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Identification of vegetable species in gastric contents using HPLC

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Abstract Identification of 16 vegetables, focusing on the influence of digestion in the stomach, was carried out on the basis of the types of flavonoids detected on chromatograms using HPLC. Among the 12 vegetables for which flavonoids were detected, the chromatographic patterns of the flavonoids in digested vegetables were the same as those of the corresponding raw vegetables, making it possible to identify the species of vegetables even after digestion. In our analysis, 5 mg of a freeze-dried sample was found to be an adequate quantity to enable the detection of flavonoids. Brief practical cases are also described.

Key words Gastric contents · Plant pigments · Flavonoid · HPLC

Introduction

In the field of forensic medicine, identification of foods present within the gastric contents is often required to elucidate not only the time of death according to the extent of digestion, but also the behaviour of the victim just prior to death. Discrimination between digested foods in the stomach was generally performed by morphological observation of the external appearance and colour of the foods [1, 2]. However, when identification of the species of vegetables present within the gastric contents is necessary, this method is clearly of limited use because of the similarity in shape and colour of vegetables following digestion [2].

Flavonoids are chemically stable plant pigments that are widely distributed in plants [3, 4]. In our ongoing studies, we previously reported an analytical method of detecting flavonoids in weeds using high-performance liquid chromatography (HPLC) [5], and we applied this method to three criminological cases [6]. Since flavonoids are present in various kinds of vegetables they would seem to be appropriate indicators for identifying the species of vegetables ingested, even after digestion has taken place. We report here on the identification of digested vegetables based on the chromatographic pattern of their flavonoids, comparing these patterns to those of the corresponding raw vegetables. Two practical cases are also described in brief.

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Materials and methods

Materials

The 16 vegetables to be analysed were obtained from a greengrocer's store and are listed in Table 1. Each of the vegetables was cut into small pieces and was freeze-dried to prevent decomposition. Standard flavonoids including myricetin (My), quercetin (Qu), kaempferol (Km), luteolin (Lu) and rhamnetin (Rh) were purchased from Extrasynthese (SSK), Geney, France. Apigenin (AP) and rutin (Ru) were purchased from Sigma (St. Louis, Mo.) and Nacalai Tesque (Kyoto, Japan), respectively. Ru and Rh were used as the reference standards to calculate the retention indices (RIs) for flavonoids. Pepsin was obtained from Katayama Chemical Industry, Osaka, Japan.

Preparation of artificial gastric juice

Artificial gastric juice was prepared by mixing 2.0 g of sodium chloride, 3.2 g of pepsin, 24 ml of 10% hydrochloric acid and dis-

Table 1 Vegetables collected for analysis

Common name	Nomenclature
Bell pepper	*Capsicum annuum*
Green pepper	*Capsicum annuum*
Welsh onion	*Allium fistulosum*
Parsley	*Petroselinum crispum*
Mitsuba	*Cryptotaenia japonica*
Garden peak	*Pisum sativum*
Chingensai	*Brassica rapa* var. *chinensis*
Head lettuce	*Lactuca sativa*
Spinach	*Spinacia oleracea*
Perilla	*Perilla ocymoides*
Mung bean sprouts	*Vigna radiata*
Japanese radish sprouts	*Raphanus sativus*
Snap bean	*Phaseolus vulgaris*
Celery	*Apium graveolens*
Cabbage	*Brassica oleracea*
Okra	*Abelmoschus esculentus*

tilled water to give a total volume of 1000 ml, in accordance with the report of Suzuki [2]. The pH value of this preparation was consequently adjusted to approximately 1.5.

Sample preparations

First, 5 mg samples of the freeze-dried vegetables were each ground to powder for use as the raw samples for detecting flavonoids. Secondly, small pieces of the raw vegetables (over 5 mg) were each immersed in 60 ml of artificial gastric juice in a laboratory dish, and then the contents of the dish were left to digest at 37° C for 3 h with gentle stirring. The digestive time was experimentally selected, taking into consideration that the gastric emptying of solid foods in vivo is completed within 4–6 h [1]. After the sample in the dish had been washed with small amounts of distilled water and again freeze-dried, 5 mg of the preparation has ground to powder for use as the digested sample.

Extraction procedure and calculation of RIs

Extraction of flavonoids was carried out based on the method reported previously [5]. Briefly, 5 mg of the raw sample was extracted with ethyl acetate after hydrolysis. The organic layer was evaporated to dryness, and the residue was dissolved in ethanol. After the solution had been passed through a filter (Columnguard FH 4), 2-μl aliquots of the ethanol solution were applied to HPLC. The digested sample was extracted in the same manner as that used for the raw samples.

To solve the problem of day-to-day variations in the retention time (RT), a retention index (RI) for each flavonoid was calculated by modifying the retention time in the gas chromatography [7]. The values of RIs were obtained from the following formula, setting RIs of Ru and Rh at 0 and 100, respectively.

$$RI(X) = 100 \times \frac{\log[RT(X)/RT(Ru)]}{\log[RT(Rh)/RT(Ru)]}$$

where RT(X) = RT of flavonoid X, RT(Ru) = RT of Ru, RT(Rh) = RT of Rh, RI(X) = RI of flavonoid X.

HPLC conditions

The apparatus used was a Waters 600E pump equipped with a Waters 486 tunable absorbance detector and an integrator a Waters 805 data station. The column was a 15 × 0.6 cm i.d. stainless tube packed with Shim-pack CLC-ODS (octadecyl silica). The eluent was methanol : acetic acid : water (43 : 10 : 47 v/v/v) at a flow rate of 1 ml/min. The wavelength selected was 365 nm.

Results and discussion

Table 2 shows the distribution of flavonoids detected in the raw samples. Various kinds of flavonoids ranging in number from 1 to 8 appeared on the chromatograms obtained from the extracts of 12 out of 16 vegetables. The values of RIs of 15, 41, 50, 69 and 73 indicate My, Qu, Lu, Km and Ap, respectively. Of the other peaks 13 with RIs of 18, 24, 28, 31, 35, 44, 60, 65, 76, 80, 95, 100 and 108 remained unidentified, while each substance was recognized as a flavonoid with a characteristic absorption wavelength of 365 nm [8]. Under the present chromatographic conditions, no flavonoids were found in 4 vegetables: snap bean, celery, cabbage and okra. The same chromatographic patterns were observed in the other 12 vegetables, even after these vegetables had been digsted with artificial gastric juice. Of the freeze-dried sample 5 mg was enough to detect the flavonoids without any decomposition, as shown in a typical example in Fig. 1, and this quantity would also be sufficient if collected from the gastric contents in practical cases.

Chromatograms of the extracts from digested bell pepper and green pepper are shown in Fig. 2. Small pieces of bell pepper and green pepper, which had changed from green to yellowish-green in colour after digestion, were apparently indistinguishable either from each other or from other green vegetables. These 2, which both belong to the same species, *Capsicum annuum*, showed similar flavonoid patterns on the chromatograms, while a peak with the RI 76 appeared only in the case of green pepper. Fig-

Table 2 Distribution of flavenoids in raw samples (*RIs15* myricetin, *41* quercetin, *50* luteolin, *69* kaempferol, *73* apigenin, *100* rhamnetin; others unidentified)

Vegetables	Retention indices of detected flavonoids
Bell pepper	41, 50
Green papper	41, 50, 76
Welsh onion	44, 69
Parsley	73
Mitsuba	50, 73, 76
Garden pea	28, 31, 41, 100
Chingensai	15, 28, 41, 69, 73
Head lettuce	18, 41, 50
Spinach	35, 41, 50, 73, 80
Perilla	31, 41, 50, 65, 73, 76, 95, 108
Mung bean sprouts	44, 60, 69, 76
Japanese radish sprouts	24, 69
Snap bean	null
Celery	null
Cabbage	null
Okra	null

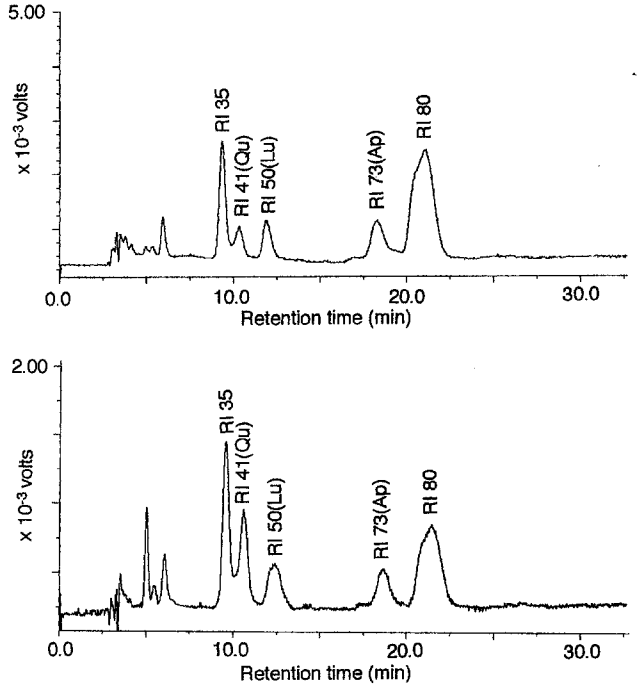

Fig. 1 Chromatograms of the extracts from raw spinach *(top)* and digested spinach *(bottom)*

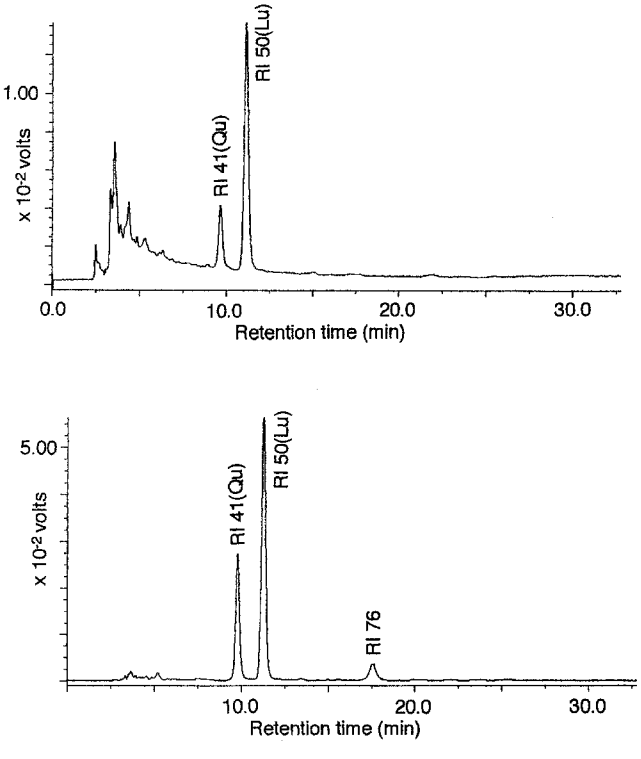

Fig. 2 Chromatograms of the extracts from digested bell pepper *(top)* and digested green pepper *(bottom)*

ure 3 shows the chromatograms of the extracts from digested chingensai and head lettuce. These 2 were clearly distinguishable from each other when judged by the types of flavonoids present, even though their external appearances are nearly the same. Eight other vegetables, Welsh

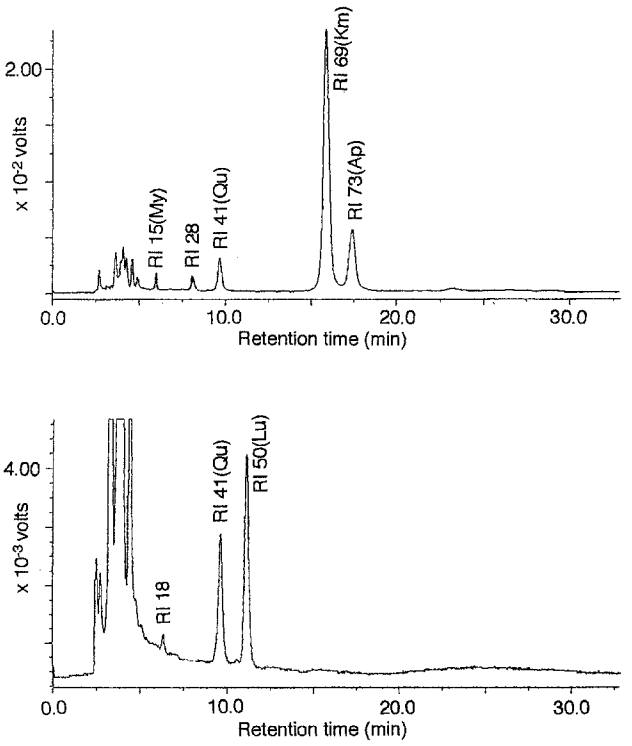

Fig. 3 Chromatograms of the extracts from digested chingensai *(top)* and digested head lettuce *(bottom)*

onion, parsely, mitsuba, garden pea, spinach, perilla, mung bean sprouts and Japanese radish sprouts were also each characterized by a different combination of flavonoids after digestive treatment. These results suggest that the species of the vegetables ingested can be identified by analysing the kinds of flavonoids present in the digested and barely recognizable samples taken from within the stomach. Further studies on those vegetables in which flavonoids were not detected on the chromatograms are deemed necessary.

Cases reports

Case 1

A 20-year-old man was found dead, having been floating in the sea for about 1 week. There were a few pieces of yellowish-green matter in the gastric contents, together with a little meat. The HPLC chromatogram of the yellowish-green matter showed three peaks corresponding to RIs 41, 50 and 76 (Fig. 4a), a pattern identical with that of green pepper (Table 2). These results were supported by a police investigation which revealed that the victim had eaten green peppers with *yakitori* (grilled chicken on a stick) the night that he went missing.

Case 2

A 53-year-old woman was found dead, having been buried beneath the sand at the sea shore for approximately 3 months. Although morphological findings could not reveal what kinds of foods were present in her stomach, two flavonoid peaks with the RIs 44 and 69 appeared on the HPLC chromatogram of the extract obtained from pieces found in the gastric contents, as shown in Fig. 4b. These

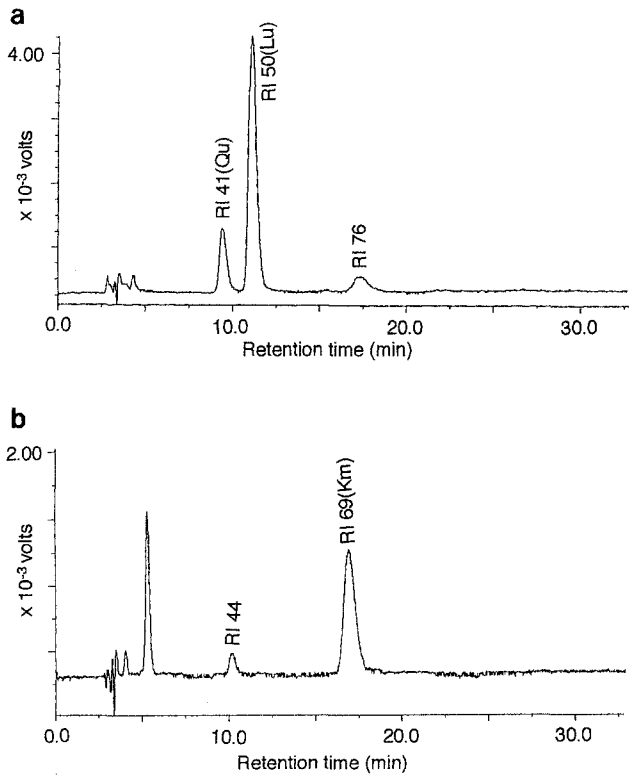

Fig. 4 a, b Chromatograms of practical forensic cases. **a** Chromatogram of the extract obtained from pieces found in the gastric contents in case 1 (identified as green pepper). **b** Chromatogram of the extract obtained from pieces found in the gastric contents in case 2 (identified as Welsh onion)

peaks corresponded with those of Welsh onion (Table 2), which was consistent with a police investigation that the victim had consumed Welsh onion with noodles at the time of the crime 3 months earlier.

Conclusions

The flavonoids contained in vegetables could be analysed by means of HPLC even after digestive treatment, and this made a differential discrimination of vegetables feasible based on the chromatographic patterns. This method can be applied to those practical forensic cases in which morphological examination of the digested vegetables cannot yield much information, thus enabling a precise judgment to be made regarding the time of death and the behaviour of the victim just prior to death.

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