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Analysis of Nitrogen Containing Compounds, *p*-Coumaroyltyramine and Feruloyltyramine, for Discrimination of Cannabis Seeds by HPLC

Tamihide MATSUNAGA,^a Masae ANO,^a Kazuhito WATANABE,^a
Ikuo YAMAMOTO,^{*,a} and Hidetoshi YOSHIMURA^b

Faculty of Pharmaceutical Sciences, Hokuriku University,^a 3-Ho, Kanagawa-machi,
Kanazawa 920-11, Japan, and Nakamura Gakuen University,^b 5-7-1 Befu,
Johanan-ku, Fukuoka 814-01, Japan

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Two peaks corresponding to authentic *p*-coumaroyltyramine (*p*-CT) and *trans*-feruloyltyramine (*trans*-FT) were detected on the HPLC chromatogram in the extract of cannabis seeds, whereas these peaks were not detected in the extracts of plant seeds of Japanese radish, radish or spinach. A part of *trans*-FT was converted to a *cis*-isomer by ultraviolet irradiation. The contents of *p*-CT and *trans*-FT were 0.2–1.3 and 5.1–26.8 mg/100 g of the seeds, respectively. These results indicate that the detection of *p*-CT and *trans*-FT in plant seeds by HPLC analysis is a useful method for the discrimination of cannabis seeds from other plant seeds.

Key words — cannabis; seed; discrimination; *trans*-feruloyltyramine; *p*-coumaroyltyramine

Introduction

Abuse of marijuana has gradually become a social problem in Japan, although use of the narcotic has been severely prohibited by the Government. More than 2000 people each year are arrested on a charge of violating the marijuana prohibition law.¹⁾ Cannabis seeds (*Cannabis sativa* L.), however, have been excluded from this regulation because of the cannabis fiber production ongoing in the country since 1953.²⁾ The seeds are also used as oriental traditional medicine (mashinin), spice, feed for birds and so on. But individuals import cannabis seeds secretly into the country and grow them to smoke their leaves; incidences of confiscation of cannabis grown for the purpose of smoking marijuana have been reported.³⁾ This suggests that the present regulations on germinable seeds should be reconsidered, except as they pertain to cannabis farmers.

We previously developed a chemical

method to distinguish cannabis seeds from other plant seeds by detection of cannabinoids such as Δ^9 -tetrahydrocannabinol and cannabinol using TLC or GC.^{4,5)} This method is very useful, because cannabinoids of the cannabis seeds are readily detectable. These cannabinoids were not biosynthesized in the seeds, however, but were derived from resin of the bract.⁵⁾ We recently found that *p*-coumaroyltyramine (*p*-CT) and *trans*-feruloyltyramine (*trans*-FT) were pharmacologically active nitrogenous constituents in the cannabis seeds (Fig. 1).⁶⁾ In the present study, we established analytical methods for *p*-CT and *trans*-FT to identify seeds of cannabis, and compared them with seeds of other plants such as Japanese radish, radish and spinach which resemble to cannabis seeds in size.

Materials and Methods

Materials — Cannabis seeds of the Minamioshihara #1 and cannabidiolic acid (CBDA) strains were harvested in the botanical

Fig. 1. Structures of *p*-CT and FT

garden of Hokuriku University and stored at room temperature in the dark. Seeds of Japanese radish (*Raphanus sativus* L. var. *longipinnatus*), radish (*Raphanus sativus* L.) and spinach (*Spinacia oleracea* L.) were purchased from a seed shop in Kanazawa. *p*-CT and *trans*-FT were prepared by the method reported previously.⁶⁾ Precoated silica gel plates (SIL UV254, 0.25 mm thickness, 20 × 20 cm) were purchased from Machery Nagel (Duren, Germany). Bond Elut C18 columns were from Analytichem International (Harbor City, CA). Other chemicals and organic solvents used were of the highest quality commercially available.

HPLC Analysis of the Fractions Obtained from Seeds — Seeds (1.0 g) were crushed in a mortar with a pestle, and extracted three times with 40 ml of ethanol at room temperature. The resulting ethanol solutions were combined and evaporated *in vacuo* to give fraction A. Fraction A was dissolved in 30 ml of ethyl ether and shaken three times with 4 ml of 5% NaHCO₃. The organic layer was further extracted three times with 4 ml of 0.1 N NaOH, and the combined aqueous layer was acidified with 1 N HCl. The acidic solution was extracted twice with 30 ml of ethyl ether and the combined organic layer was evaporated *in vacuo* to give fraction B.

Fractions A and B were individually treated before application to HPLC as follows: the residue was dissolved in 2 ml of methanol–water (1:4, v/v) and applied to a Bond Elut C18 column pre-equilibrated with the same solution. The column was washed with 6 ml of methanol–water (1:4, v/v), followed by elution with 2 ml of methanol–water (4:1, v/v). An aliquot (2–5 μl) was subjected to HPLC.

Determination of *p*-CT and *trans*-FT in Cannabis Seed — Fraction A, added with 100 μg of *p*-nitrophenol as an internal standard, was dissolved in 30 ml of ethyl ether. The sample for HPLC was prepared using the procedure of solvent extraction and Bond Elut C18 column treatment described above.

Calibration Curves for *p*-CT and *trans*-FT — A solution of various concentrations of *p*-CT and *trans*-FT added with 100 μg of *p*-nitrophenol was evaporated *in vacuo*. The sample for HPLC was prepared using the procedure of solvent extraction and Bond Elut C18 column treatment described above. The quantitative ranges of *p*-CT and *trans*-FT were 2 to 150 μg and 4 to 300 μg under these conditions, respectively.

Instruments and Operating Conditions — HPLC (Hitachi 655 type) conditions were as follows: column, DuPont ZORBAX ODS (4.6 mm i.d. × 15 cm); absorption 310 nm (Hitachi 655A-21 type); mobile phase, acetonitrile–methanol–water (5:40:55, v/v/v) and 0.1% acetic acid–methanol (58:42, v/v); flow rate, 1.0 and 0.5 ml/min; and column temperature, 20–25°C. The extracts were subjected to silica gel TLC (methanol–chloroform, 1:9, v/v): *cis*- and *trans*-FT, *R_f* 0.5 (blue color under UV lamp, 365 nm).

Results and Discussion

The HPLC chromatograms of fractions A obtained from cannabis, Japanese radish, radish and spinach seeds are shown in Fig. 2A. Two peaks corresponding to *p*-CT and *trans*-FT were detected at 9.0 and 10.5 min, respectively, on the chromatogram of the cannabis seeds, whereas these peaks were not found in

Fig. 2. HPLC Chromatograms of Fraction A (A) and Fraction B (B) Obtained from Seeds

(a) cannabis (Minamioshihara #1). (b) Japanese radish. (c) radish. (d) spinach. Column, DuPont ZORBAX ODS (4.6 mm i.d. $\times$ 15 cm); mobile phase, acetonitrile-methanol-water (5:40:55, v/v/v); flow rate, 1.0 ml/min; absorption, 310 nm.

the seeds of the three other seeds. However, the baseline on the chromatogram was increased by substances in the seeds. Thus, fraction A was further purified by liquid-liquid partition and Bond Elut C18 column to obtain fraction B, and subjected to HPLC (Fig. 2B). The baseline and the extra peaks were decreased. The peaks of *p*-CT and *trans*-FT were still detected in the cannabis seeds at

9.0 and 10.5 min, respectively, while, no peak except for void volume was detected in other seeds. Fraction B was then subjected to silica gel TLC. A spot ($R_f=0.5$) on TLC corresponding to *trans*-FT was found in cannabis seeds (Fig. 3), while it was not detected in the seeds of other plants (data not shown).

Fukuda *et al.* reported that *trans*-FT was converted to *cis*-isomer by UV irradiation,

Fig. 3. Silica Gel TLC of Fraction B Obtained from Cannabis Seeds

(A) *trans*-FT (500 ng). (B) fraction B obtained from cannabis seeds of Minamioshihara #1 (10 μ l). Developing solvent, methanol–chloroform (1:9, v/v). Spots of R_f values 0.50, 0.44 and 0.38 on TLC fluoresced with blue, red and light blue colors, respectively, when exposed to UV light (365 nm).

reaching a constant content ratio.⁷⁾ We further studied the effect of UV irradiation on *trans*-FT in cannabis seeds. By UV (253.6 nm)

irradiation on the solution of *trans*-FT for 0.5 min, a new peak (Peak II) appeared at 7 min on the HPLC chromatogram in addition to the Peak I of *trans*-FT at 10.5 min (Fig. 4). The peak ratio (Peak II/I) was increased time-dependently by UV irradiation and became constant (Peaks II/I=1.9) after 8 min (Fig. 4). Peak II was characterized as *cis*-isomer of FT by mass spectrum of the compound corresponding to this peak isolated by preparative HPLC. The fraction B obtained from cannabis seeds exposed to UV light for 8 min compared with UV-irradiated authentic *trans*-FT. Peak II at 7 min was detected on the HPLC chromatogram of the seeds (Fig. 5B). The ratio of Peak II/I of the seeds was the same as that of the authentic *trans*-FT. These results indicate that the appearance of Peak II by UV irradiation is useful for the identification of *trans*-FT.

To determine the contents of *p*-CT and *trans*-FT in cannabis seeds, the fraction A added with *p*-nitrophenol as an internal standard was partially purified by liquid–liquid partition and Bond Elut C18 column. The mobile phase was changed to 0.1% acetic acid–methanol (58:42, v/v), because the

Fig. 4. HPLC Chromatograms of UV-Irradiated *trans*-FT

Ethanol solution of *trans*-FT was exposed to UV light (253.6 nm) for (A) 0 min. (B) 0.5 min. (C) 2 min. (D) 8 min and (E) 16 min. HPLC conditions are the same as described in the legend to Fig. 2.

Fig. 5. HPLC Chromatograms of UV-Irradiated *trans*-FT and Fraction B Obtained from Cannabis Seeds

(A) *trans*-FT and (B) fraction B obtained from cannabis seeds of Minamioshihara #1 were exposed to UV light (253.6 nm) for 8 min. HPLC conditions are the same as described in the legend to Fig. 2.

retention time of *p*-nitrophenol was identical to that of *p*-CT with the former solvent system (data not shown). With the latter solvent system, *p*-nitrophenol, *p*-CT and *trans*-FT were eluted from the column at 20, 25 and 28 min, respectively, at a flow rate of 0.5 ml/min. Table 1 shows the contents of *p*-CT and *trans*-FT in the seeds of Minamioshihara #1 and CBDA strains. These results indicate that the

seeds preferentially contain *trans*-FT rather than *p*-CT. However, further extensive studies are required to clarify whether variation of *trans*-FT content in the seed is attributable to the harvest year and/or term of storage.

We earlier reported that *trans*-FT is a new component in cannabis seeds.⁶⁾ In the present study, no peak or spot on the chromatograms of HPLC and TLC corresponding to the authentic *trans*-FT was detected in ethanol extracts of other plant seeds. However, *N*-cinnamoyl- β -phenethylamine derivatives, including *p*-CT and *trans*-FT, have also been isolated from several plants: *Tinospora tuberculata* (from the stems),⁷⁾ *Evodia belaehe* B. (bark),⁸⁾ *Zea mays* (kernels),⁹⁾ *Solanum melongena* L. (roots),¹⁰⁾ *Capsicum annum* var. *grossum* (roots),¹¹⁾ *Allium chinense* G. (bulbs)¹²⁾ and *Asimina trioba* (bark).¹³⁾ Therefore, not only analyses of *p*-CT and *trans*-FT but also of cannabinoids are required for discrimination between cannabis seeds and other plant seeds.

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Table 1. Content of *p*-CT and *trans*-FT in Cannabis Seeds

Strain	Months after harvest	<i>p</i> -CT (mg/100 g of seeds)	<i>trans</i> -FT (mg/100 g of seeds)
Minamioshihara #1	0	1.3	8.3
	12	0.8	12.3
	36	0.9	5.1
CBDA	0	0.8	26.8
	12	0.2	5.9

p-CT and *trans*-FT in the cannabis seeds were determined by the method described in Materials and Methods. HPLC conditions: mobile phase, 0.1% acetic acid-methanol (58:42, v/v); flow rate, 0.5 ml/min; and absorption, 310 nm.

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