FOOD COMPOSITION AND ADDITIVES

Fast Cleanup Method for the Analysis of Sudan I–IV and Para Red in Various Foods and Paprika Color (Oleoresin) by High-Performance Liquid Chromatography/Diode Array Detection: Focus on Removal of Fat and Oil as Fatty Acid Methyl Esters Prepared by Transesterification of Acylglycerols

YOKO UEMATSU, MAMI OGIMOTO, JUNICHIROU KABASHIMA, KUMI SUZUKI, and KOUICHI ITO
The Tokyo Metropolitan Institute of Public Health 3-24-1, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Japan

A fast and effective cleanup method was developed for the analysis of Sudan I, II, III, IV, and Para Red (Sudan dyes) in various foods and paprika color (oleoresin) by high-performance liquid chromatography (LC) with a diode array detector (DAD). Removal of fat or oil in fatty sample was a critical point for reducing the volume of the final sample solution in order to obtain a sufficient level of the analytes. Separation of fat or oil from the dyes with a silica gel solid-phase extraction (SPE) column seemed unfeasible, because elution profiles of oil, fat, and the dyes were similar. Finally, fat and oil were separated from the dyes by elution from the SPE column with *n*-hexane, not as intact compounds but as fatty acid methyl esters prepared by direct transesterification of acylglycerols in fat and oil, leaving the dyes on the column. The dyes were eluted with *n*-hexane–diethyl ether (9 + 1). Gradient elution with water and tetrahydrofuran was used for separation on a C18 column by LC. Measurement of spectral of 0.5 μg/g of Sudan dyes in foods and 1 μg/g in paprika color (oleoresin) with the DAD was achieved.

In 2003, the illegal presence of the dye Sudan I in chili powder and in foods containing chili powder was reported in Europe. Since then, there have been many reports of the presence of Sudan I, II, III, and IV in foods all over the world. In 2005, the presence of Para Red was reported (1). Use of these dyes as food additives is illegal in Japan, Europe, United States, and most other countries. In 2006, a method for the determination of Sudan I, II, III, and IV and Para Red (Sudan dyes, structures are illustrated in Figure 1) in foods by high-performance liquid chromatography (LC) with detection at 500 nm was notified by the Health, Labor, and Welfare Ministry of Japan (the Notified Method; 2). However, a method for the confirmation of LC peaks, e.g., by measurement of ultraviolet-visible (UV-Vis) spectra with a diode array detector (DAD) or by mass spectrometry (MS), appeared only as reference without description of cleanup. Measurement of UV-Vis spectra of Sudan dyes by LC-DAD was attempted since they would provide useful information about the compounds based on maximum absorption wavelength (λmax) and the shape of the spectra, and they can be used as a tool for confirmation of LC peaks of Sudan dyes in foods, distinguishing from natural colors in the sample. However, measurement of the spectra with a DAD required higher concentrations of analytes than detection at a single wavelength. Sample solutions subjected to LC should contain a sufficient level of the analytes, which is sometimes difficult for fatty foods because fat or oil that remains in the final solution restricts reducing the volume of the solution. Several methods for the analysis of Sudan dyes in hot chili and other foods were reported (3–9). In these methods, extraction by organic solvents alone was used for sample preparation. For fatty foods, substantial fat or oil was extracted together with the Sudan dyes. However, no further cleanup procedures were described to remove fat or oil before LC analysis except gel-permeation chromatography (GPC), which was only applicable to Sudan I in a limited kind of foods (10).

In this paper, we developed a fast and effective cleanup method for spectral measurement of Sudan dyes in a wide variety of foods and paprika color (oleoresin) by LC-DAD, with a focus on removal of fat and oil. Fat and oil were removed as fatty acid methyl esters (FAMEs) prepared by transesterification of acylglycerols in the fat and oil.

Experimental

Samples

Chili sauce, chili powder, Chinese hot chili pepper seasoning, Korean pickles seasoned with hot chili pepper, salted cod ovum seasoned with hot chili pepper, grilled eel, deep fried chicken seasoned with hot chili pepper, potato chips seasoned with hot chili pepper, commercial curry roux,

Received September 29, 2006. Accepted by SG November 15, 2006.
Corresponding author's e-mail: Youko_Uematsu@member.metro.tokyo.jp

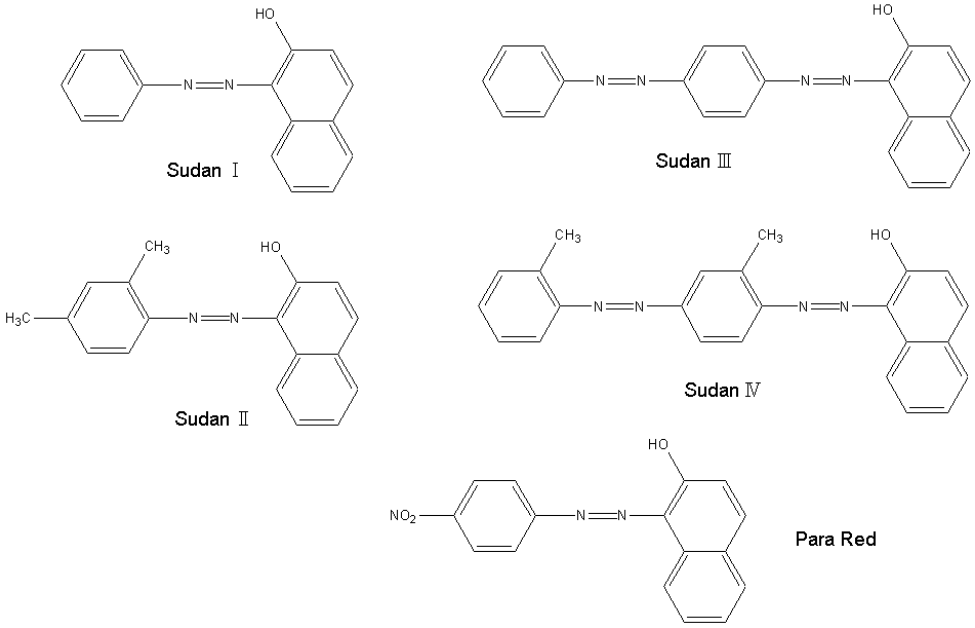

Figure 1. Chemical structures of Sudan dyes.

palm oil, paprika oleoresin, each one product, and 3 paprika color preparations (2 liquid and 1 powder).

Standards and Reagents

(**a**) *Sudan I–IV*.—Sigma-Aldrich Japan K.K. (Tokyo, Japan).

(**b**) *Para Red*.—Acros (through Kanto Chemical Co., Inc., Tokyo, Japan).

(**c**) *Tetrahydrofuran (THF)*.—LC grade (Nacalai Tesque, Inc., Kyoto, Japan).

(**d**) *Sodium methoxide methanol solution*.—28% (Wako Pure Chemical Industries Ltd., Osaka, Japan).

(**e**) *Disodium hydrogen citrate 1.5 H_2O*.—Kanto Chemical Co., Inc.

(**f**) *Methyl stearate*.—Doosan Serdary Research Laboratories, SRL (through Funakoshi Co., Ltd., Tokyo, Japan).

(**g**) *Tristearin*.—>95% (Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan).

(**h**) *Solid-phase extraction (SPE) columns*.—Bond Elut SI (silica gel), 500 mg (LRC type), and Mega Bond Elut SI, 1 g/6 mL (Varian Inc., Palo Alto, CA; through GL Sciences Inc., Tokyo, Japan).

Instruments

(**a**) *LC system*.—Model LC10A equipped with a DAD (Shimadzu Co., Kyoto, Japan).

(**b**) *Gas chromatograph*.—GC-6890 with a flame ionization detector (Agilent Technologies, Inc., Palo Alto, CA).

(**c**) *UV-Vis spectrophotometer*.—Model UV-2200 (Shimadzu Co.).

(**d**) *Sonicator*.—Model 2210 (Branson, Daubury, CT).

Standard Solutions

A 10 mg portion of each standard dye was dissolved in 10 mL THF (stock standard solution). Portions of each stock standard solution were mixed and diluted with methanol to obtain working standard solutions (0.01–100 μg/mL).

Sample Preparation

The sample was homogenized; dry or powder sample was mixed with an equal amount of water before homogenization. A 1 g sample (2 g for a sample to which water was added) of the homogenate was weighed in a 50 mL centrifuge tube with a screw cap. For samples that contained large amounts of fat or oil (samples in which fat or oil content was more than 25%, e.g., potato chips, curry roux, or palm oil), 0.5 g (1 g for a sample to which water was added) of the homogenate was weighed. Samples that contained solid fat were heated in a water bath until the fat liquefied. One mL ethanol and 4 mL THF were added to the homogenate and mixed. Then, 5 mL 5% sodium methoxide methanolic solution was added to the mixture and shaken vigorously for 1–2 min. Samples that contained solid fat were occasionally heated at 60°C during the reaction. Ten mL *n*-hexane was added and mixed, resulting in a single liquid phase. Then, 10 mL 15% disodium hydrogen citrate solution was added, mixed, and centrifuged for 5 min at 3000 rpm to separate the 2 phases. The upper phase was transferred to a flask, and the solvent in the phase was removed under reduced pressure. The residue was dissolved in a small amount of *n*-hexane, and was transferred to an SPE column (silica gel, 500 mg) previously conditioned with 10 mL *n*-hexane. The column was washed with 10 mL *n*-hexane, followed by elution of Sudan dyes with 10 mL *n*-hexane + diethyl ether (9 + 1). The solvent in the eluate was removed under reduced pressure. The residue was dissolved

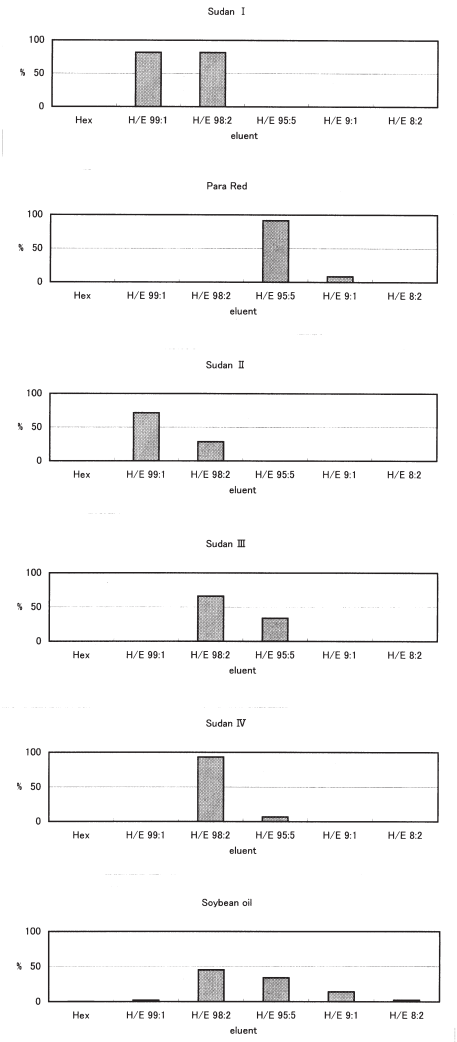

Figure 2. Elution profile of Sudan dyes and oil from an SPE (silica gel) column; eluent volume: 10 mL; Hex, H: *n*-hexane; E: diethyl ether.

in a small amount of THF, and methanol was added to make up to 0.5 mL. For the samples where 0.5 g was weighed, the residue was made up to 0.25 mL.

For palm oil, 1 g silica gel and twice the volume of the solvents was used for SPE, and the volume was made up to 0.25 mL.

Determination and Confirmation of Sudan Dyes

The sample solution prepared according to the procedure described above was injected into the LC column, and concentrations of the dyes were determined with the calibration curves obtained by injecting standard solutions. LC peaks were confirmed by comparing the DAD spectra with those of standard dyes.

LC Conditions

(**a**) *Column*.—Cosmosil 5C18-AR, 150 × 4.6 mm id, 5 μm particle diameter (Nacalai Tesque, Inc.).

(**b**) *LC gradient profile*.—Mobile Phase A, distilled water; Mobile Phase B, THF: 0–1 min (A 60%, B 40%) → 15 min (A 10%, B 90%) → 23 min (A 10%, B 90%) → 24 min (A 60%, B 40%).

(**c**) *Flow rate*.—1.0 mL/min.

(**d**) *Injection volume*.—20 μL.

(**e**) *Column oven temperature*.—35°C.

(**f**) *Monitoring wavelength*.—520 nm.

(**g**) *Wavelength range of DAD spectra*.—380–650 nm.

Gas Chromatography (GC) Conditions

(**a**) *Column*.—DB-wax, 30 m × 0.25 mm id, 0.5 μm film thickness (J&W Scientific, Folsom, CA).

(**b**) *Column oven temperature program*.—100°C (2 min) → 10°C/min to 210°C (6 min).

(**c**) *Injector temperature*.—200°C.

(**d**) *Detector temperature*.—250°C.

(**e**) *Injection*.—1 μL, by the split injection method (split ratio 30:1).

Measurement of Color Value ($E^{10\%}_{1\ cm}$)

Oleoresin and oil-soluble paprika color were diluted with acetone, and the absorbance at λmax was measured using a spectrophotometer (11). Powder and water-soluble paprika color was dispersed in water and diluted stepwise with methanol followed by acetone, and then the absorbance was measured. The λmax was not observed in the spectra of chili powder, and absorbance at 460 nm (λmax of paprika oleoresin) was measured.

Results and Discussion

Concept of the Method

Attempts to separate fat/oil intact from Sudan dyes.—Sudan dyes (each 5 μg) were dissolved in a small amount of *n*-hexane and transferred to an SPE column (silica gel, 500 mg), and the elution profiles of Sudan dyes were investigated, by continuous elution with 10 mL of various solvents [*n*-hexane; *n*-hexane–diethyl ether (99 + 1), (98 + 2), (95 + 5), (9 + 1), and (8 + 2); Figure 2]. Sudan dyes start eluting with *n*-hexane–diethyl ether (99 + 1) and finish at (9 + 1). A similar experiment was conducted for soybean oil. A 50 mg portion of soybean oil was transferred to the column with a small amount of *n*-hexane, and the weight of residue of each eluate was measured after solvent was evaporated (Figure 2). Soybean oil showed an elution profile similar to the Sudan dyes. Therefore, removal of fat or oil intact by cleanup with SPE (silica gel) was unsuccessful.

Removal of fat/oil as FAMEs.—FAMEs were prepared by transesterification of acylglycerols in fat or oil contained in the sample. The prepared FAMEs were eluted from the SPE (silica gel) column with *n*-hexane. Sudan dyes, which were extracted in the same phase as the FAMEs after transesterification and were transferred to the SPE column together with the FAMEs in the following process, were retained and then eluted with *n*-hexane–diethyl ether (9 + 1;

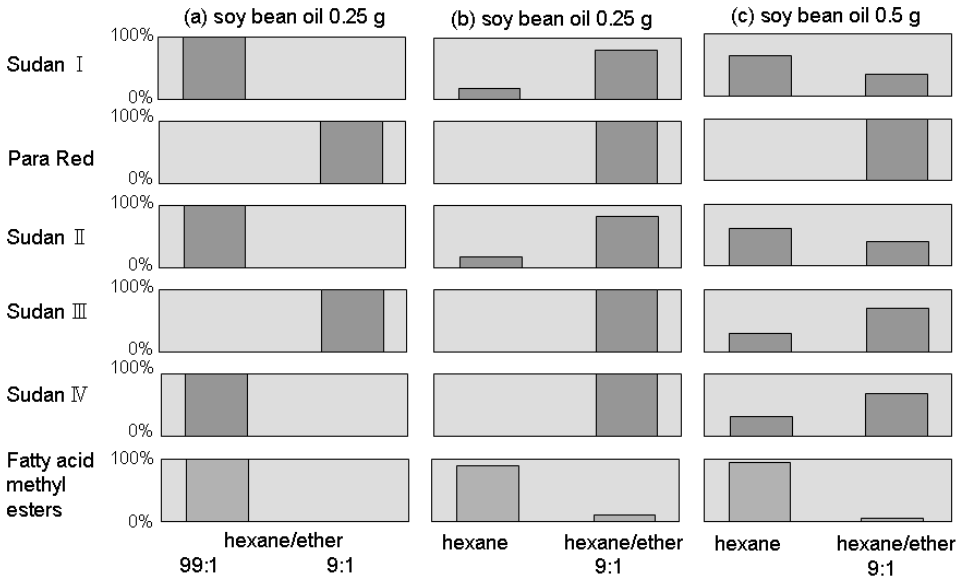

Figure 3. Elution profiles of Sudan dyes and FAMEs from an SPE (silica gel) column; eluent volume: 10 mL.

described in detail later). In this way, fat or oil was separated from Sudan dyes.

Selection of Test Samples

Foods in which the illegal presence of Sudan dyes was reported and chili powder-containing foods were selected as test samples. A wide variety of foods (from foods with little fat or oil, such as pickles and chili powder, to foods with large amounts of fat or oil, such as potato chips and oil) were included. In addition, paprika oleoresin and paprika color preparation were chosen as examples of difficult samples, because they contained high levels of natural color components that exhibited peaks near the retention time of the Sudan dyes in the liquid chromatogram and could potentially interfere with the detection of the Sudan dyes.

Sampling and Extraction

THF was used for extraction solvent because of high solubility of Sudan dyes. However, a mass formed when THF was added directly to nonfatty dry samples, such as chili powder or powder-type paprika color. Therefore, water and methanol were added to the sample before THF was added to facilitate extraction of dyes enclosed in sample matrixes. Solid fat in foods was liquefied by heating in order to facilitate the transesterification reaction.

Transesterification

Transesterification was performed directly in the homogenized food without preseparation of fat or oil (12). Transesterification, and the following extraction of the FAMEs together with Sudan dyes, was carried out in a 50 mL centrifuge tube for simple manipulation. Reagent and solvent volumes were adjusted to suit the tube volume. Since at least 0.5–1 μg/mL Sudan dyes in the final solution was required for measurement of spectra by LC-DAD, at least 0.5 g of the sample should be transesterified. A preliminary test was carried out to determine the amount of reagent and solvent sufficient for the transesterification of 0.5 g triglycerides. Tristearin and soybean oil were used for test samples, and they were transesterified using the same volumes of reagent and solvents described in the *Experimental* section. The resulting amounts of FAMEs were determined by GC with a calibration curve obtained with standard methyl stearate. Then the amounts of FAMEs were converted to triglyceride amounts, and yields of transesterification were calculated as the ratio of the triglyceride amount to the sample amount subjected to transesterification. Since triglyceride content in the sample would be less than 100%, the obtained yield would be slightly lower than the real yield.

When transesterification was carried out at ambient temperature, the yield was 92% for soybean oil and 50% for tristearin. The cause of the poor yield for tristearin may have been because tristearin did not fully dissolve in the solvent, and the reaction was not complete. When the reaction was carried out at 60°C, the yield went up to 92%; therefore, the temperature for transesterification was set at 60°C for samples containing solid fat. The amounts of sample, reagent, and solvent were chosen as described in the *Experimental* section.

Extraction of FAMEs and Sudan Dyes with n-Hexane

Resulting FAMEs were extracted with *n*-hexane. The Sudan dyes were all extracted in the *n*-hexane phase. Therefore, the procedure of transesterification and the following extraction removed the polar compounds in the sample, as well. Extraction first with THF, followed by re-extraction with *n*-hexane, reduced the amount of polar compounds in the solution transferred to the SPE column, and shortened the time for SPE, compared to extraction with acetonitrile used in the Notified method. (These compounds

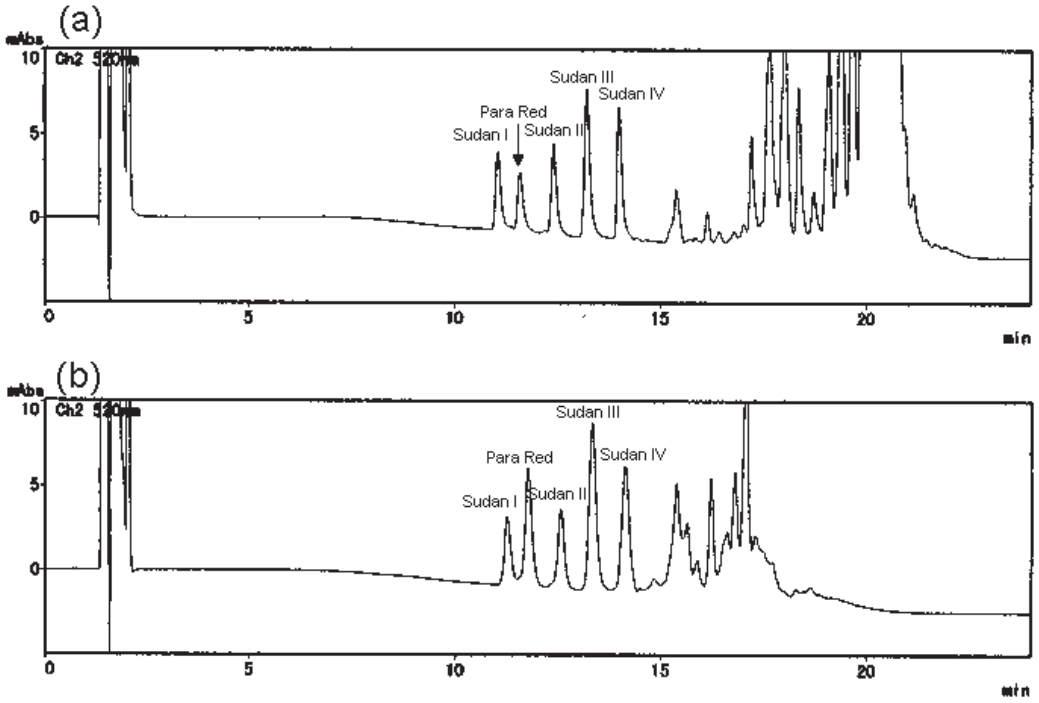

Figure 4. Chromatograms obtained from foods spiked with standard Sudan dyes monitored at 520 nm: (a) chili powder, (b) palm oil; spiked level: 0.5 μg/g food.

often precipitate on the surface of the SPE column and hinder elution.)

Cleanup with Silica Gel Columns

A portion of soybean oil spiked with Sudan dyes was transesterified, and the elution profiles of the dyes and FAMEs from the SPE column were investigated (Figure 3). With 10 mL of *n*-hexane–diethyl ether (99 + 1), 100% of the prepared FAMEs were eluted, whereas Sudan I, II, and IV were coeluted as well (0.25 g of soybean oil was used; Figure 3a). With 10 mL of *n*-hexane, although 10% of the FAMEs remained on the column, the coeluted amount of Sudan I and II was small (19% and 18%, respectively), and other dyes did not coelute (0.25 g of soybean oil was used; Figure 3b). Therefore, *n*-hexane was chosen for the elution of FAMEs. Although the loss of dyes might reduce the recovery to 80%, this level would be acceptable for trace analysis. The loss was minimized by increasing the amount of silica gel to 1 g, and, accordingly, 1 g was used for the palm oil sample.

When the soybean oil amount was increased to 0.5 g, more than 50% of Sudan I and II and parts of Sudan III and IV were eluted with *n*-hexane together with the FAMEs, probably because the FAMEs themselves functioned as a part of the eluent (Figure 3c). Therefore, the amount of fat or oil subjected to transesterification (from which prepared FAMEs were intended to be applied to the column) was less than 0.25 g. Accordingly, the amount of foods with high fat or oil (samples with a fat or oil contents greater than 25%, e.g., curry roux and potato chips in which fat or oil contents were 37.5 and 34.5%, respectively, according to the package labels) subjected to transesterification was less than 0.5 g. For palm oil, 0.5 g was transesterified and 1 g silica gel and twice as much volume of solvents were used for SPE, in order to obtain sufficient levels of analyte in the sample solution injected for LC. For other samples, 1 g was used.

LC Solutions

A few drops of THF were added to the residue in the eluate from the column, and the volume was made up to 0.25 or 0.5 mL with methanol. With this small volume, sufficient levels of analytes were obtained for measurement of spectra with the DAD. Since FAMEs dissolve well in THF, a part of the FAMEs that may have eluted in *n*-hexane–diethyl ether (9 + 1) without being removed from the column by *n*-hexane and remained in the eluate was no problem. Without transesterification, fat or oil remained in the sample extract, which was not soluble in a few drops of THF, and it was difficult to reduce the volume of the final solution (0.25 mL for the LC solution).

LC Conditions

Instead of isocratic elution as used in the Notified method and some of the previously reported methods, gradient elution was used. With isocratic elution, the peaks of Sudan III and IV, which eluted at a late retention time, became broadened, and, therefore, the peak heights were not sufficient for measurement of spectra of low levels of the dyes. Gradient elution was preferable because the peak was higher than that with isocratic elution (peak height restricts the detection limit more than peak area; natural colors, which eluted at later

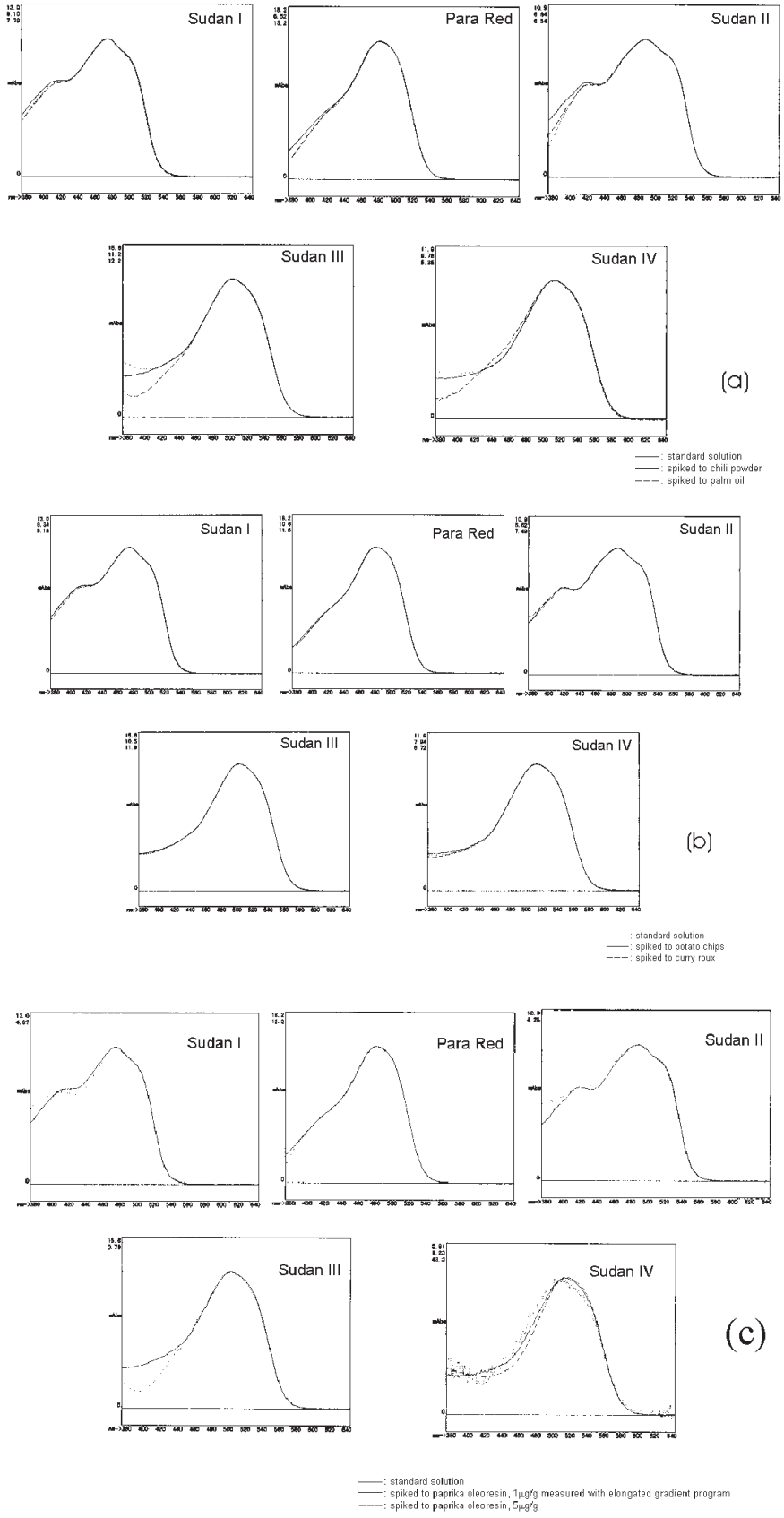

Figure 5. (a) Spectra of Sudan dyes spiked in chili powder and palm oil; spiked level: 0.5 μg/g food, normalized spectra. (b) Spectra of Sudan dyes spiked in potato chips seasoned with hot chili pepper, and commercial curry roux; spiked level: 0.5 μg/g food, normalized spectra. (c) Specta of Sudan dyes spiked in paprika oleoresin; spiked level: 1 μg/g oleoresin, normalized spectra.

Table 1. Similarities of spectra among spiked samples and standard solutions

	Sudan I	Para Red	Sudan II	Sudan III	Sudan IV
Spiked level: 0.5 μg/g					
Chili sauce	1.0000	1.0000	1.0000	1.0000	1.0000
Chili powder	0.9998	0.9991	0.9976	0.9992	0.9997
Chinese hot chili pepper seasoning	1.0000	1.0000	1.0000	0.9996	0.9996
Korean pickles seasoned with hot chili pepper	1.0000	1.0000	1.0000	1.0000	1.0000
Salted cod ovum seasoned with hot chili pepper	1.0000	1.0000	1.0000	0.9924	0.9990
Grilled eel	0.9999	0.9998	0.9999	0.9971	0.9992
Fried chicken seasoned with hot chili pepper	1.0000	1.0000	1.0000	1.0000	0.9999
Potato chips seasoned with hot chili pepper	1.0000	0.9999	0.9999	1.0000	0.9999
Commercial curry roux	0.9999	0.9999	0.9999	1.0000	0.9998
Palm oil	0.9997	0.9992	0.9990	0.9943	0.9956
Spiked level: 1 μg/g					
Paprika oleoresin	0.9988	0.9998	0.9987	0.9917	0.9962[a]
					0.9973[b]
Paprika color (liquid-type 1)	0.9939	0.9930	0.9984	0.9942	0.9958[a]
					0.9978[b]
Paprika color (liquid-type 2)	1.0000	0.9995	1.0000	0.9968	0.9979
Paprika color (powder type)	1.0000	0.9997	0.9999	0.9982	0.9944

[a] Measured with a modified LC gradient program.

[b] Spectra of 5 μg/g dye.

retention times than Sudan dyes, should be removed from the column in an appropriate elution period; and dye peaks that were narrow in width enabled separation from natural colors eluted at retention times close to the Sudan dyes. THF was used as part of the mobile phase because of its strong elution power, which worked to elute the natural color, and for good separation of azo dyes from each other (13). Sudan dyes eluted within 15 min (Figure 4). A wavelength of 520 nm was used for monitoring because the absorbance of Sudan dyes was relatively higher than natural colors at this wavelength compared with the detection at 500 nm in the Notified method (as described later in the section *Application of the Method to Paprika Oleoresin and Paprika Color*).

Calibration Curves, Limit of Detection for Spectral Measurement, and Recoveries

Peaks of natural colors were observed in the chromatograms of some samples. These peaks did not interfere with the detection of spiked Sudan colors. Chromatograms of chili powder and palm oil, in which relatively higher natural color peaks were observed, are illustrated in Figure 4. Calibration curves for the standard solutions were linear between 0.01–5 μg/mL at 520 nm detection wavelength [correlation coefficient (r) > 0.9996].

The lowest concentration, 0.01 μg/mL, corresponded to 0.005 μg/g, as referenced against the sample. However, at least 0.5–1 μg/mL was needed for acceptable spectra (with smooth edges) with the instruments used in this study, which corresponded to 0.5 μg/g referring to the sample. Spectra of Sudan dyes spiked in the listed samples at the level of 0.5 μg/g and treated as described in the *Experimental* section were measured, and compared to those of the standard solutions (similarities, calculated with the software of the LC system, are indicated in Table 1). For most samples, similarities were close to 1.0000, which suggested that interfering substances were removed by the cleanup procedure.

For some samples similarities were slightly low, probably due to the coeluting natural color components that exhibited λmax around 400 nm. Spectra of Sudan dyes spiked in natural-color-rich or high-fat samples, i.e., chili powder, palm oil, potato chips, and curry roux, at the level of 0.5 μg/g are illustrated in Figure 5a and b as normalized spectra. The obtained similarities would be sufficient for the confirmation of Sudan dyes in the sample.

Recovery testing was conducted by spiking 1 and 5 μg/g Sudan dyes (as 100 μg/mL THF solution) in the homogenate. THF was removed in a stream of nitrogen, and the method was performed as described in the *Experimental* section. Recoveries were 78–93% for the 5 μg/g spiking level and 73–94% for the 1 μg/g spiking level, which would be satisfactory for trace analysis (Table 2). Repeatability (precision) of the method was expressed as standard deviation (SD) of 3 trials in the recovery test. The SDs were 0.8–8.2% for

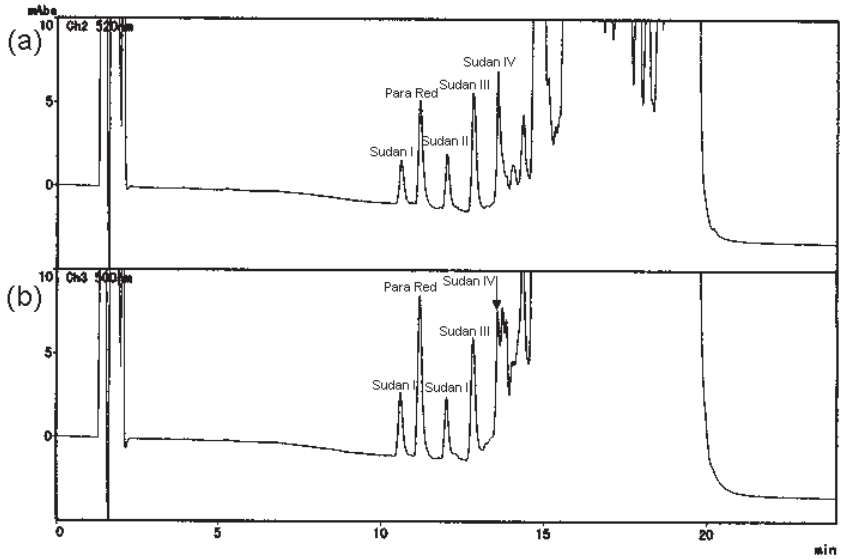

Figure 6. Chromatograms obtained from paprika oleoresin spiked with standard Sudan dyes, monitored at (a) 520 nm and (b) 500 nm; spiked level: 1 μg/g oleoresin.

Table 2. Recovery[a] of Sudan dyes from various foods, paprika oleoresin, and paprika color

	Sudan I	Para Red	Sudan II	Sudan III	Sudan IV
Spiked level: 5 μg/g					
Chili sauce	92 ± 5.0	91 ± 3.0	93 ± 5.4	92 ± 4.8	92 ± 5.0
Chili powder	86 ± 4.4	78 ± 3.7	86 ± 5.2	82 ± 4.6	80 ± 5.6
Chinese hot chili pepper seasoning	89 ± 1.6	88 ± 1.2	89 ± 2.2	91 ± 4.9	90 ± 4.4
Korean pickles seasoned with hot chili pepper	86 ± 4.3	84 ± 4.6	87 ± 3.2	89 ± 7.5	89 ± 8.2
Salted cod ovum seasoned with hot chili pepper	84 ± 2.8	81 ± 2.8	85 ± 3.2	79 ± 1.4	78 ± 2.1
Grilled eel	82 ± 7.5	88 ± 7.0	84 ± 7.8	89 ± 6.1	90 ± 6.8
Fried chicken seasoned with hot chili pepper	90 ± 8.1	86 ± 3.6	89 ± 6.6	88 ± 5.8	87 ± 6.1
Potato chips seasoned with hot chili pepper	87 ± 0.8	86 ± 2.0	89 ± 1.1	88 ± 2.8	86 ± 1.8
Commercial curry roux	89 ± 2.6	88 ± 3.0	93 ± 2.5	90 ± 5.3	91 ± 6.7
Palm oil	87 ± 3.1	85 ± 5.7	89 ± 3.0	87 ± 5.6	86 ± 6.3
Paprika oleoresin	53 ± 3.3	93 ± 3.7	55 ± 4.9	73 ± 7.7	85 ± 2.4
Paprika color (powder)	83 ± 8.0	74 ± 4.2	84 ± 8.5	84 ± 9.4	82 ± 9.3
Spiked level: 1 μg/g					
Chili sauce	93 ± 5.5	85 ± 5.4	89 ± 4.7	93 ± 4.1	89 ± 5.3
Chili powder	77 ± 6.9	73 ± 5.6	81 ± 7.0	85 ± 7.6	83 ± 6.4
Chinese hot chili pepper seasoning	85 ± 5.9	82 ± 7.1	85 ± 7.8	87 ± 3.1	90 ± 4.7
Korean pickles seasoned with hot chili pepper	94 ± 4.0	85 ± 6.8	91 ± 8.3	87 ± 6.7	86 ± 3.7
Salted cod ovum seasoned with hot chili pepper	77 ± 3.9	74 ± 2.4	79 ± 3.6	75 ± 2.2	76 ± 4.2
Grilled eel	81 ± 8.7	82 ± 4.5	82 ± 8.9	92 ± 6.9	87 ± 5.6
Fried chicken seasoned with hot chili pepper	81 ± 1.7	82 ± 1.2	83 ± 6.8	89 ± 6.1	86 ± 2.7
Potato chips seasoned with hot chili pepper	82 ± 7.2	80 ± 3.8	80 ± 1.0	90 ± 3.2	88 ± 3.7
Commercial curry roux	83 ± 5.5	82 ± 2.6	84 ± 5.4	92 ± 1.9	86 ± 6.3
Palm oil	73 ± 9.8	82 ± 4.8	73 ± 7.3	92 ± 7.5	89 ± 5.0

[a] Recovery reported as % ± SD (n = 3).

the 5 μg/g spiking level, and 1.2–8.9% for the 1 μg/g spiking level, showing good repeatability of the measurements.

Application of the Method to Paprika Oleoresin and Paprika Color

Analysis of illegally added artificial dyes in natural color extracts is generally demanding, because these dyes exhibit similar properties (elution profile from columns, solubility, or λmax) compared to natural colors. In fact, this is the objective of illegal addition of artificial dyes. Furthermore, concentrations of natural color components are high compared to that in foods; color values at λmax (460 nm) were 1495($E^{10\%}_{1cm}$) for the oleoresin, 1150($E^{10\%}_{1cm}$) and 499($E^{10\%}_{1cm}$) for the liquid-type paprika colors, and 147($E^{10\%}_{1cm}$) for the powder-type paprika color. These values were 10–100 times higher than those for food; 6.2($E^{10\%}_{1cm}$) for chili powder at 460 nm (no λmax was observed) and 14.6($E^{10\%}_{1cm}$) for palm oil at 451 nm, although relatively high levels of natural color were present (as observed in LC) in these foods. Chromatograms of paprika oleoresin monitored at 520 and 500 nm are illustrated in Figure 6, indicating that 520 nm was preferable to 500 nm because peaks of natural colors were relatively low. Spectra of Sudan IV spiked at 1 μg/g in paprika oleoresin and one of the liquid-type paprika colors [color value: 1150($E^{10\%}_{1cm}$)] were distorted because of interference from natural color components. Peaks of Sudan IV were separated from the peaks of natural colors with an elongated LC gradient program (0 min B, 30%; 1 min B, 30%; 60 min B, 60%; 63 min B, 90%; 73 min B, 90%), with good similarities of spectra compared to the standard spectra (Table 1), although the edges of the obtained spectra were not smooth because the peaks were too low for spectral measurement (Figure 5c). Samples spiked at 5 μg/g and measured with the original LC gradient program showed satisfactory spectra (Table 1; spectra are illustrated in Figure 5c). Recovery test results for paprika oleoresin and powder-type paprika color spiked with 5 μg/g of Sudan dyes are shown in Table 2. Recoveries of Sudan I and II from paprika oleoresin were less than 60%, probably because large amounts of natural substances in the oleoresin worked as a part of the washing eluent (*n*-hexane) in SPE, increasing the polarity of the eluent and resulting in loss of a part of these dyes (data not shown). Recoveries of Sudan III and IV and Para Red from the paprika oleoresin were 73–93%, and recoveries for all dyes from the powder-type paprika color were 74–84%, showing good recoveries of the experiments together with good repeatability for all measurement expressed as the standard deviation of the 3 trials (2.4 to 9.4%).

Conclusions

Fat or oil in samples often prevents reducing the volume of the final sample solution, and their removal is a critical point for the analysis of low levels of analyte. However, this is sometimes difficult, especially when the analytes associate with fat or oil in the solvent extraction or during elution from the SPE column. Separation can be achieved by changing the behavior of either the analyte or the fat and oil. This idea was applied to the analysis of Sudan dyes in samples containing fat or oil. Behavior of fat or oil was changed by transesterification. Elution profiles of fat or oil from a silica gel SPE column were similar to those of Sudan dyes, whereas FAMEs resulting from transesterification eluted with *n*-hexane, leaving Sudan dyes on the column. Transesterification followed by extraction by *n*-hexane was effective for removing polar compounds as well. Sample extraction and the following reaction were conducted in a 50 mL centrifuge tube, which required small amounts of solvents and resulted in fast and simple manipulation. Sufficient levels of analyte for measurement of spectra (0.5–1 μg/mL) in the final solution subjected to LC-DAD were obtained from 0.5 μg/g Sudan dyes in foods and 1 μg/g paprika color and oleoresin.

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