Simultaneous Determination of Medicinal Ingredients in So-called Health-promoting Food Using Liquid Chromatography Tandem Mass Spectrometry with a Pentafluorophenyl Stationary Phase

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An analytical method using liquid chromatography tandem mass spectrometry equipped with electrospray ionization (ESI) was demonstrated for the determination of medicinal ingredients, such as fenfluramine (FF), N-nitrosofenfluramine (NFF), sibutramine (SIB), sildenafil (SDF), vardenafil (VDF), tadalafil (TDF) and xanthoanthrafil (XAF), in so-called health-promoting food. These analytes were clearly separated with acetonitrile-water (40 : 60) containing 4 mM formic acid and 8 mM ammonium formate used in the mobile phase on a pentafluorophenyl (PFP) column under isocratic conditions. The retention times of FF, SIB, SDF and VDF on the PFP column were longer than those on the C18 column under the same mobile phase conditions. Within wide ranges, all peaks were proportional and the coefficient of determination (r^2) showed more than 0.9950 in a linear regression analysis. The limit of quantification (LOQ) of the developed method was 0.8–42.2 μg/l (S/N = 10). The recoveries of analytes admixed with commercially available health-promoting food ranged from 80.2 to 113.3% and were acceptable for quantitative analysis. Analytes of more than 2 μg in a health-promoting food sample (0.5 g) were able to be identified by the European Communities (EC) criteria.

Key words —— pentafluorophenyl column, health-promoting food, medicinal ingredient, liquid chromatography tandem mass spectrometry

INTRODUCTION

Preventive medicine in general has expanded its market in Japan along with the maturation of the aging society. People wish to take so-called health-promoting foods in an attempt to prevent life-style related diseases. Under most circumstances these foods are harmless, but some contain toxic agents. The presence of medicinal ingredients intentionally added to health-promoting foods has been reported. In 2002, the impairment of health, hepatopathy in many cases which was fatal in some, occurred in peoples who took health-promoting food made in China,[1)] which contained N-nitrosofenfluramine (NFF), a nitroso derivative of fenfluramine (FF), an anorexic drug. At present widely distributed health-promoting foods which are potentially dangerous can be categorized into two, *i.e.*, those advertising weight loss and those advertising the improvement of sexual dysfunction. FF, NFF and sibutramine (SIB) are occasionally added to weight-loss food. Sildenafil (SDF), vardenafil (VDF), tadalafil (TDF) and xanthoanthrafil (XAF) are occasionally added to health-promoting food advertising the improvement of sexual dysfunction. To prevent health impairment by medicinal ingredients in health-promoting food, it is necessary to analyze the ingredients in health-promoting food with a rapid, simple and highly sensitive method. Most analytical methods for medicinal ingredients in so-called health-promoting food that have been published use high performance liquid chromatography (HPLC)

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with UV detection. However, these methods require confirmation of medicinal ingredients by using thin layer chromatography and HPLC/photo-diode array, as selectivity of UV detection is comparatively low.[2] So, these methods need a lot of efforts and time to determine existence of medicinal ingredients. Recently, methods using mass spectrometer have been published. However, these methods require confirming the identity between a mass spectrum measured in a sample and the spectrum of standard.[3,4] It is difficult to do determination and identification at once.

High-performance liquid chromatography with the tandem mass spectrometry detection technique (LC/MS/MS) is a powerful method. It is generally believed that the utilization of LC/MS/MS practically guarantees specificity even under conditions where the sample preparation is simplified or even eliminated, and no or very little prechromatographic separation is required.[5,6] Contrary to common belief, a number of examples illustrated the need for extensive assay validation and careful assessment of LC/MS/MS assay specificity, including studies of matrix effect and ion suppression.[7,8]

Electrospray ionization (ESI), generally part of the LC/MS/MS equipment, is ideally suited for the introduction of polar, thermally labile compounds into a mass spectrometer. The higher the proportion of organic solvent, the stronger is the signal intensity in ESI. Better spray performance appears to be due to the lower surface tension of the organic solvent.[9,10] Consequently, to ensure the robustness of the LC/MS/MS procedure, an analytical method was required to isolate all analytes from each other, to use the mobile phase of a higher proportion of the organic solvent and to be performed in an isocratic mode. The Octadecylsilica (ODS) stationary phase has been widely used for LC/MS/MS analysis of health-promoting foods.[3,4] However, SDF, VDF, TDF and XAF are hydrophilic, and it is difficult to analyze them simultaneously with FF, NFF and SIB under isocratic conditions. It is thus necessary to develop a new method capable of analyzing simultaneously a large number of compounds in health-promoting foods in order to screen health-promoting foods containing the above ingredients. The pentafluorophenyl (PFP) stationary phase has been reported to exhibit specific interaction with halogen-containing compounds and basic compounds.[11–20] Consequently, it will retain some ingredients in a different fashion compared with the ODS stationary phase. Therefore, in this paper, we tried to develop a simultaneous determination method by LC/MS/MS with a PFP column.

MATERIALS AND METHODS

Reagents, Standards, and Supplies —— The HPLC-grade of acetonitrile was purchased from Kanto Chemical Ltd. (Tokyo, Japan). Formic acid and ammonium formate were special grade and were purchased from Nacalai Tesque Ltd. (Kyoto, Japan). FF, NFF, SIB, SDF, VDF, TDF and XAF were provided by the National Institute of Health Sciences in Japan (Tokyo, Japan). All chemicals were used without any further purification.

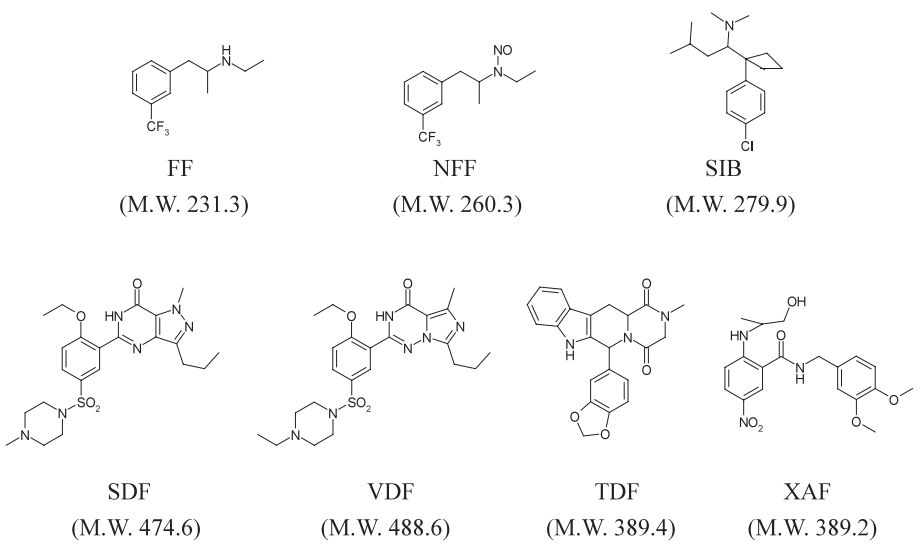

Fig. 1. Chemical Structures of the Analytes

Chemical structures of analytes are shown in Fig. 1. All aqueous solutions were prepared using distilled deionized water (DDW), which was obtained using a Milli-Q SP TOC Ultra-Pure Reagent Water System (Millipore, Billerica, Massachusetts, U.S.A.). Stock standard solutions of all analytes were prepared at a concentration of 100 mg/l in the solvents which were used for the mobile phase.

LC/MS/MS Analysis —— Analyte separation was achieved using an Aliance 2695 system (Waters Co., Milford, Massachusetts, U.S.A.) equipped with a Supelco Discovery HS F5 (50 mm × 2.1 mm i.d., 3 μm particles) analytical column obtained from Sigma-Aldrich Corporation (St. Louis, Missouri, U.S.A.). Identification and quantification were accomplished using a Quattro Micro API (Waters Corp., Milford, Massachusetts, U.S.A.) equipped with ESI, which utilized nitrogen as the sheath gas and helium as the reagent gas. Control of the HPLC system and communication with the mass spectrometer were accomplished using Masslynx software (Waters Corp.) and integration of the chromatographic peaks was accomplished using Targetlynx LC/MS/MS software (Waters Corp.). For all determinations, HPLC was operated in an isocratic mode with a flow rate of 0.2 ml/min. The mobile phase was prepared as follows. Aliquots (8 ml) of 500 mM formic acid and 1000 mM ammonium formate were transferred to a 100 ml volumetric flask. After adjusting the volume with DDW, this solution was mixed with acetonitrile (400 ml) and DDW (500 ml) and this mixture was filtered through a vacuum filtering apparatus that incorporated a 0.5 μm 47 mm hydrophilic polytetrafluoroethylene (PTFE) membrane filter (Advantec MFS, Inc., Tokyo, Japan). The mobile phase ratio employed was 40 : 60 (acetonitrile : buffer solution). The column temperature was maintained at 40°C. The HPLC column was routinely equilibrated overnight prior to use. Following ESI operating conditions were adopted: source temperature, 120°C; desolvation temperature, 350°C; cone gas flow, 50 l/hr; desolvation gas flow, 600 l/hr; capillary voltage, 3.15 kV; multiplier voltage, 650 V. The mass spectra for analyte precursor and daughter ions are shown in Table 1.

Sample Extraction —— Health-promoting food samples were ground with a pestle, samples (0.5 g) were placed in a 10 ml centrifuge tube, and 5 ml of the mobile phase was added. The centrifuge tube was capped and sonicated for 10 min in an ultrasonic bath US-4 (SND Co., Ltd., Suwa, Japan). After centrifugation (3000 rpm, 5 min) in a KN-70 (KUBOTA Corp., Tokyo, Japan), the supernatant was transferred to a 20 ml volumetric flask and sediment was extracted twice again with 4 ml of the mobile phase. All supernatants were combined and the volume was adjusted with the mobile phase. These solutions were then filtered through a PTFE filter. An aliquot (2 μl) of each solution was injected onto the column.

RESULTS AND DISCUSSION

Optimization of LC/MS/MS System

Working with 1 mg/l standards with the exception of TDF that was 10 mg/l, we began finding an optimizing condition as described below at an injection volume of 5 μl. The mobile phase ratio employed was 50 : 50 (acetonitrile : DDW) at a flow rate of 0.2 ml/min. All analytes created $[M+H]^+$ ions in the positive ionization mode. SDF, VDF, TDF and XAF also created $[M-H]^-$ ions in the negative ionization mode. After identifying $[M+H]^+$ ions and $[M-H]^-$ ions, selected ion recording (SIR, a single MS mode), conditions were optimized by injecting each analyte. Ionization evaluation of all analytes indicated that positive ionization, *i.e.*, creating $[M+H]^+$ ions, was much more effective than negative ionization, which formed $[M-H]^-$ ions except for XAF. Since the relative intensity of XAF in the negative ionization mode to the positive ionization mode was 1.16, the intensities of XAF at both ionization modes were practically similar. From these results, the positive mode was chosen for ionization. Likewise, the conditions of multiple reactions monitoring (MRM, a tandem MS mode) were optimized by injecting each analyte. For the identification of analytes according to EC criteria,[21] the condition was chosen under which two daughter ions were clearly created. Mass spectra of analytes in ESI by precursor ion scan are shown in Fig. 2.

Relationship between the Concentration of Ammonium Formate and the Capacity Factor

Optimization of the chromatographic system was realized by examining the relationship between the concentration of ammonium formate of the mobile phase and the capacity factor (k') of the analytes. The mobile phase ratio employed was 30 : 70 and 60 : 40 (acetonitrile : buffer solution). The final concentrations of ammonium formate tested were 1, 2, 4 and 8 mM in the mobile phase. MS detection was carried out by SIR. As shown in Fig. 3,

Table 1. Summary of Mass Spectra for Analyte Precursor Ions and Daughters Ions

Analyte	Precursor ion m/z	Analysis Condition			Retention Window[d] (min)
		Daughter ions[a]	CV[b] (V)	CE[c] (V)	
FF	232.1	109.0 82.9	30	60	0.00– 3.50
NFF	261.1	109.0 82.9	20	65	0.00– 3.00
SIB	280.2	88.9 125.0	25	60	2.00– 4.00
SDF	475.2	57.8 100.0	50	40	2.50– 4.50
VDF	489.2	151.0 312.2	55	40	4.00– 7.00
TDF	390.1	169.1 134.9	20	20	6.50–11.00
XAF	390.2	106.9 151.1	20	55	11.00–22.00

a) Upper column is an ion for quantitation. Lower column is an ion for identification. *b*) CV: Cone Voltage. *c*) CE: Collision Energy. *d*) Retention Window: opening time and ending time of acquiring data.

analytes were classified into two groups in one of which (Group 1) log k' was negatively proportional to the logarithmically transformed concentration of ammonium formate, and in the other (Group 2) log k' was constant irrespective of the ammonium formate concentration. The former Group 1 consisted of FF, SIB, SDF and VDF, and NFF, TDF and XAF made up Group 2. It has been shown that log k' was negatively proportional to the logarithmically transformed concentration of ammonium formate in the mobile phase on ion exchange chromatography.[22] Thus, an interaction between an analyte in Group 1 and PFP in the stationary phase appeared to be similar to those on ion exchange chromatography. It was possible to predict the retention time of the analyte. The concentration of ammonium formate in the mobile phase was set at 8 mM, since the retention time of SIB which was strongly retained on PFP column was shortest at this concentration.

Relationship between the Concentration of Acetonitrile and the Capacity Factor

Fig. 4 shows the relationship between the concentration of acetonitrile in the mobile phase and the k' of analytes with the PFP and ODS columns. Acetonitrile compositions were varied in a 10% (v/v) stepwise manner from 20 to 90%. Using this mobile phase, the retention time of the analyte on the PFP column was compared with that on the ODS column. MS detection was carried out using SIR.

As shown in Fig. 4b, a relationship between the log k' values and acetonitrile concentration in the mobile phase was almost linear on the ODS column over all of the investigated concentration ranges for all analytes. Several studies have been reported on a relationship between the k' values of analytes, and volume fraction ϕ of organic solvents in water-organic solvent mixtures in reversed phase HPLC. Snyder *et al.*[23] give this relationship as

$$\log k' = -S\phi + \log k_w \quad (1)$$

where k_w represents the k' value of a compound with pure water as the mobile phase and S is related to the solvent strength of a pure organic solvent. For acetonitrile-water, S should therefore be constant for a given column and different types of solutes. Our results on the ODS column satisfied the equation (1).

On the other hand, as shown in Fig. 4a, the relationship between the log k' values and acetonitrile concentration was not strictly linear on the PFP column, suggesting that the interaction of the analytes with the PFP column was more complicated than with the ODS column. The retention times of Group 1 analytes (FF, SIB, SDF, VDF) on the PFP column were longer than on the ODS column. Thus, it is possible to analyze simultaneously seven analytes at a concentration of acetonitrile of 40% on the PFP column.

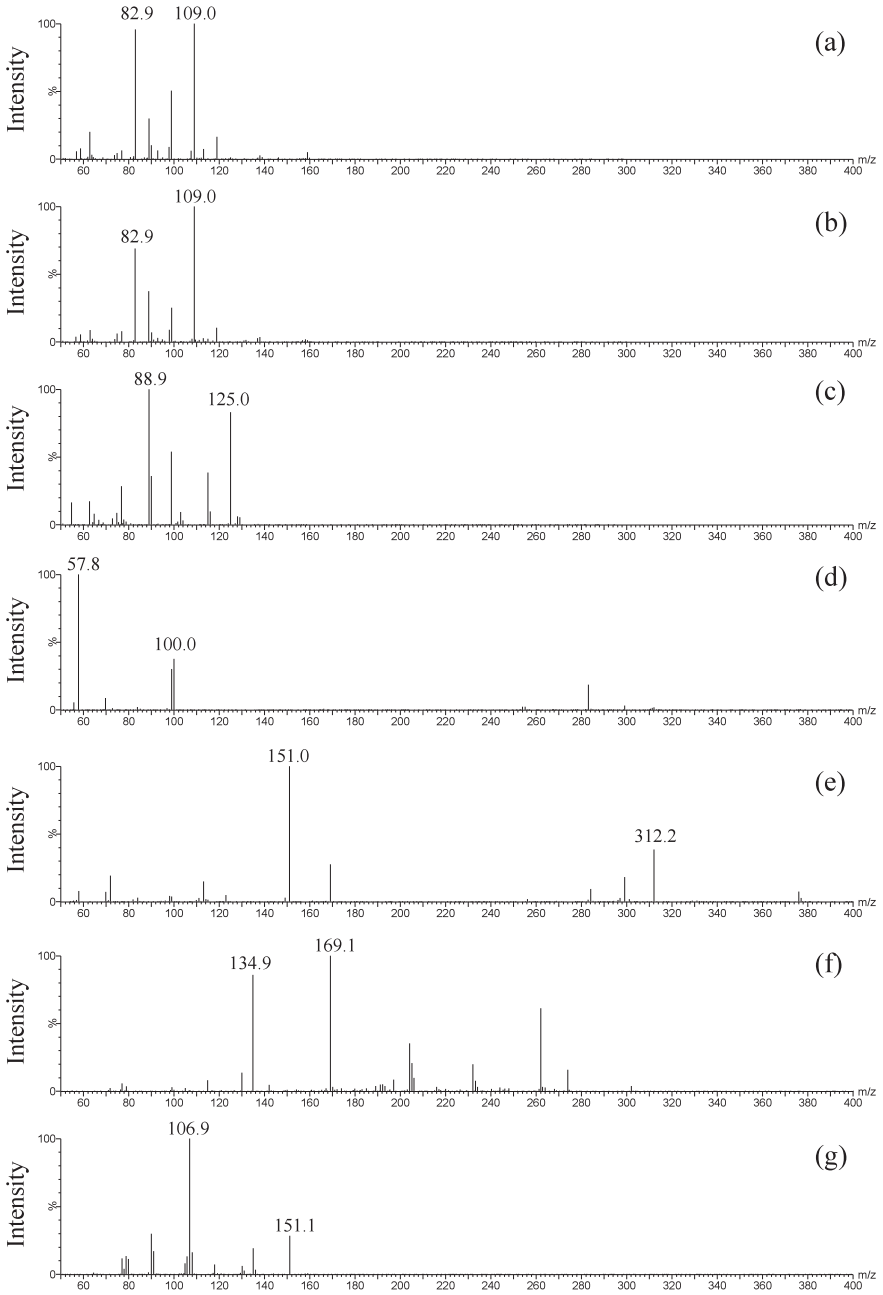

Fig. 2. Mass Spectra of Analytes in ESI by Precursor Ion Scan

(a) FF, (b) NFF, (C) SIB, (d) SDF, (e) VDF, (f) TDF, (g) XAF. Standard solution was injected at 2 µl (Each concentration was 10 µg/ml except for 1 µg/ml FF). Conditions: Supelco Discovery HS F5 column, 5 cm × 2.1 mm i.d. 3 µm, acetonitrile-water (40 : 60) containing 4 mM formic acid and 8 mM ammonium formate as the mobile phase at a flow rate of 0.2 ml/min. Column Temperature: 40°C.

Linear Dynamic Range and Limit of Detection

The linearity of the calibration curve was investigated in the analyte concentration ranges of 10–10000 (ng/ml) with the exception of FF, the ranges for which were 1–1000 ng/ml. As shown in Table 2, calibration curves showed good linearity with the coefficient of determination (r^2) exceeding 0.9950 for all analytes tested. Linear dynamic ranges were 10–10000 ng/ml for SDF, VDF and XAF, 1–1000 ng/ml for FF, and 50–10000 ng/ml for NFF, SIB and TDF.

Table 2 also shows the limit of quantification (LOQ) for evaluating the sensitivity of this procedure. LOQ (ng/ml) was defined as the lowest concentration of analytes having a minimum signal-to-noise ratio (S/N) of 10, in addition to meeting the LC/MS/MS special fingerprint identification and retention time criteria. As shown in Table 2, the LOQs

of analytes in this procedure were 0.8 for SDF, 1.0 for FF and VDF, 7.2 for XAF, 14.2 for SIB, 15.2 for NFF and 42.2 for TDF. Mass spectrometric methods are suitable for confirmatory methods, because it can identify proper fragment ions of analyts. The condition of MS/MS was chosen so as to meet the EC criteria, which demand that spectral data must contain an ion definitively designated as the parent molecule and at least two ions of breakdown products, the spectra of which are completely separable. For the quantification purposes, only two ions of the breakdown product were monitored. Based on these criteria, the LOQs of analytes in the present method seemed to be higher than those of analytes in the method usually employed (data not shown).

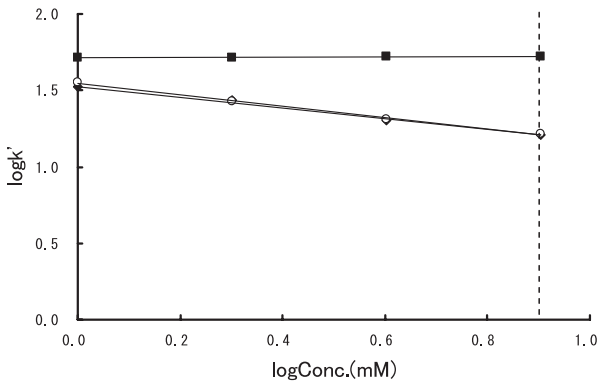

Fig. 3. Representative Relationship between log k' Values and the Logarithm of Ammonium Formate Concentration in the Mobile Phase with the PFP Column

k' : the capacity factor. Standard solution was injected at 2 µl, each concentration was 1 µg/ml. Conditions: Supelco Discovery HS F5 column, 5 cm × 2.1 mm, i.d. 3 µm, acetonitrile-water (30 : 70) containing 4 mM formic acid and 1, 2, 4 or 8 mM ammonium formate, respectively, as the mobile phase, flow rate 0.2 ml/min, column temperature 30°C. ■: NFF, ◆: SDF, ○: VDF. The broken line shows the adopted condition in this study.

Matrix Effect, Recoveries and Identification

It is very important to evaluate the matrix effect on the quantitative determination of analytes in health-promoting food. Without such cautions, undetected endogenous compounds present in these samples might co-elute with an analyte of interest, affect the efficacy of its ionization, and as a result decrease or increase MS responses. To determine the matrix effect, two health-promoting foods (one was advertising weight loss, the other was advertising improvement of sexual dysfunction) were extracted and admixed with two different doses of the standard compound, *i.e.*, the nominal concentrations of FF being 10 ng/ml and 100 ng/ml, and 100 ng/ml and 1000 ng/ml for the others. As shown

Table 2. Linearity of Calibration Curve and Limit of Quantification (LOQ) of Analyte

Analyte	r^2 Value[a)]	LOQ[b)] (ng/ml)
FF	1.0000*	1.0
NFF	0.9997**	15.2
SIB	0.9996**	14.2
SDF	1.0000	0.8
VDF	0.9950	1.0
TDF	0.9966**	42.2
XAF	0.9999	7.2

a) r: coefficient of determination. *b*) LOQs are provided as S/N > 10. Linearity Range is 10–10000 ng/ml except for *(1–1000 ng/ml) and **(50–10000 ng/ml).

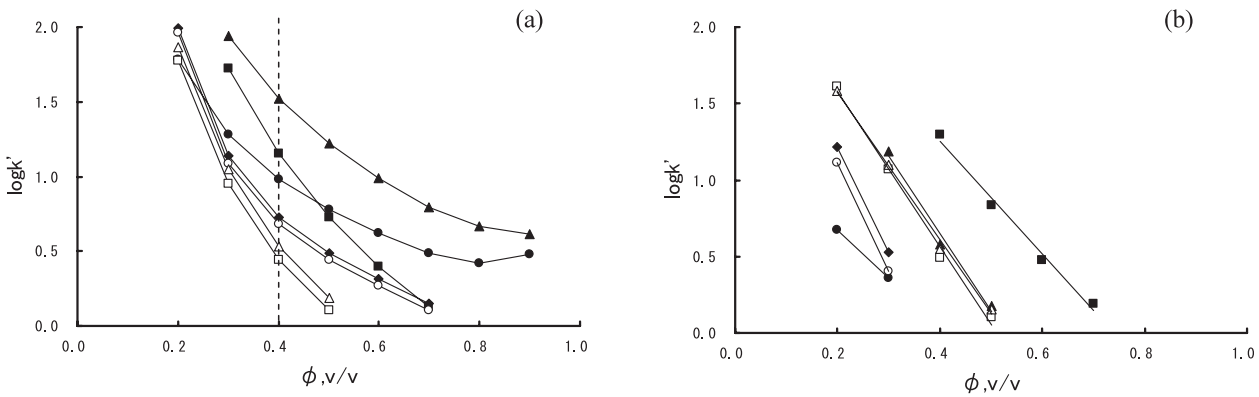

Fig. 4. Relationship between log k' Values and the Acetonitrile Concentration in the Mobile Phase with PFP and ODS Columns

(a) PFP column (Supelco Discovery HS F5, 5 cm × 2.1 mm i.d., 3 µm), (b) ODS column (Supelco Ascentis C18, 5 cm × 2.1 mm i.d., 3 µm), k': capacity factor, ϕ: the volume fraction of organic solvent in the mobile phase. Standard solution was injected at 2 µl, each concentration was 1 µg/ml except for 0.1 µg/ml FF. Conditions: acetonitrile-water containing 4 mM formic acid and 8 mM ammonium formate. Acetonitrile composition varied by steps of 10% (v/v) from 20 to 90%, flow rate 0.2 ml/min, column temperature 30°C. ●: FF, ■: NFF, ▲: SIB, ◆: SDF, ○: VDF, □: TDF, △: XAF. Broken line shows the adopted condition in this study.

Table 3. Matrix Effect (ME) and Recovery (RE) Data for Analytes in Two Different Admixed Healthfood Supplements

Analyte	Nominal Conc. (ng/ml)	ME[a] (%)		RE[b] (%)	
		Sample 1[c]	Sample 2[d]	Sample 1[c]	Sample 2[d]
FF	10	100.5 (4.0)	97.5 (3.1)	113.3 (3.7)	99.6 (4.3)
	100	104.9 (1.3)	98.4 (2.1)	106.6 (1.5)	92.1 (3.8)
NFF	100	103.0 (6.4)	99.1 (2.0)	99.1 (5.3)	94.6 (2.4)
	1000	99.8 (2.8)	102.3 (2.1)	98.7 (1.3)	92.2 (1.6)
SIB	100	101.0 (2.9)	97.6 (1.9)	99.3 (4.0)	94.2 (4.7)
	1000	99.4 (2.8)	97.8 (0.7)	101.0 (1.2)	90.4 (3.7)
SDF	100	101.4 (2.1)	98.7 (1.3)	102.3 (2.2)	95.2 (1.6)
	1000	100.1 (1.9)	100.9 (1.3)	101.7 (1.3)	90.5 (4.9)
VDF	100	103.0 (2.2)	97.8 (1.4)	104.3 (2.9)	94.4 (2.3)
	1000	100.7 (1.4)	102.4 (1.2)	101.4 (1.3)	88.4 (4.6)
TDF	100	99.5 (5.0)	99.6 (7.2)	85.5 (13.2)	80.2 (12.2)
	1000	99.0 (2.2)	97.3 (3.5)	97.8 (3.3)	86.4 (5.7)
XAF	100	100.6 (5.3)	97.3 (3.7)	99.3 (4.8)	86.4 (2.9)
	1000	99.9 (1.2)	97.0 (1.4)	103.2 (1.7)	93.3 (2.8)

Matrix effect and recovery were tested using samples admixed with two different doses of analyte (n = 6). The value in parenthesis is relative standard deviation. *a*) The Matrix effect is expressed as the ratio of the mean peak area of analyte admixed after extraction to the mean peak area of the same analyte standards multiplied by 100. A value of > 100% indicates ionization enhancement, and a value of < 100% indicates ionization suppression. *b*) Recovery calculated as the ratio of the mean peak area of an analyte admixed before extraction to the mean peak area of the same analyte standards multiplied by 100. *c*) Sample 1 is a so-called health-promoting food advertising weight loss. *d*) Sample 2 is a so-called health-promoting food advertising the improvement of sexual dysfunction.

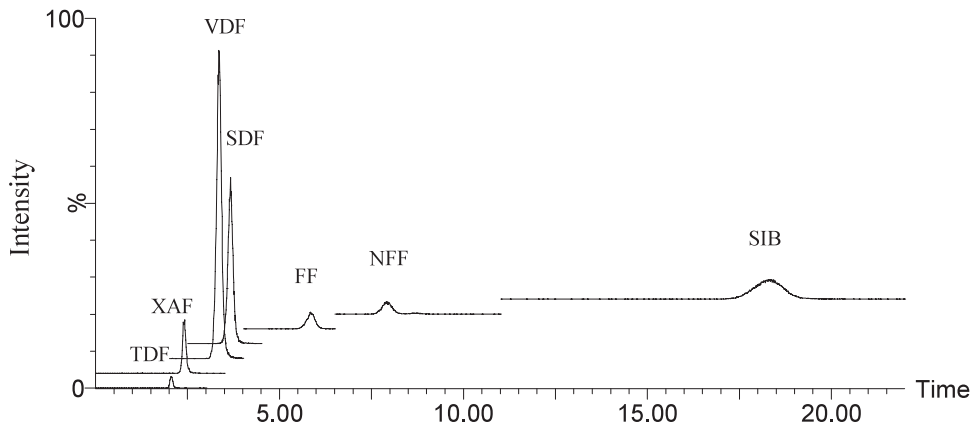

Fig. 5. Mass Chromatogram of a Commercially Available So-called Health-promoting Food Admixed with Analytes

Conditions: Supelco Discovery HS F5 column, 5 cm × 2.1 mm i.d., 3 μm, acetonitrile-water (40 : 60) containing 4 mM formic acid and 8 mM ammonium formate as the mobile phase at a flow rate of 0.2 ml/min, column temperature 40°C, injection volume 2 μl, each nominal concentration 1 μg/ml except for 0.1 μg/ml FF.

in Table 3, the matrix effect ranged from 97.0 to 104.9%. Consequently, it was clearly demonstrated that the matrix effect did not occur in this method.

Recovery tests were also carried out on two health-promoting foods used for the evaluation of the matrix effect, where two different doses of the standard compound were admixed. Recovery was calculated as the ratio of the mean peak area of an analyte admixed before extraction to the mean peak area of the same analyte standards multiplied by 100. The mass chromatogram is shown in Fig. 5. Interfering peaks were not observed for any analytes. As shown in Table 3, the recovery rate of analytes ranged from 80.2 to 113.3%, which were acceptable for analysis. According to the EC criteria, identification and quantification must be carried out at the same time. The present results satisfied the EC criteria except for FF, NFF, TDF and XAF at a low

admixed concentration level. Since the concentration of analytes in actual health-promoting foods are generally higher than that of the recovery test, this method is practically useful for identification.

In conclusion, a convenient and reliable method was demonstrated for the simultaneous identification and quantification of medicinal ingredients in health-promoting foods. By using a PFP column, polar analytes could be retained adequately and all analytes could be analyzed simultaneously, which was difficult to achieve by using a conventional ODS column. ESI-MS was a "soft" ionization technique that yielded a simple spectrum consisting of predominant protonated $[M+H]^+$ ions from analytes in the present study. The MS/MS analysis of these unique $[M+H]^+$ ions enhanced the specificity of the method. The present method was selective, and samples could be analyzed directly without any pretreatment except for extraction. There was no interference from other co-existing substances analyzed so far. Our findings suggest that the present method would provide a useful approach for screening analyses in health-promoting foods.

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