A Rapid and Reliable Solid-Phase Extraction Method for High-Performance Liquid Chromatographic Analysis of Opium Alkaloids from *Papaver* Plants

Kayo YOSHIMATSU,*[,a] Fumiyuki KIUCHI,[a] Koichiro SHIMOMURA,[a,1)] and Yukiko MAKINO[b]

[a] *Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation; 1–2 Hachimandai, Tsukuba, Ibaraki 305–0843, Japan: and* [b] *Narcotics Control Department, Kanto-Shin'etsu Regional Bureau of Health and Welfare; 2–4–14 Nakameguro, Meguro-ku, Tokyo 153–0061, Japan.*

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A rapid and reliable solid-phase extraction method for HPLC analysis of opium alkaloids from *Papaver* plants was established. Fifty mg of dried and powdered plant sample was extracted with 5 ml of 5% acetic acid for 30 min under sonication. After centrifugation, 3 ml of the supernatant was loaded on a reversed-phase cation-exchange solid-phase extraction cartridge. After seriate washings with 0.1 M hydrochloric acid and methanol, alkaloids were eluted with a mixture of 28% ammonia and methanol (1 : 19). The eluate was concentrated under nitrogen stream at 40 °C and the residue was dissolved in 50% aqueous methanol for high performance liquid chromatographic analysis. With this solid-phase extraction method, the recovery of morphine, codeine, oripavine, thebaine, papaverine, noscapine and sanguinarine was from 99.94 to 112.18% when the standard alkaloids were added to the plant samples. Opium alkaloids of a variety of genus *Papaver* plants cultivated in a field and phytotron were analyzed by this method.

Key words solid-phase extraction; opium alkaloid; genus *Papaver*; high performance liquid chromatographic analysis

Opium poppy, *Papaver somniferum* L. contains pharmaceutically important alkaloids such as morphine, codeine, thebaine, papaverine and noscapine.[2)] Although morphine is the most effective analgesic, it also has strongly addictive properties and is a raw material for illegal heroin production. Opium alkaloids including morphine, codeine and thebaine are narcotic drugs under international control.[3)] Cultivation of opium poppy (*P. somniferum* L.) and great scarlet poppy (*P. bracteatum* Lindl.), which produces thebaine, is strictly controlled under the Opium Law and the Narcotics and Psychotropics Control Law, respectively, in Japan. *P. somniferum* has a long cultivation history worldwide and there are many varieties having various colors and shapes of flowers, shapes of capsules, and alkaloid composition and content.[2—4)] Despite the strict control of the plants, seeds of *P. somniferum* have been imported and sold as an ornamental gardening flower. To control illegal cultivation of the plants, rapid and reliable extraction methods of opium alkaloids from many plant samples are required, because distinction of these plants from legal ornamental species is difficult at their vegetative growth stage.

The alkaloids in genus *Papaver* were extracted by liquid–liquid extraction,[5)] with methanol,[6)] or a mixture of methanol and 28% ammonia.[7)] However, liquid–liquid extraction is laborious and requires biologically toxic chloroform, the use of which is decreasing because of environmental protection, and methanolic extracts contain impurities that affect separation of alkaloids. The alkaloids in gum opium and poppy straw were also extracted with 2.5% acetic acid and purified by use of a base-deactivated silica-based stationary phase column.[8)] In this case, toxic organic solvents, dichloromethane and isopropanol, were required to elute the alkaloids from the purification column. Solid-phase extraction has been applied for analysis of narcotic drugs in biological fluids such as urine and plasma,[9—11)] and shown to provide clean extracts without sample loss through emulsion formation. Additionally, halogenated solvents, chloroform and dichloromethane, are not needed, however, there is no report on solid-phase extraction of opium alkaloids from the plant materials as far as we know. The present paper describes a rapid and reliable solid-phase extraction method for opium alkaloids from *Papaver* plants for HPLC analysis.

Experimental

Plant Materials Capsules of *Papaver somniferum*, *P. setigerum*, *P. bracteatum* and *P. pseudo-orientale* cultivated in a field or a phytotron (20 °C/14 or 16 h light, 17 °C/10 or 8 h dark, 60% relative humidity) at Division of Tsukuba, Research Center for Medicinal Plant Resources, National Institute of Biomedical Innovation, were used as plant materials. They were either air-dried or lyophilized and ground prior to extraction. Capsules of field-grown *P. somniferum* var. Ikkanshu and leaves of juvenile *P. somniferum* (India variety) grown in a phytotron were used for extraction experiments.

Extraction Solvent A powdered capsule (*ca.* 150 mg) was weighed and placed in a 2 ml eppendorf tube, and extracted with a solvent (1.5 ml) indicated in Table 1 under sonication for 30 min. Then the mixture was stirred with vortex mixer for 1 min, and centrifuged (18000×*g*, 10 min). The supernatant (20 μl) was injected into an HPLC column.

HPLC Conditions The alkaloids were analyzed with a TOSOH (Tokyo, Japan) HPLC system (auto sampler, AS-8020; column oven, CO-8020, pump system, CCPM-II; photodiode array detector, PD-8020) equipped with a TSKgel ODS-120 A column (TOSOH, 4.6 i.d.×250 mm). The conditions were as follows: mobile phase, acetonitrile (solvent A)−10 mM sodium 1-heptanesulphonate in water (adjusted to pH 3.5 with phosphoric acid) (solvent B); gradient, 0—15 min from 20 to 30% solvent A, 15—20 min from 30 to 40% solvent A, 20—25 min 40% solvent A, 25—30 min from 40 to 50% solvent A, 30—35 min from 50 to 20% solvent A; flow rate, 1 ml/min; column temperature, 40 °C; detection, UV at 284 nm. Each peak corresponding to morphine, codeine, oripavine, thebaine, papaverine, noscapine and sanguinarine was confirmed by measuring UV spectrum from 200 to 400 nm using photodiode array detector. Correlation coefficients (r) of the standard curves of the alkaloids were from 0.9998 to 0.9999. Retention times of the authentic alkaloids did not change (differences were between 0.1 to 2.5%) even after several hundreds of analyses.

Liquid–Liquid Extraction (Partition) Liquid–liquid extraction of opium alkaloids was carried out according to a method reported previously[12)] with a slight modification. Briefly, powdered capsules (*ca.* 50 mg) were extracted with 5% acetic acid (5 ml) under sonication and then mixed

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∗ To whom correspondence should be addressed. e-mail: yoshimat@nibio.go.jp

Table 1. Influence of Extraction Solvent on the Extraction of Opium Alkaloid from Field-Cultivated *P. somniferum* var. Ikkanshu Capsules

Entry	Extraction solvent	Alkaloid$^{a)}$ % dry weight (% CV$^{b)}$)				
		Mor	Cod	The	Pap	Nos
1	H_2O	0.224 (7.6)	0.031 (34.3)	0.007 (0.0)	0.015 (11.5)	0.013 (27.7)
2	1% AcOH	0.272 (17.6)	0.025 (22.3)	0.007 (8.7)	0.023 (24.4)	0.023 (49.0)
3	5% AcOH	0.307 (22.7)	0.029 (27.6)	0.010 (6.0)	0.032 (19.0)	0.019 (34.4)
4	0.1 M HCl	0.232 (19.5)	0.030 (46.7)	0.011 (39.6)	0.029 (21.0)	0.021 (24.1)
5	0.1 M Na-citrate$^{c)}$	0.282 (5.7)	0.037 (27.1)	0.011 (43.3)	0.017 (0.0)	0.020 (13.2)
6	50% EtOH	0.456 (22.1)	0.040 (82.5)	0.018 (38.5)	0.072 (51.2)	0.078 (71.6)
7	1% AcOH+EtOH (1 : 1)	0.367 (21.5)	0.026 (13.7)	0.013 (26.6)	0.054 (26.7)	0.050 (25.2)
8	5% AcOH+EtOH (1 : 1)	0.357 (38.5)	0.029 (75.1)	0.011 (58.7)	0.045 (49.2)	0.047 (65.5)
9	0.1 M HCl+EtOH (1 : 1)	0.335 (11.7)	0.031 (42.7)	0.014 (14.3)	0.061 (11.6)	0.056 (11.7)
10	0.1 M Na-citrate+EtOH (1 : 1)	0.331 (9.2)	0.028 (13.4)	0.021 (13.5)	0.044 (15.6)	0.033 (43.7)

a) Mor, morphine; Cod, codeine; The, thebaine; Pap, papaverine; Nos, noscapine. *b)* CV, coefficient of variation (n=3). *c)* pH 6.0.

by a vortex mixer. After filtration, 3 ml of the filtrate was washed with chloroform (3 ml, acid-$CHCl_3$). The aqueous phase was made alkaline with 28% ammonia, and extracted with a mixture of chloroform and isopropanol (3 : 1) three times (2, 1 and 1 ml). The combined organic extract (base-organic) was concentrated to dryness under nitrogen stream, and the residue was dissolved in an appropriate volume of 50% aqueous methanol and analyzed by HPLC. The chloroform phase (acid-$CHCl_3$), which was used to wash 5% acetic acid extract, was recovered, concentrated and dissolved in an appropriate volume of 50% aqueous methanol and similarly analyzed by HPLC.

Solid-Phase Extraction A reversed-phase solid-phase extraction cartridge (Oasis HLB®, Waters Co., Massachusetts, U.S.A.) and a reversed-phase cation-exchange solid-phase extraction cartridge (Oasis MCX®, Waters, Massachusetts, U.S.A.) were used for solid-phase extraction. Powdered plant samples (*ca.* 50 mg) were extracted with 5 ml of an extraction solvent [water, 5% acetic acid, 0.1 M sodium citrate buffer (pH 6.0)] under sonication for 30 min. After mixing (vortex mixer, 1 min) and centrifugation (18000×*g*, 10 min), 3 ml of the supernatant was purified by solid-phase extraction cartridge (either Oasis HLB® or Oasis MCX®) according to the product application protocols[10] or their modification indicated in Tables 2 and 3. In brief, solid-phase extraction cartridges were conditioned with methanol (3 ml) and then equilibrated with water (3 ml). Three ml of crude extract was loaded onto the solid-phase extraction cartridge and the cartridge was washed with a washing solvent (W, 3 ml). The alkaloids were eluted with an elution solvent twice (E1, 2 ml; E2, 1 ml) and each eluate was concentrated to dryness under nitrogen stream, dissolved in an appropriate volume of 50% aqueous methanol and analyzed by HPLC.

Recovery Test Powdered plant samples (*ca.* 50 mg) were extracted with 5% acetic acid (5 ml) as mentioned above. After centrifugation (18000×*g*, 10 min), 2.8 ml of the supernatant was taken and mixed with authentic alkaloids dissolved in 75% methanol (0.2 ml) and then loaded on a conditioned Oasis MCX® solid-phase extraction cartridge. After washing with 0.1 M hydrochloric acid and methanol, the alkaloids were eluted with a mixture of 28% ammonia and methanol (1 : 19). The solvent was removed under nitrogen stream and the residue was dissolved in 50% aqueous methanol and analyzed by HPLC as described above. As a control experiment, 3 ml of the supernatant obtained from the same plant sample was purified and analyzed as described above. The recovery of added alkaloids was calculated as a difference of the amount of each alkaloid between the added and control experiments.

Results and Discussion

Influence of Extraction Solvent *P. somniferum* var. Ikkanshu capsules were extracted with various aqueous solutions (water, acetic acid, hydrochloric acid, sodium citrate buffer) or mixtures of these solutions and ethanol, and the crude extracts were analyzed by HPLC (Table 1). Five alkaloids, morphine, codeine, thebaine, papaverine and noscapine, were detected in the capsules, neither oripavine nor sanguinarine could be detected. The amount of the alkaloids detected varied with the different extraction solvents. The highest amount of morphine and papaverine were obtained with 5% acetic acid, while the amounts of codeine and thebaine were highest with 0.1 M sodium citrate buffer among the aqueous solutions (entry 1—5). The calculated amount of the extracted alkaloids increased when ethanol was added to the corresponding aqueous solution (entry 6—10). However, increase of interfering peaks and rising of the base line were observed in the HPLC chromatogram (Fig. 1); that might be a reason for increased calculation value of the alkaloids. Five percent acetic acid and 0.1 M sodium citrate buffer were chosen as an extraction solvent and used for further experiments.

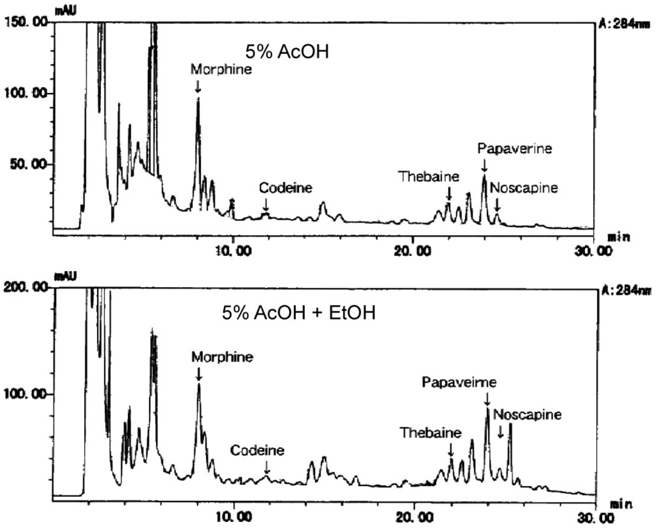

Fig. 1. HPLC Chromatograms of the Alkaloids Extracted from *P. somniferum* var. Ikkanshu Capsule with 5% Acetic Acid (Upper) and 5% Acetic acid–Ethanol (1 : 1) (Lower)

Comparison of Extraction and Purification Efficiency Influences of extraction conditions on the analytical values of alkaloid contents in leaves and capsules of *P. somniferum* are shown in Tables 2 and 3. Five alkaloids, morphine, codeine, thebaine, papaverine and noscapine were detected in the leaves of juvenile India variety grown in a phytotron (Table 2) as in the Ikkanshu capsules (Tables 1, 3). When purified by liquid–liquid extraction method, most parts of thebaine, papaverine and noscapine were transferred into the chloroform washing (acid-$CHCl_3$) (Fig. 2a). The highest value of morphine in the capsules was obtained for extraction and purification with Oasis HLB® according to the supplier's standard method (Table 3, entry 5). However, interfering sub-

Table 2. Influences of Extraction Conditions on the Analytical Values of Opium Alkaloid Contents from the Leaves of Phytotron-Grown Juvenile Plant of *P. somniferum* (India Variety)

Entry	Extraction[a] method	Step[b]	Fraction	Alkaloid[c] % dry weight				
				Mor	Cod	The	Pap	Nos
1	Partition		Acid-$CHCl_3$	—	0.001	0.002	0.009	0.026
			Base-Organic	0.004	0.029	0.003	0.002	0.007
2	Crude		5% AcOH	—	0.026	—	0.012	0.038
3	Oasis HLB®	L	5% AcOH	0.001	—	—	—	0.001
		W	H_2O	0.002	0.001	—	—	—
		E1	3% triethylamine	—	0.011	—	—	—
		E2	3% triethylamine	—	0.015	—	—	—
4	Oasis HLB®	L	H_2O	—	—	—	—	—
		W	H_2O	—	—	—	—	—
		E1	3% triethylamine	—	—	—	—	0.007
		E2	3% triethylamine	—	0.018	—	—	—
5	Oasis HLB®	L	0.1 M Na-citrate	—	—	—	—	—
		W	H_2O	—	—	—	—	—
		E1	3% triethylamine	—	—	—	—	0.005
		E2	3% triethylamine	—	0.021	—	—	—
6	Oasis HLB®	L	0.1 M Na-citrate	—	—	0.000	—	—
		W1	2% NH_4OH+5% MeOH	—	—	—	—	—
		W2	2% NH_4OH+20% MeOH	0.009	—	—	—	—
		E1	1% AcOH+25% MeOH	—	0.029	0.002	—	—
		E2	1% AcOH+25% MeOH	—	0.001	0.004	—	—

a) Partition, liquid–liquid extraction; Crude, unpurified 5% AcOH extract; Oasis HLB®, plant sample was extracted with a solvent (indicated in step L) and the extract was purified by solid-phase extraction cartridge. *b*) L, loading; W, washing (1, first; 2, second); E, elution (1, first; 2, second) steps. *c*) Mor, morphine; Cod, codeine; The, thebaine; Pap, papaverine; Nos, noscapine.

Table 3. Influences of Extraction Conditions on the Analytical Values of Opium Alkaloids in the Capsules of Field-Grown *P. somniferum* var. Ikkanshu

Entry	Extraction[a] method	Step[b]	Fraction	Alkaloid[c] % dry weight				
				Mor	Cod	The	Pap	Nos
1	Partition		Acid-$CHCl_3$	—	—	0.004	0.038	0.027
			Base-Organic	0.243	0.029	0.002	0.002	—
2	Oasis HLB®	L	0.1 M Na-citrate	na[d]	na	na	na	na
		W	2% NH_4OH+5% MeOH	—	—	—	—	—
		E1	2% NH_4OH+20% MeOH	0.174	—	—	—	—
		E2	MeOH	0.067	0.029	0.007	0.022	0.015
3	Oasis HLB®	L	0.1 M Na-citrate	na	na	na	na	na
		W	2% NH_4OH+5% MeOH	—	—	—	—	—
		E1	MeOH	0.210	0.022	0.010	0.022	0.017
		E2	MeOH	0.005	—	0.001	0.001	0.001
4	Oasis HLB®	L	0.1 M Na-citrate	na	na	na	na	na
		W	2% NH_4OH+5% MeOH	—	—	—	—	—
		E1	1% AcOH+50% MeOH	0.197	0.021	0.007	0.019	0.014
		E2	1% AcOH+50% MeOH	0.008	0.002	0.001	0.003	0.004
5	Oasis HLB®	L	0.1 M Na-citrate	na	na	na	na	na
		W	5% MeOH	—	—	0.001	—	—
		E1	MeOH	0.387	0.030	0.012	0.023	0.016
		E2	MeOH	0.006	—	—	0.001	—
6	Oasis MCX®	L	5% AcOH	na	na	na	na	na
		W1	0.1 M HCl	—	—	—	—	—
		W2	MeOH	—	—	—	—	—
		E1	1.4% NH_4OH+95% MeOH	0.253	0.033	0.006	0.017	0.017
		E2	1.4% NH_4OH+95% MeOH	0.005	—	—	0.001	—

a) Partition, liquid–liquid extraction; Oasis HLB® and Oasis MCX®, plant sample was extracted with a solvent (indicated in step L) and the extract was purified by solid-phase extraction cartridge. *b*) L, loading; W, washing (1, first; 2, second); E, elution (1, first; 2, second) steps. *c*) Mor, morphine; Cod, codeine; The, thebaine; Pap, papaverine; Nos, noscapine. *d*) na, not analyzed.

stances that affect morphine separation could not be removed (Fig. 2c). On the other hand, good separation of alkaloids including morphine was obtained when the sample was extracted and purified by Oasis MCX® (Table 3, entry 6, Fig. 2d). Therefore, this method was employed to test the recovery of the alkaloids. A mixture of authentic alkaloids was added to a crude extract of capsules and the alkaloids were purified by Oasis MCX® cartridge. No elution of the authentic alkaloids was observed in the loading and the washings, and good recovery (99.94—112.18%) was obtained (Table 4).

Alkaloids in *Papaver* Plant Capsules The alkaloids in capsules of various *Papaver* plants were extracted and purified by Oasis MCX® and analyzed by HPLC (Table 5). Alkaloid composition and contents were obviously varied among the *P. somniferum* varieties. It is reported that Japanese Ikkanshu variety of *P. somniferum* lacked oripavine whereas a variety grown in the Tasmania contained 0.1% oripavine in its dried capsules.[13] In our study, although oripavine could not be detected in three varieties (phytotron-grown India-1 and Turkey and field-grown Ikkanshu), it was detected in two varieties (India-2 and France). Extremely high content of morphine (1.6% dry weight) together with codeine, oripavine and thebaine was detected in a capsule of France variety; nevertheless, papaverine and noscapine, which are component alkaloids in the other varieties, were not detected. Lower content of morphine (0.134% dry weight) than *P. somniferum* was detected in unripe capsules of *P. setigerum* together with low levels of codeine, papaverine and sanguinarine.

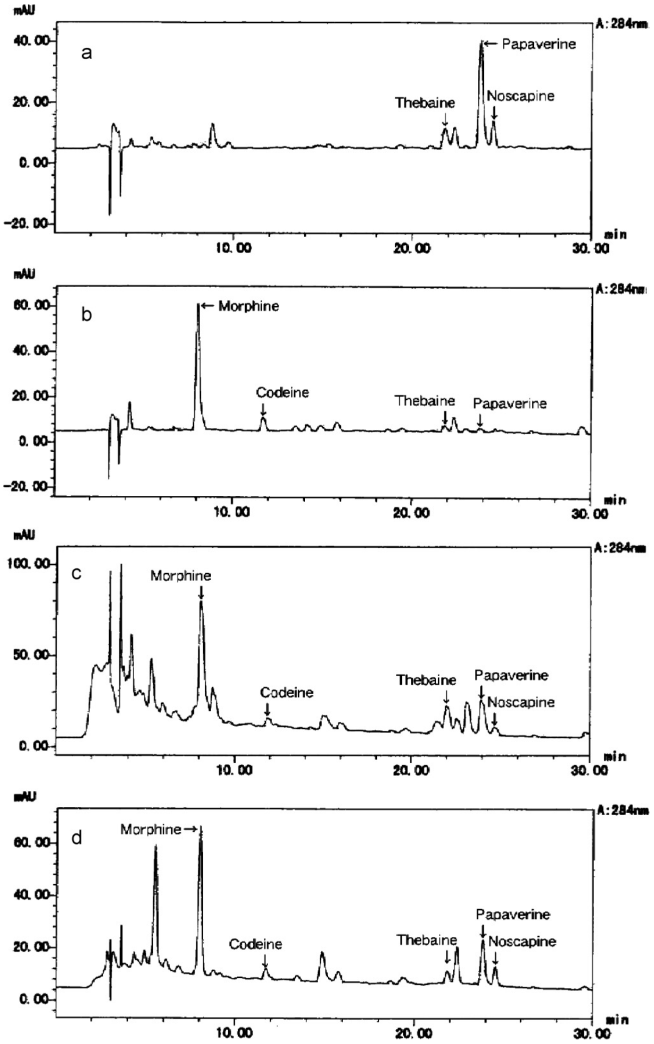

Fig. 2. HPLC Chromatograms of the *P. somniferum* var. Ikkanshu Capsule Extracts

(a) Table 3 entry 1 Acid-$CHCl_3$, (b) Table 3 entry 1 Base-Organic, (c) Oasis HLB® recommended method (Table 3 entry 5 Elution 1), (d) Oasis MCX® (Table 3 entry 6, Elution 1).

Cultivation of *P. bracteatum* has been controlled under the Narcotics and Psychotropics Control Law in Japan, while *P. pseudo-orientale* has been cultivated as an ornamental flower. Both species are perennial, having a rosette with long, thick, bristly, and pinnately dissected leaves, and a long, almost always unbranched flower axis, and displaying erect flower bud.[14] Flowers of *P. bracteatum* have red petals with long, dark, stripe-like basal marks, whereas *P. pseudo-orientale* has flowers of orange-red petals and the basal marks may or may not be present. One of the most obvious morphological features in *P. bracteatum* is the existence of bracts. However, several bracts may occur in some individuals of *P. pseudo-orientale*. These morphological similarities between *P. bracteatum* and *P. pseudo-orientale* make it difficult to distinguish these two species. In addition, production of F1 hy-

Table 4. Recovery of Opium Alkaloids by Oasis MCX® from the Crude Extract of Capsules of Field-Grown *P. somniferum* var. Ikkanshu

Alkaloid	Added weight μg	Recovered weight μg[a]	Recovery %[b]
Morphine	12.666	12.659±1.005	99.94
Codeine	11.085	11.937±0.617	107.68
Oripavine	14.286	15.694±0.907	109.86
Thebaine	17.286	17.348±1.354	100.36
Papaverine	14.447	15.914±1.227	110.15
Noscapine	15.097	16.936±1.306	112.18
Sanguinarine	12.145	13.247±0.879	109.07

a) The data are shown as a mean±standard deviation of three analyses. *b*) Recovery %=recovered weight/added weight·100.

Table 5. Alkaloid Contents in the Capsules of *Papaver* Plants

Place	Plant	Type	Alkaloid[a] % dry weight						
			Mor	Cod	Ori	The	Pap	Nos	San
Phytotoron	*P. somniferum* (India-1)	Ripe	0.150	0.207	nd[b]	0.055	0.127	0.152	nd
	P. somniferum (India-2)	Ripe	0.256	0.033	0.014	0.229	0.014	0.007	nd
	P. somniferum (Turkey)	Ripe	0.289	0.033	nd	0.063	0.014	0.007	nd
	P. somniferum (France)	Ripe	1.569	0.197	0.013	0.093	nd	nd	nd
Field	*P. somniferum* (Ikkanshu)	Ripe	0.253	0.033	nd	0.006	0.018	0.017	nd
	P. setigerum	Unripe	0.134	0.005	nd	nd	0.004	nd	0.009
	P. bracteatum (14-95)	Unripe	nd	nd	nd	0.331	nd	nd	0.003
	P. pseudo-orientale (2-4)	Unripe	nd	nd	nd	nd	nd	nd	0.002
	P. pseudo-orientale (130-95)	Unripe	nd	nd	nd	nd	nd	nd	0.001

a) Mor, morphine; Cod, codeine; Ori, oripavine; The, thebaine; Pap, papaverine; Nos, noscapine; San, sanguinarine. *b*) nd, Not detected.

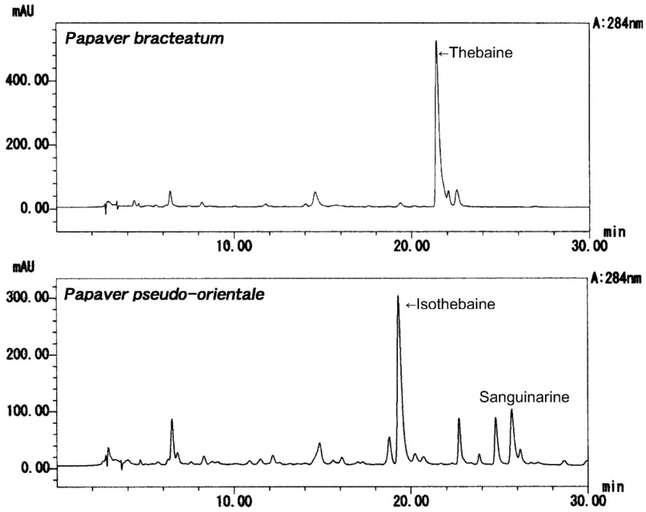

Fig. 3. HPLC Chromatogram of the Unripe Capsule Extract Purified by Oasis MCX® from *P. bracteatum* (Upper) and *P. pseudo-orientale* (Lower)

brid between *P. bracteatum* and *P. pseudo-orientale* was reported.[7]

Phytochemical analysis of these plants might be a potent tool to distinguish the two species, because a difference of the predominant alkaloid, thebaine in *P. bracteatum* and isothebaine in *P. pseudo-orientale*, has been demonstrated.[15] In this study, HPLC chromatograms of their alkaloid extracts prepared by Oasis MCX® differed each other and were distinctive (Fig. 3). Thebaine was the major alkaloid in unripe capsules of *P. bracteatum* while it was not detected in *P. pseudo-orientale*. A trace of sanguinarine was detected in the both species.

Conclusion

Papaver plant materials contain many biosynthetically related substances, which interfere separation and quantification of opium alkaloids by HPLC. That is why time-consuming liquid–liquid extraction has been carried out for the analysis of opium alkaloids from the plant materials. However, care had to be exercised to avoid the formation of emulsion when the mixture of alcohol and chloroform was added and mixed with the alkalized extraction solution.[5] The solid-phase extraction method presented here is rapid and reliable, and makes it possible to save both time and labor for extraction of opium alkaloids.

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