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Study on "Signal" Constituents for the Evaluation of Animal Crude Drugs. II.[1] Organic Acids

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An analytical method of determining organic acids in animal crude drugs using selective and sensitive postcolumn-pH buffered electroconductivity detection HPLC was established and the organic acid contents in 10 drugs were determined. Nine organic acids: acetic acid, butylic acid, formic acid, isobutylic acid, lactic acid, malic acid, propionic acid, pyroglutamic acid and succinic acid were found. Lactic acid content in Lumbricus varied greatly among the samples from 0.83 to 13.08 mg/g, and the contents of acetic acid, butylic acid, isobutylic acid and propionic acid in Cervi Parvum Cornu also showed large variation. Contents of the organic acids in other animal crude drugs did not vary greatly among the samples. These contents were believed to change during the time course of preservation and distribution on the market, so the content in animal crude drugs stored under various conditions, and the appearance, Loss on drying and pH were also studied. No change was observed in any of these factors in any drug stored in tightly closed containers, except for the lactic acid content in Amydae Carapax which showed a slight increase. However, various changes in the factors were observed in all crude drugs stored in 40°C–75% relative humidity (RH) open containers.

Key words animal crude drug; organic acid; postcolumn-pH buffered; electroconductivity detection; HPLC

Knowledge of "signal" constituents and the establishment of an analytical method to evaluate animal crude drugs used in various Chinese medicines are important. We researched these constituents and proved in an earlier report[2] that "signal" constituents were the free amino acids and total amino acids. In this study, the organic acid and organic acids contents in 10 animal crude drugs were determined using postcolumn-pH buffered electroconductivity detection HPLC.[3] Organic acids were considered to be one of the changeable constituents with time during the course of preservation and distribution on the market. Organic acids in animal crude drugs were stored under various conditions to determine whether they were "signal" constituents.

Experimental

Samples These samples of each of Bezoar Bovis, Ostreae Testa, Lumbricus, Cicadae Periostracum and Amydase Carapax were available commercially on the Japanese market. The Bezoar Bovis samples were from Australia, North America and Brazil obtained from Nihon Funmatsu Yakuhin Co., Ltd. (Osaka, Japan) and met the requirements of the Pharmacopoeia of Japan (JP). Ostreae Testa samples also met the JP requirements, one was from Japan and obtained from Nakajima Shoyaku Co., Ltd. (Kyoto, Japan), and the others were from an unknown location and obtained from Koshiro Seiyaku Co., Ltd. (Osaka, Japan). The Lumbricus samples were from Thailand and obtained from Nihon Funmatsu Yakuhin Co., Ltd., Cicadae Periostracum samples were from China and obtained from Takasago Yakugyo Co., Ltd. (Osaka, Japan), Tochimoto Tenkaido Co., Ltd. (Osaka, Japan) and Kinokuniya Kanyakkyoku Co., Ltd. (Tokyo, Japan). One of the Amydae Carapax samples was from India obtained from Takasago Yakugyo Co., Ltd., and the others were from an unknown location and obtained from Tochimoto Tenkaido Co., Ltd. and Kinokuniya Kanyakkyoku Co., Ltd. Three samples of each of Asini Gelatinum, Cervi Parvum Cornu, Hippocampus, Kokurozin and Koukuzin were from China and were provided by Tenjin Li Sheng Pharmaceutical Factory (Tenjin, China).

Reference Standard Butylic acid, isobutylic acid, lactic acid, L(−)-malic acid, sodium acetate, sodium formate, sodium propionate and succinic acid of special grade were obtained from Katayama Chemical (Osaka, Japan). L-Pyroglutamic acid was obtained from Tokyo Chemical Industries (Tokyo, Japan). Organic acid standards were dissolved in water for use.

Reagents The water used in this study was prepared in a Milli-RO 60 system using reverse osmosis and an ionization cartridge (Nihon Millipore, Tokyo, Japan). The chemicals used were analytical grade. *p*-Toluenesulfonic acid monohydride (*p*-TSA) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Disodium ethylenediaminetetraacetate (EDTA Na_2) and Tris were obtained from Katayama Chemical.

Apparatus The HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-6AD pumps, a SCL-8B system controller, a SIL-6B auto-injector and a CTO-6A column heater. Electroconductivity was monitored with a CM-1000 electroconductivity detector (Toso, Tokyo, Japan). Data from the HPLC system was recorded continuously by a CR5A data processor (Shimadzu). A PCS5-052 (5 × 50 mm) guard column and a SCS5-252 (5 × 250 mm) column were obtained from Yokogawa Electric Co., Ltd. (Tokyo, Japan), and an IonPac ICE-AS1 (9 × 250 mm) was obtained from Nippon Dionex Co., Ltd. (Fukuoka, Japan). pH was determined by a F-8_{AT} pH meter (Horiba, Kyoto, Japan). A disposable Centrifree™ Micropartition System, MPS-3 (Grace Japan Co., Ltd., Tokyo, Japan) was used for the ultrafiltration.

Preparation of Sample Solutions for Organic Acids A powdered sample (0.2 g) was mixed with 4 ml water, shaken well and sonicated for 30 min. After centrifugation at 3500 rpm for 5 min, 1 ml of the supernatant liquid and 1 ml 10 mM *p*-TSA were mixed and shaken well. After ultrafiltration, the filtrate was used as the sample solution for organic acids.

Analysis of Organic Acids The detector was adjusted the polarity and the range to plus and 20 μS/FS, respectively. The eluent was 10 mM *p*-TSA. The buffer solution was composed of 10 mM *p*-TSA, 0.2 mM EDTA Na_2 and 40 mM Tris. The column temperature was 40 °C. The flow rate of the eluent and buffer solutions were 0.8 ml/min, respectively. One hundred μl of the standard and sample solutions were injected into the column.

Storage Conditions and Items Tested in Stability Study of Crude Drugs The powdered samples were stored under the following conditions: 25 °C tightly closed containers, 40 °C tightly closed containers and 40 °C–75% relative humidity (RH) open containers, and the items were studied for periods of 1, 3 and 6 months focussing on the contents of organic acid, appearance, Loss on drying and pH. Appearance was judged visually. Loss on drying was measured by the general JP tess. A powdered sample (0.2 g) was mixed with 4 ml water, shaken well and sonicated for 30 min, centrifuged at 3500 rpm for 5 min, and the supernatant liquid was used as the sample solution for pH.

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Results and Discussion

Analysis of Organic Acids Organic acids have been determined by various methods.[4-6] In this study, the postcolumn-pH buffered electroconductivity detection method developed by Hayashi and Nishimura was used for selective and sensitive determination of organic acids in animal crude drugs which contain many impurities. Although they used *p*-TSA, boric acid and bis(hydroxyethyl)iminotris(hydroxymethyl)methane to prepare the solvent and the buffer solutions,[3] we used *p*-TSA, EDTA Na_2 and Tris for the buffer solution because of the ease of preparation and the stability of the electroconductivity baseline.

Primary investigation showed that acetic acid, butyric acid, formic acid, isobutylic acid, lactic acid, malic acid, propionic acid, pyroglutamic acid and succinic acid were included in animal crude drugs. These organic acids were separated from each other satisfactorily under the established conditions (Fig. 1).

Calibration curves were linear over the concentration range of 0 to 160 mg/ml for acetic acid, 0 to 400 mg/ml for butylic acid, 0 to 80 mg/ml for formic acid, 0 to 400 mg/ml for isobutylic acid, 0 to 160 mg/ml for malic acid, 0 to 200 mg/ml for propionic acid, 0 to 400 mg/ml for pyroglutamic acid, and 0 to 160 mg/ml for succinic acid. The reproducibility was good with a coefficients of variation (CV) value between 0.25 and 0.82% ($n=5$). There were few impurities in animal crude drugs which disturbed the determination by the established method.

Organic Acids in Animal Crude Drugs Table 1 shows the organic acid contents in animal crude drugs used in this investigation.

The content of each organic acid in Lumbricus varied greatly among the samples, particularly lactic acid which was from 0.83 to 13.08 mg/g. The reason for this variation was believed to be because of the difference in their pretreatment and preservation, but the details were not clear. The contents of acetic acid, butylic acid, isobutylic acid and propionic acid in Cervi Parvum Cornu varied greatly among the samples, while the contents in other animal crude drugs did not vary as much. Total organic acid contents were as much as about 16 and 13 mg/g in

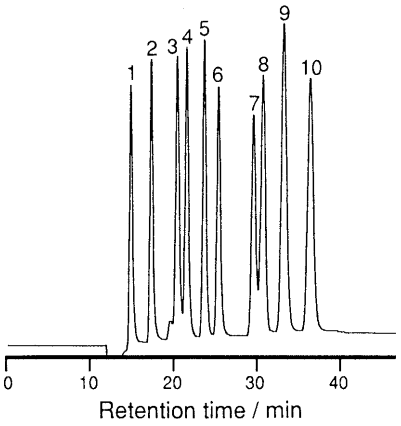

Fig. 1. Typical Chromatogram of Organic Acids Obtained from Standard Solution

Organic acid concentrations of standard solution (mg/ml) 1: phosphoric acid, 2: malic acid (86.7), 3: succinic acid (81.6), 4: lactic acid (174.6), 5: formic acid (40.9), 6: acetic acid (78.7), 7: propionic acid (99.5), 8: pyroglutamic acid (206.2), 9: isobutylic acid (197.5), 10: butylic acid (197.5).

Table 1. Organic Acid Contents[a)]

Crude drug[b)]	Acetic acid	Butylic acid	Formic acid	Isobutylic acid	Lactic acid
Bezoar bovis	0.07—0.15	ND	0.14—0.15	ND	ND
Ostreae testa	ND	ND	0.03—0.05	ND	ND
Asini gelatinum	1.60—2.27	ND	0.75—0.77	ND	1.25—1.38
Lumbricus	0.36—1.09	ND	0.12—0.28	ND	0.83—13.08
Cicadae periostracum	0.15—0.58	ND	0.03—0.07	ND	ND
Amydae carapax	0.32—0.43	ND	0.10—0.14	ND	1.07—1.54
Cervi parvum cornu	0.38—1.79	ND—0.13	0.15—0.18	ND—0.13	3.13—4.91
Hippocampus	1.02—1.33	ND	0.23—0.27	ND	4.19—5.28
Kokurozin[c)]	2.57—2.66	0.19—0.24	0.47—0.54	0.12—0.14	7.28—8.04
Koukuzin[d)]	1.66—1.81	ND	0.62—0.68	ND	11.38—12.66
Detection limit	0.03	0.05	0.02	0.05	0.07

Crude drug[b)]	Malic acid	Propionic acid	Pyroglutamic acid	Succinic acid	Total
Bezoar bovis	ND	ND	ND	ND—0.08	0.27—0.32
Ostreae testa	ND	ND	ND	ND	0.03—0.05
Asini gelatinum	0.12—0.13	0.08—0.11	0.36—0.42	0.07—0.08	4.25—5.15
Lumbricus	0.09—0.99	ND—0.22	ND—0.75	0.06—1.18	1.46—17.32
Cicadae periostracum	0.24—0.94	ND	ND	0.04—0.28	0.49—1.87
Amydae carapax	0.03—0.07	ND	ND	0.05—0.16	1.57—2.34
Cervi parvum cornu	0.03—0.08	ND—0.16	0.13—0.53	0.04—0.23	5.39—6.46
Hippocampus	0.07—0.11	0.07—0.09	0.26—0.42	0.31—0.40	6.43—7.67
Kokurozin[c)]	0.23—0.26	0.18—0.21	1.10—1.18	0.41—0.51	12.69—13.62
Koukuzin[d)]	0.27—0.34	0.04	0.80—0.83	0.29—0.34	15.57—16.70
Detection limit	0.03	0.04	0.07	0.02	

mg/g dried weight. *a*) The data are shown as min.—max. values. *b*) From Namba's writing (colored Illustration of Wakan-yaku), except for Kokurozin and Koukuzin. *c*) The external genitalia of male *Equus asinus* L. *d*) The external genitalia of male *Canis familiaris* L. ND: not detected.

Koukuzin and Kokurozin, respectively. The contents of acetic acid, butylic acid, isobutylic acid, propionic acid and pyroglutamic acid in Kokurizin, formic acid in Asini Gelatinum and lactic acid in Koukizin were also high. Koukuzin, Kokurozin and Hippocampus contained lactic acid in an amount 60 to 75% of their total contents. Acetic acid accounted for about 40% of the total contents of Asini Gelatinum, and about 55% of the total contents of Cicadae Periostracum was malic acid.

Stability of Animal Crude Drugs Volatile Acids: acetic acid, butylic acid, formic acid, isobutylic acid and propionic acid, and non-volatile acids: lactic acid, malic acid, pyroglutamic acid, and succinic acid, were found in animal crude drugs. These organic acids were believed to be free acids and/or salts. Moreover, it was presumed that the organic acid contents in animal crude drugs were decreased by evaporation and decomposition, and increased by fermentation, decomposition and/or the oxidation of the tissue and so on. Therefore, the behavior of organic acids in these drugs stored under various conditions was studied. Usual factors tested of appearance, Loss on drying and pH were also evaluated (Table 2).

No change was observed in all the tested items in any of the samples stored in the tightly closed container (25 °C, 40 °C), except for lactic acid content in Amydae Carapax which showed a slight increase. Various changes were observed, however, in all the samples stored in the 40 °C–75% RH open containers.

Organic Acids in Animal Crude Drugs: The decrease in acetic acid was caused mainly by evaporation of this volatile free acid; its quantity did not change in any of the samples stored in the tightly closed containers, but decreased greatly in some of the samples stored in the open containers. Especially, it was presumed that an acetic acid existed as a free acid in Asini Gelatinum, Lumbricus, Hippocampus, Kokurozin and Koukuzin, because their contents decreased to nearly zero in six months. The increase of acetic acid was assumed to be caused primarily by decomposition and/or oxidation of the tissue in the presence of moisture and air: its quantity did not change

Table 2. Various Changes in Items (40 °C–75%RH Open Container)[a]

Crude drug[b]	Term	AA	BA	FA	IA	LA	MA	PA	PyA	SA	Appearance	Loss on drying	pH
Bezoar bovis	Initial	0.11	ND	0.14	ND	ND	ND	ND	ND	0.04	—	4.33	7.45
	1 month	0.24	ND	0.12	ND	ND	ND	ND	ND	0.04	—	6.56	7.38
	3 month	0.10	ND	0.13	ND	ND	ND	ND	ND	0.07	—	6.67	7.40
	6 month	0.04	ND	0.15	ND	ND	ND	ND	ND	0.08	—	6.72	7.42
Ostreae testa	Initial	ND	ND	0.04	ND	ND	ND	ND	ND	ND	—	0.00	9.50
	1 month	ND	ND	0.04	ND	ND	ND	ND	ND	ND	—	0.34	9.47
	3 month	ND	ND	0.06	ND	ND	ND	ND	ND	ND	—	0.33	9.45
	6 month	ND	ND	0.08	ND	ND	ND	ND	ND	ND	—	0.30	9.43
Asini gelatinum	Initial	2.03	ND	0.76	ND	1.31	0.12	0.10	0.40	0.07	—	10.35	5.28
	1 month	0.73	ND	0.44	ND	1.39	0.04	ND	0.40	0.07	···	13.37	5.14
	3 month	0.13	ND	0.21	ND	0.72	ND	ND	0.41	0.08	···	12.88	5.15
	6 month	0.08	ND	0.22	ND	0.81	ND	ND	0.40	0.08	···	12.93	5.05
Lumbricus	Initial	0.79	ND	0.21	ND	8.61	0.69	0.11	0.38	0.80	—	5.37	5.84
	1 month	0.47	ND	0.20	ND	8.64	0.60	0.02	0.49	0.85	—	9.87	5.81
	3 month	0.26	ND	0.17	ND	6.58	0.57	ND	0.67	0.83	—	10.05	5.79
	6 month	0.08	ND	0.19	ND	6.36	0.61	ND	0.85	0.80	—	10.00	5.56
Cicadae periostracum	Initial	0.32	ND	0.05	ND	ND	0.64	ND	ND	0.16	—	8.36	6.70
	1 month	0.57	ND	0.07	ND	ND	0.57	ND	ND	0.15	—	10.07	6.37
	3 month	0.23	ND	0.10	ND	ND	0.45	ND	ND	0.14	—	10.07	6.24
	6 month	0.10	ND	0.12	ND	ND	0.49	ND	ND	0.15	—	10.31	6.32
Amydae carapax	Initial	0.36	ND	0.13	ND	1.38	0.05	ND	ND	0.11	—	9.28	7.91
	1 month	0.82	ND	0.13	ND	1.86	0.06	ND	ND	0.08	—	9.24	8.23
	3 month	0.99	ND	0.21	ND	2.02	0.05	ND	ND	0.09	—	9.34	8.17
	6 month	1.24	ND	0.27	ND	1.84	0.06	ND	ND	0.06	—	9.40	8.20
Cervi parvum cornu	Initial	0.96	0.04	0.17	0.04	3.97	0.06	0.05	0.33	0.13	—	6.70	7.29
	1 month	1.17	0.03	0.16	0.03	3.95	0.05	0.05	0.29	0.10	—	9.93	7.23
	3 month	0.98	ND	0.19	ND	3.02	0.05	0.03	0.37	0.09	—	10.11	7.18
	6 month	0.78	ND	0.25	ND	3.01	0.05	ND	0.27	0.09	—	10.13	7.24
Hippocampus	Initial	1.16	ND	0.25	ND	4.82	0.09	0.08	0.33	0.35	—	5.46	6.54
	1 month	0.66	ND	0.22	ND	4.18	0.06	ND	0.33	0.28	×	13.94	6.32
	3 month	0.08	ND	0.19	ND	4.60	0.06	ND	0.32	0.29	×	13.71	6.28
	6 month	0.04	ND	0.26	ND	4.24	0.07	ND	0.31	0.28	×	13.93	6.19
Kokurozin[c]	Initial	2.61	0.21	0.50	0.13	7.64	0.24	0.19	1.14	0.48	—	4.65	6.42
	1 month	1.17	ND	0.42	ND	7.56	0.16	0.05	0.92	0.43	×	12.87	6.40
	3 month	0.14	ND	0.43	ND	7.80	0.16	ND	1.01	0.43	×	13.04	6.38
	6 month	0.06	ND	0.42	ND	7.55	0.15	ND	1.17	0.49	×	12.90	6.29
Koukuzin[d]	Initial	1.75	ND	0.65	ND	12.17	0.31	0.04	0.81	0.31	—	4.76	5.83
	1 month	0.53	ND	0.30	ND	11.13	0.17	ND	0.74	0.21	×	11.41	5.83
	3 month	0.10	ND	0.32	ND	9.08	0.20	ND	0.75	0.21	×	11.45	5.78
	6 month	0.04	ND	0.27	ND	9.28	0.21	ND	0.81	0.23	×	11.24	5.70

AA, acetic acid; BA, butylic acid; FA, formic acid; IA, isobutylic acid; LA, lactic acid; MA, malic acid; PA, propionic acid; PyA, pyrioglucamic acid; SA, succinic acid. Unit: Organic acid: mg/g dried weight, Loss on drying: %. *a*) The data are shown as the mean of 3 samples. *b*—*d*) see Table 1. ND: not detected, —: no change, ×: change of color, ···: change in quality.

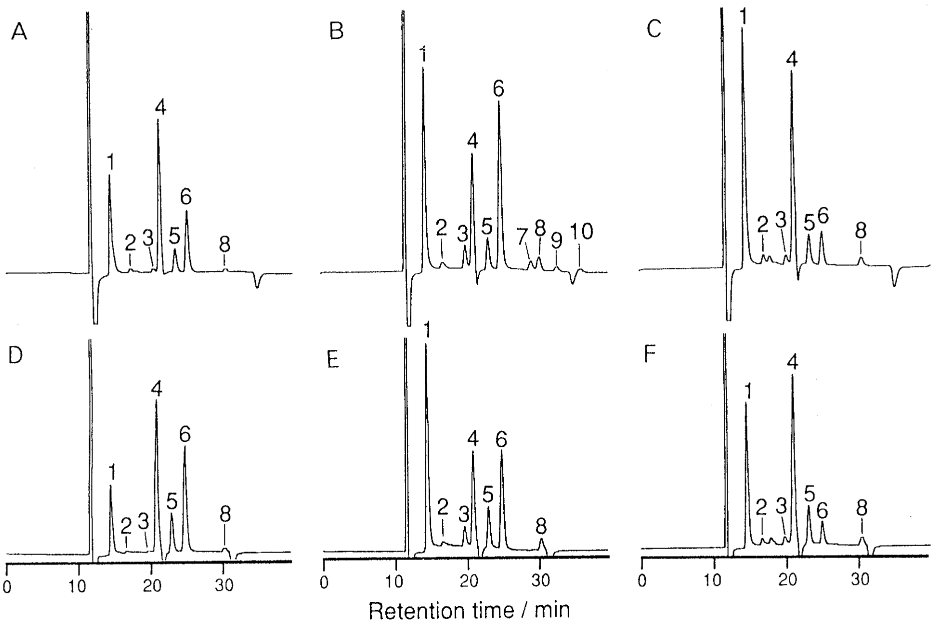

Fig. 2. Changes of Organic Acids in Cervi Parvum Cornu

A: initial chromatogram of sample No. 1, B: initial chromatogram of sample No. 2, C: initial chromatogram of sample No. 3, D: chromatogram of sample No. 1 after six months of storage, E: chromatogram of sample No. 2 after 6 months of storage, F: chromatogram of the sample No. 3 after 6 months of storage. 1—10: see Fig. 1.

in any of the samples stored in the 40 °C tightly closed containers, but increased in some of those stored in the 40 °C–75% RH open containers. The decrease in formic acid was believed due to evaporation, and the increase mainly to decomposition and/or oxidation of the tissue. Formic acid was assumed to be present in Asini Gelatinum, Kokurozin and Koukuzin as a formate, because its quantity became nearly constant after large decreases of about 30%, 85%, and 45%, respectively, of the initial values. Lactic acid decrease was believed caused by decomposition because it was a non-volatile acid, while increase of this acid was due to decomposition and/or the oxidation of the tissue; the increase of lactic acid of Amydae Carapax, however, was thought caused by fermentation because it was observed under all conditions. Evaporation was believed to be the cause of decrease of butylic acid, isobutylic acid and propionic acid, and these acids was presumed to be existed as free acids. The decrease of malic acid and succinic acid was due mainly to decomposition, and the increase of pyroglutamic acid by decomposition and/or oxidation of tissue.

The contents of acetic acid, butylic acid, isobutylic acid and proponic acid in Cervi Parvum Cornu, which varied greatly among the samples, showed no change in the samples stored in the tightly closed containers (25°C, 40 °C), but changes were observed in those samples stored in the 40 °C–75% RH open containers. Acetic acid contents in sample Nos. 1 and 2 increased and then decreased, while that in sample No. 3 decreased slightly. The contents of butylic acid, isobutylic acid and propionic acid in sample No. 2 decreased and finally were not observed at all (Fig. 2).

These differences were believed to be due to the difference in preservation conditions: sample No. 2 was stored without air ventilation because it contained the volatile acids: propionic acid, butylic acid and isobutylic acid; and sample Nos. 1 and 3 were kept in air ventilated conditions because no volatile acids were observed.

Appearance: Asini Gelatinum became hard and did not dissolve in water. The color tone of Hippocampus, Kokurozin and Koukuzin became slightly darker; humidity causes this change in color tone. There was no change in appearance of other samples.

Loss on Drying: Drastic increase of loss in weight which was thought to be caused by the absorption of moisture was observed during the first month of storage in all samples except for Amydae Carapax; thereafter it remained nearly constant.

pH: Ostreae Testa showed a nearly constant value. Amydae Carapax showed a tendency to be alkaline until the first month and to be nearly constant after that. Asini Gelatinum, Lumbricus, Cicadae Periostracum, Kokurozin and Koukuzin tended to be slightly acidic.

Conclusion

1. A selective and sensitive method of analyzing organic acids in animal crude drugs was established and the contents in 10 drugs were determined. Nine organic acids: acetic acid, butylic acid, formic acid, isobutylic acid, lactic acid, malic acid, propionic acid, pyroglutamic acid and succinic acid were found in animal crude drugs used in this investigation.

2. The change of organic acid content in the drugs stored under various conditions was studied, and no change was observed in any of the samples stored in tightly closed containers except for lactic acid of Amydae Carapax. However, the organic acid contents decreased and increased in the samples stored in 40 °C–75% RH open containers.

3. It was possible to compare an animal crude drug's history with others by examining the change of organic acid content in samples stored in 40 °C–75% RH open

containers. The organic acids were thus proved to be "signal" constituents for the evaluation of animal crude drugs.

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