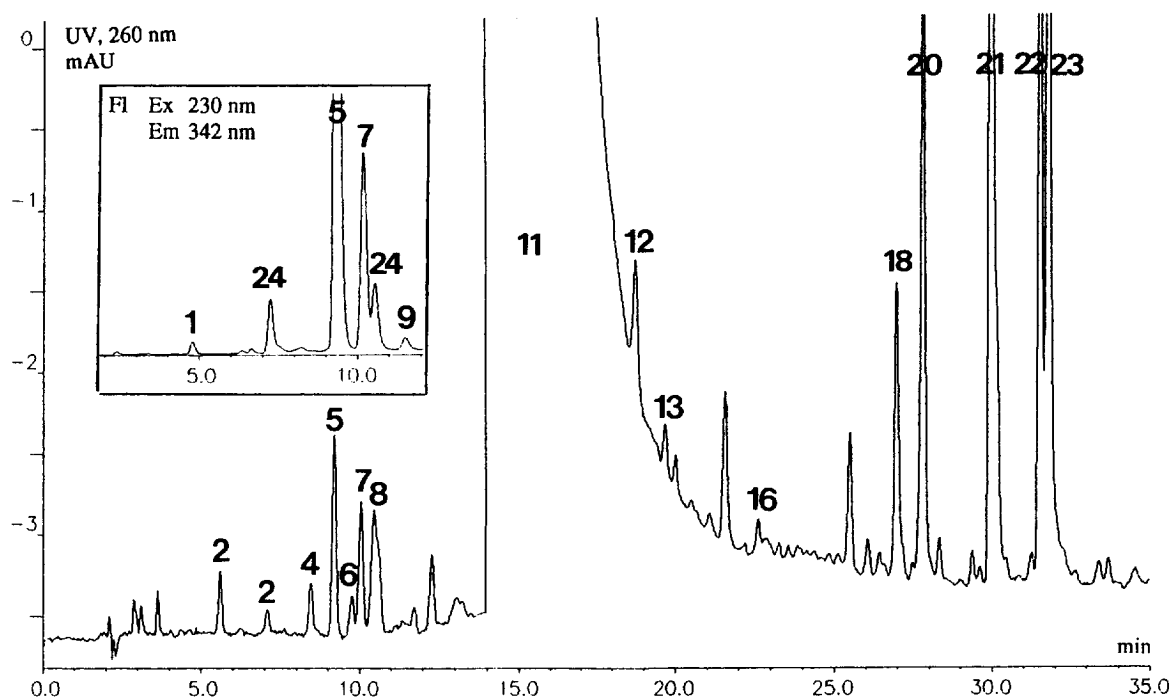


Fig. 3. Chromatogram obtained from EMS related SD-Trp lot (67 236 201) (10 mg/ml) (for conditions see Table 2, for peak identification see Table 1, for amounts see Table 4).

Table 4
Contaminants in Showa Denko Trp lot 67 236 201 (for abbreviations see Tables 1 and 3)

Metabolites	Oxidation compounds	Carbonyl condensation compounds	EMS-related compounds
<i>Kyn-pathway</i>	<i>Indole-oxidized</i>	<i>Pictet-Spengler</i>	
Kyn (4) 9 ppm	PIC (1) 20 ppm ^a	THCC (12) 45 ppm	3-PAA (7) 220 ppm
NFK (6) 11 ppm	Oia A,B (8) 73 ppm	MTHCC (13) 15 ppm	1,1'-EBT (18) 105 ppm
	DiOia A,B (2) 28 ppm	IMTHCC (22,23) detectable ^c	IMT (21) detectable ^c
<i>5-HT-pathway</i>	<i>Phenyl-oxidized</i>	<i>Dimerization</i>	
5-OH-Trp (5) 100 ppm	5-OH-Trp (5) 100 ppm	2,2'-EBT (17) <1 ppm ^b	
5-HT (3) <0.1 ppm ^h	OH-Trp-isomers (24) detectable ^{a,c}	1,1'-EBT (18) 105 ppm	
<i>IAA-pathway</i>	<i>Pyrrole-cleaved</i>		
Tra (10) - ^d	Kyn (4) 9 ppm		
ILA (14) <1 ppm ^h	NFK (6) 11 ppm		
IAA (16) 10 ppm			
<i>Biosynthesis-intermediates</i>			
AA (9) 13 ppm			
Ind (19) 30 ppm			

^a Fluorescence detection.

^b Practical limit of detection (fluorescence).

^c Quantification not possible without standard.

^d Coelution with Trp-peak.

derived by the Pictet–Spengler reaction of Trp and indole-3-acetaldehyde [8], a metabolite of Trp. Since Pictet–Spengler reactions are acid catalysed, formation of these carbonyl condensation compounds might have occurred either during fermentation (pH 7.0) or after adjustment of pH to 1.7 [31] prior to the cation-exchange clean-up step.

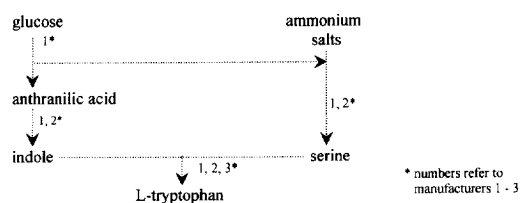
3.2.3. Metabolites

Among the selected metabolites, 5-OH-Trp (5) was the major component (Fig. 3, Table 4). This may be due to the similarity of the physicochemical properties of 5-OH-Trp and Trp, which renders a complete separation during the purification procedure difficult. NFK (6) and Kyn (4) can be formed either by non-specific oxidation or by enzymatic Trp metabolism and were detected in SD-Trp in small amounts. Non-amino acid Trp-metabolites were determined in very small amounts [IAA (16)] or were not detectable (ILA). Toyo'oka et al. [7] describe the identification of traces of indolealdehyde. AA (9) was used as the starting material for the biosynthesis of SD-Trp (Fig. 4) and was determined in low amounts in the final product. The almost absence of non-amphoteric metabolites or degradation compounds may be traced back to the SD-purification process (Fig. 4), which includes both anion- and cation-exchange clean-up.

3.2.4. EMS related compounds

To date, the association of the structurally identified EMS-related contaminants to EMS lacks statistical significance. Nevertheless, 1,1'-EBT (18) and 3-PAA (7) might be used as markers for potentially EMS related Trp. Knowledge of the formation conditions of the mentioned contaminants may help to prevent their formation by a prospective manufacturing practice or to set demands for a safe purification process.

During laboratory scale experiments simulating the operation conditions of the commercial SD plant, Sakimoto and Torigoe [31] found no 1,1'-EBT in the fermentation broth of the EMS related *B. amyloliquefaciens* strain V, but determined reasonable amounts of this impurity after cation-exchange chromatographic process at pH 1.7 regardless of the *B. amyloliquefaciens* strain used. The 2,2'-Trp dimer which is formed under strongly acid conditions



manufacturer	1	2 = Showa Denko K.K.	3
microorganism	unknown	<i>B. amyloliquefaciens</i>	<i>Escherichia coli</i>
substrates	glucose, ammonium salts	anthranilic acid, ammonium salts	indole, D,L-serine
purification process	unknown	centrifugation, reverse osmosis, anion IEC, cation IEC, activated carbon,	centrifugation, evaporation crystallization, activated carbon

Fig. 4. Manufacturing processes of three different pharmaceutical grade Trp raw materials.

(pH<0) could not be detected in the implicated SD-Trp. These facts elucidate the conditions that promote formation of 1,1'-EBT and will be described in detail by Eulitz et al. (publication in preparation).

Toyoda et al. [32] suggest that 3-PAA could be formed under the fermentation and purification conditions used by SD from AA and serine. This is supported by the findings of Eulitz et al. (publication in preparation), who were able to synthesize 3-PAA from dehydroalanine and aniline. Synthesis of IMT (21) might yield further useful information about the conditions under which EMS related substances are formed.

The German Pharmacopoeia DAB 10 [23] demands for the RP-HPLC purity of pharmaceutical grade Trp that a peak coeluting with 1,1'-EBT must not exceed 8 ppm (mg 1,1'-EBT/kg Trp); the sum of UV_{220 nm}-detectable contaminant peaks eluting prior to Trp must not exceed 100 ppm; those eluting after Trp, 300 ppm (both calculated with N-Ac-Trp as internal standard). No specifications are made for 3-PAA or other single substances.

3.3. Quality status of recent L-Trp materials and preparations

SD-Trp and Trp raw material of two further manufacturers were chosen to compare the amount and pattern of impurities. Manufacturer 1 (M 1) uses glucose and ammonium salts as substrates for a de

novo synthesis of Trp, manufacturer 3 (M 3) performs a biotransformation by *Escherichia coli* Trp-synthetase with Ind and D,L-serine as substrates [33]. Even purification procedures, as far as they were given out by the manufacturer, are markedly different (Fig. 4). In both recently manufactured lots, 3-PAA and 1,1'-EBT were not detectable (<1 ppm). Although M 1 uses a de novo synthesis of Trp, metabolites, except for 5-OH-Trp, were below 1 ppm in the final product. Surprisingly no Ind (<0.1 ppm) was detectable in the final product of M 3. Both M 1 and M 3 Trp lots, contained traces of Pictet–Spengler derived carbonyl condensation compounds (THCC and MTHCC) and minor peaks coeluting with IMTHCC (*cis, trans*) (22,23) and IMT (21), known from SD-Trp. All results indicate that the purification process, not the fermentation, governs the pattern of contaminants in biotechnologically derived Trp. Most remarkable is the comparison of the total amount of contaminants calculated as Trp equivalents eluting prior to or after the Trp peak. While Trp of M 1 and M 3 contained less than 100 ppm prior to as well as after the Trp peak, the examined SD-Trp lot contained 340 ppm prior to and 1990 ppm after the Trp peak (Fig. 5). This supports the assumption that, in addition to the application of 3-PAA and 1,1'-EBT as markers for EMS-suspicious Trp, the introduction of contaminant-sum-parameters

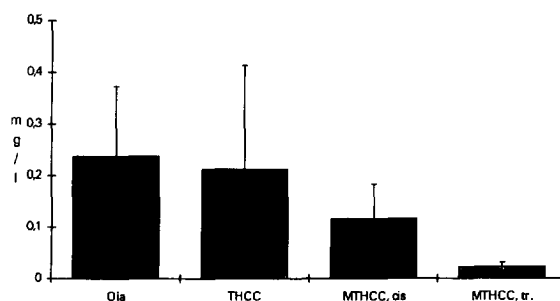


Fig. 6. Contaminants in L-Trp-containing infusions, result of eight examined infusion solutions of different composition and Trp content (0.6–2.3 g/l).

to purity control is useful, taking into account that the etiologic agent of EMS might still be unknown.

Since 1990, Trp use in Germany has only been licensed for certain dietary preparations and parenteral feeding by the German authorities. Eight infusion solutions of different composition and Trp content (0.6–2.3 g/l) were analysed to identify Trp-related impurities. Oia and Pictet–Spengler derived carbonyl condensation compounds (THCC and MTHCC *cis, trans*) were detected in varying amounts (Fig. 6). It remains to be established whether their amount is influenced by storage time and storage conditions. In a glucose containing infusion solution, an UV-absorbing, fluorescent contaminant peak eluting prior

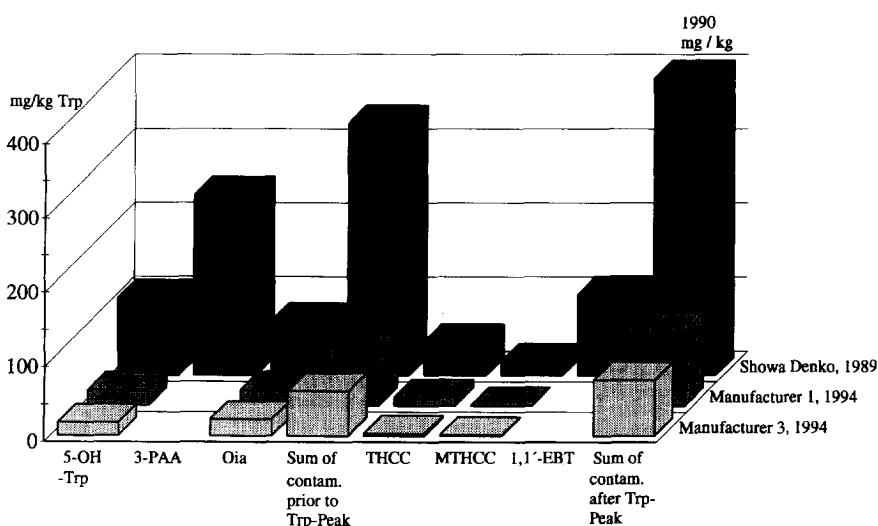


Fig. 5. Contaminants in EM-related SD-Trp and in two recently manufactured Trp lots.

to Trp was detected. This finding has to be confirmed by the examination of several products of this composition. This unknown compound might be a Pictet–Spengler adduct of Trp and glucose; its synthesis is in progress.

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References

- [1] E.A. Belongia, C.W. Hedberg, G.J. Gleich, K.E. White, A.N. Mayeno, D.A. Loegering, S.L. Dunnette, P.L. Pirie, K.L. MacDonald and M.T. Osterholm, *N. Engl. J. Med.*, 323 (1990) 357.
- [2] E.M. Kilbourne, *Epidemiol. Rev.*, 14 (1992) 16.
- [3] P.A. Hertzman, H. Falk, E.M. Kilbourne, S. Page and L.E. Shulman, *J. Rheumatol.*, 18 (1991) 867.
- [4] R.H. Hill, S.P. Caudill, R.M. Philen, S.L. Bailey, W.D. Flanders, W.J. Driskell, M.L. Kamb, L.L. Needham and E.J. Sampson, *Arch. Environ. Contam. Toxicol.*, 25 (1993) 134.
- [5] A.N. Mayeno, F. Lin, C.S. Foote, D.A. Loegering, M.M. Ames, C.W. Hedberg and G.J. Gleich, *Science*, 250 (1990) 1707.
- [6] M.J. Smith, E.P. Mazzola, T.J. Farrell, J.A. Sphon, S.W. Page, D. Ashley, S.R. Sirimanne, R.H. Hill and L.L. Needham, *Tetrahedron Lett.*, 32 (1991) 991.
- [7] T. Toyooka, T. Yamazaki, T. Tanimoto, K. Sato, M. Sato, M. Toyoda, M. Ishibashi, K. Yoshihira and M. Uchiyama, *Chem. Pharm. Bull.*, 39 (1991) 820.
- [8] A. Müller, E. Busker, K. Günther and B. Hoppe, *Bioforum*, 14 (1991) 350.
- [9] Y. Goda, J. Suzuki, T. Maitani, K. Yoshihira, M. Takeda and M. Uchiyama, *Chem. Pharm. Bull.*, 40 (1992) 2236.
- [10] T. Simat, K. Meyer and H. Steinhart, *J. Chromatogr. A*, 661 (1994) 93.
- [11] R.M. Silver, A. Ludwicka, M. Hampton, T. Ohba, S.A. Bingel, T. Smith, R.A. Harley, J. Maize and M.P. Hayes, *J. Clin. Invest.*, 93 (1994) 1473.
- [12] L.A. Love, J.I. Rader, L.J. Crofford, R.B. Raybourne, M.A. Principato, S.W. Page, M.W. Trucksess, M.J. Smith, E.M. Dugan, M.L. Turner, E. Zelazowski and E.M. Sternberg, *J. Clin. Invest.*, 91 (1993) 804.
- [13] J. Adachi, T. Naito, Y. Ueno, Y. Ogawa, I. Ninomiya and Y. Tatsuno, *Arch. Toxicol.*, 67 (1993) 284.
- [14] H. Sidransky, E. Verney, J.W. Cosgrove, P.S. Latham and A.N. Mayeno, *Toxicol. Appl. Pharmacol.*, 126 (1994) 108.
- [15] W.J. Driskell, D.L. Ashley, J. Grainger, S.R. Sirimanne, E.P. Mazzola, S.W. Page, L.L. Needham and R.H. Hill, *Bull. Environ. Contam. Toxicol.*, 48 (1992) 679.
- [16] J. Ito, Y. Hosaki, Y. Torigoe and K. Sakimoto, *Food Chem. Toxicol.*, 30 (1992) 71.
- [17] J. Adachi, M. Gomez, C.C. Smith and E.M. Sternberg, *Arch. Toxicol.*, 69 (1995) 266.
- [18] J. Adachi, T. Mio, Y. Ueno, T. Naito, A. Nishimura, S. Fujiwara, K. Sumino and Y. Tatsuno, *Arch. Toxicol.*, 68 (1994) 500.
- [19] H. Sidransky, E. Verney, J.W. Cosgrove and P.S. Latham, *Toxicology*, 86 (1994) 135.
- [20] R.M. Philen, R.H. Hill, W.D. Flanders, S.P. Caudill, L. Needham, L. Sewell, E.J. Sampson, H. Falk and E.M. Kilbourne, *Am. J. Epidemiol.*, 138 (1993) 154.
- [21] A.N. Mayeno and G.J. Gleich, *Trends Biotechnol.*, 12 (1994) 346.
- [22] U. Hagemann, Bundesinstitut für Arzneimittel und Medizinprodukte, Berlin, personal communication.
- [23] K. Feiden, Bundesministerium für Gesundheit, *Dtsch. Apoth. Ztg.*, 134 (1994) 3361; (Banz. 20.07.1994, p. 8505).
- [24] S. Yamada and H. Akimoto, *Tetrahedron Lett.*, 36 (1969) 3105.
- [25] W.E. Savige, *Aust. J. Chem.*, 28 (1975) 2275.
- [26] K. Uchida, N. Enomoto, K. Itakura and S. Kawakishi, *Arch. Biochem. Biophys.*, 279 (1990) 14.
- [27] B.I. Knight and A.G. Davidson, *J. Pharm. Biomed. Anal.*, 11 (1993) 85.
- [28] M.W. Trucksess, F.S. Thomas and S.W. Page, *J. Pharm. Sci.*, 83 (1994) 720.
- [29] M. Friedman and J.-L. Cuq, *J. Agric. Food Chem.*, 36 (1988) 1079.
- [30] T. Simat, K. Meyer, B. Stöver and H. Steinhart, in G. Allegrì Filippini and C.V. Costa (Editors), *Recent Advances in Tryptophan Research*, Plenum, New York, in press.
- [31] K. Sakimoto and Y. Torigoe, in W. Kochen and H. Steinhart (Editors), *L-Tryptophan – Current Prospects in Medicine and Drug Safety*, Walter de Gruyter, Berlin, 1994, p. 295.
- [32] M. Toyoda, Y. Saito, M. Uchiyama, A.L. Troy, M.W. Trucksess and S.M. Page, *Biosci. Biotech. Biochem.*, 58 (1994) 1318.
- [33] H. Plischke and W. Steinmetzer, *German Pat.*, DE 3 630 878 (1988); *Chem. Abstr.*, 109 (1988) 188 845a.
- [34] CDC, *MMWR*, 39 (1990) 589.