Determination of Simmondsins and Simmondsin Ferulates in Jojoba Meal and Feed by High-Performance Liquid Chromatography

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Methods are described for the extraction and high-performance chromatographic separation and quantitation of simmondsins and simmondsin ferulates in jojoba meal, detoxified jojoba meal, and animal feed mixtures. The simmondsin ferulates in the meal occur only in the *trans* configuration; however, if no precautions are taken, it is likely that both *trans* and *cis* isomers are detected. The influence of radiation on *trans*−*cis* isomerization is described.

Keywords: *Simmondsins; simmondsin ferulates; trans*−*cis isomerization; jojoba meal; HPLC*

INTRODUCTION

Jojoba meal is obtained after removal of the oil present in the seeds of the jojoba plant (*Simmondsia chinensis*), which is native to the Sonora Desert (U.S.). Because of its economic value, the plant has been cultivated in many arid and semiarid countries all over the world. The oil has important aesthetic and technical qualities that make it a widespread basic cosmetic ingredient and lubricant. Because of its high protein content, de-oiled jojoba meal has been tried as animal feed. However, the meal has been reported as toxic when used as a livestock feed ingredient (Booth et al., 1974; Verbiscar et al., 1980). The toxic effects seem to be especially related to the inhibition of feed intake caused by simmondsin, demethylsimmondsin, and didemethylsimmondsin (Elliger et al., 1973, 1974; Cokelaere et al., 1992), the relative activities of which are not yet fully known. The meal also contains simmondsin ferulates: the main ferulate is simmondsin 2′-ferulate (Elliger et al., 1973; Van Boven et al., 1993), and three other ferulates have been isolated and identified recently (Van Boven et al., 1994a, 1995). Tests by Cokelaere et al. (1996) in rats revealed that simmondsin 2′-ferulate has two-thirds the activity of simmondsin, corresponding to an activity that is equimolar with that of simmondsin. Hydrolysis of simmondsin 2′-ferulate to simmondsin occurs in the gastrointestinal (GI) system. Different mechanisms of action for the inhibitory feed intake effect of simmondsin have been described. The effect can be due either to a central action of simmondsin or to a local action of simmondsin in the GI system. Some authors suggest that the action is due to the aglycon formed by enzymatic deconjugation in the gut (Booth et al., 1974; Elliger et al., 1973; Verbiscar et al., 1980). More recently, Cokelaere et al. (1993a,b) have proved a cholecystokinin (CCK)-mediated mechanism of action.

Different authors (Verbiscar and Baningan, 1978; Verbiscar et al., 1980, 1981, 1991) describe methods to eliminate simmondsins and simmondsin ferulates from the meal. To follow the “detoxification” procedures of the meal and to allow standarized tests with normal and detoxified jojoba meal, a precise method for the quantitative determination of simmondsins and simmondsin ferulates is needed. Methods for the determination of simmondsin and simmondsin 2′-ferulate in jojoba meal have been described by Verbiscar et al. (1978) and Abott et al. (1988). These methods are suited for the determination of simmondsins and simmondsin ferulates in fresh extracts of jojoba meal. Chromatograms showed supplementary peaks, particularly when the extracts were exposed to sunlight for some time. Simmondsin ferulates are esters from simmondsin or simmondsin analogues with ferulic acid. As described by Van Boven et al. (1994a, 1995), the plant produces only the *trans* isomers. It was therefore suggested that simmondsin ferulates quickly isomerize under ultraviolet (UV) radiation.

The development of methods for extraction, separation, and determination of simmondsins and *cis*- and *trans*-simmondsin ferulates in jojoba meal are described in this paper. The isomerization of the different *trans* isomers, isolated from jojoba meal, was examined under the influence of both daylight and long UV radiation. The structure of simmondsin 2′-*cis*-ferulate was confirmed by mass spectrometry (MS) following isolation of the pure compound by the described HPLC procedure after previous UV-photoisomerization.

MATERIALS AND METHODS

Reagents and Materials. Simmondsin, 4-demethylsimmondsin, didemethylsimmondsin, simmondsin 2′-*trans*-ferulate, simmondsin 3′-*trans*-ferulate, 5-demethylsimmondsin 2′-*trans*-ferulate, and 4-demethylsimmondsin 2′-*trans*-ferulate were used as references and were isolated from jojoba meal as described by Van Boven et al. (1993, 1994a,b, 1995). The structure of the different simmondsins and simmondsin *trans*-ferulates are shown in Figure 1. The *cis* isomers were prepared by exposure of the *trans* isomers to long-wavelength UV radiation (365 nm). All solvents used were analytical grade. Hollow fiber syringe filters, with 45-μm pores and 3.9-cm^2 surface area (Microgon, Brussels, Belgium), were used to filter solutions prior to their injection onto the HPLC column. A rotary mixer (Labin, Belgium) was used for the extraction with 100-mL glass extraction tubes. Glass columns (20 × 1.0 cm i.d.; 50-mL reservoir) were used for the column extraction method.

Samples. Different de-oiled jojoba samples from Israel and America were used for the quantitative determinations. Feed

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R2' = H	R3' = H	R4 = CH_3	R5 = CH_3	simmondsin
R2' = H	R3' = H	R4 = H	R5 = CH_3	4-demethylsimmondsin
R2' = H	R3' = H	R4 = H	R5 = H	didemethylsimmondsin
R2' = fer. ac. moiety	R3' = H	R4 = CH_3	R5 = CH_3	simmondsin 2'-ferulate
R3' = fer. ac. moiety	R2' = H	R4 = CH_3	R5 = CH_3	simmondsin 3'-ferulate
R2' = fer. ac. moiety	R3' = H	R4 = CH_3	R5 = H	5-demethylsimmondsin 2'-ferulate
R2' = fer. ac. moiety	R3' = H	R4 = H	R5 = CH_3	4-demethylsimmondsin 2'-ferulate

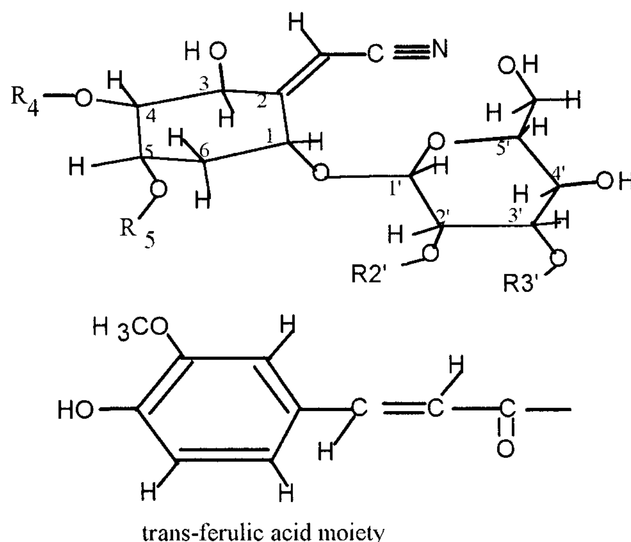

Figure 1. Structure of simmondsins and simmondsin *trans*-ferulates.

mixtures containing 3% de-oiled jojoba meal were chosen as models for the determination in feed samples, because this concentration was also used in a previous study in rats on the influence of jojoba meal on growth, organ function, and on fertility after long-term jojoba meal supplementation (Cokelaere et al., 1993b).

Thin-Layer Chromatography. Thin-layer chromatography (TLC) was performed on silica gel plates (Polygram Sil G/UV254, Machery-Nagel, Germany), with a mixture of methanol and chloroform (30/70, v/v) as solvent. The spots were localized by long-wavelength UV radiation (365 nm) and by spraying the plates with 1-naphtol reagent. The latter was prepared by adding 10.5 mL of a 15% ethanolic solution of 1-naphtol to a mixture of 40.5 mL of ethanol, 4 mL of water, and 6.5 mL of sulfuric acid. After spraying, the plates were heated at 100 °C in an oven for 5 min.

HPLC. A model L-6200 high-performance liquid chromatograph (Merck-Hitachi, Germany), equipped with a variable wavelength L-4250 UV−vis detector (Merck-Hitachi, Germany) and operated at 218 nm for systems IA and II and at 225 nm for system IB (see following description of systems), was used. The extracts were applied by a Rheodyne model 7125 (Berkeley, CA) sample loop injector in a volume of 200 μL. Three different systems were tested. System IA consisted of a Si-60 5-μm particle size column (25 × 0.4 cm i.d; E. Merck, Germany), and the eluent was a mixture of acetonitrile and water (90/10, v/v) that was used at a flow rate of 1.0 mL/min. System IB consisted of the same column as in system IA, with a mixture of methanol and 1,2-dichlorethane (20/80, v/v) as the eluent. System II consisted of a Lichrosorb C18 column (25 × 0.4 cm i.d.), with a mixture of water and methanol (70/30, v/v) at a flow rate 1.0 mL/min as eluent. All solvents were deaerated with helium. The columns were protected by a Si-60 RP-18 endcapped (5 μm) guard column (Merck, Germany). Peak areas and retention times for the two chromatographic systems were determined with a D-2500 Merck-Hitachi Chromato-Integrator.

Mass Spectrometry. Liquid surface-assisted ionization mass-spectrometry (L-SIMS) was performed with a Kratos Concept 1H instrument with a 7-kV Cs beam. The spectra, in the positive ion mode, were obtained by dissolving the samples in a glycerol matrix and placing them on a copper probe tip prior to their bombardement with Cs atoms.

Exposure of Simmondsin *trans*-ferulates to UV Radiation. Solutions of 1 mg of simmondsin *trans*-ferulates in 100 mL of a mixture of acetonitrile and water (90/10, v/v) were placed in daylight at the laboratory window. Identical solutions were exposed to long-wavelength UV radiation (365 nm); the bottom of the lamp was 15 cm from the surface of the solutions. After 10, 30, and 60 min, and 2, 4, and and 8 h, 20-μL samples were analyzed by HPLC with the three different systems mentioned.

Preparation and Isolation of Simmondsin 2′-*cis*-Ferulate. A solution of 1.0 mg of simmondsin 2′-*trans*-ferulate per milliliter of methanol was stirred on a magnetic stirrer and irradiated for 4 h by long-wavelength UV radiation. The *cis* isomer that was formed was separated from the *trans* isomer on the silaca gel column with a mixture of acetonitrile and water (90/10) as eluent (system IA). The 50-μL samples were injected onto the chromatograph. The two separated isomers were collected, and the solvent was evaporated under a stream of nitrogen. The obtained residue, corresponding to the newly formed peak, was examined by mass spectrometry.

Calibration. Calibration graphs of simmondsin, 5-demethylsimmondsin, didemethylsimmondsin, simmondsin 2′-*trans*-ferulate, simmondsin 3′-*trans*-ferulate, 4-demethylsimmondsin 2′-*trans*-ferulate, and 5-demethylsimmondsin 2′-*trans*-ferulate were obtained by plotting peak areas against weight. For this reason, quantities of 1, 2, 4, 16, 32, and 64 μg of the different references in 20 μL of HPLC eluent were injected onto the chromatograph that was operated under the conditions of the three systems. Primary stock solutions were prepared by dissolving 10.0 mg of the different references in 10.0 mL of methanol. Stock solutions were kept at −20 °C. Individual standard solutions were made by diluting the primary solutions with the respective HPLC solvents to obtain the concentrations needed for the calibration graphs. The stock solutions of the simmondsins were stable for several months when kept at −20 °C; the stock solutions of the simmondsin ferulates in the refrigerator, however, were stable only for ~1 month. Dilutions of the simmondsin ferulates were prepared at the beginning of each day. Both stock solutions and dilutions were protected from daylight.

Extraction of Jojoba Meal. Two methods (A and B) of sample preparation were investigated. Samples were extracted either by column extraction (method A) or by extraction

Table 1. R_f Values and Chromogenic Behavior in the TLC Analysis of the Different Simmondsins and Simmondsin Ferulates and the Retention Times in the Different HPLC Systems

compound	R_f value	naphthol spray	fluorescence	system IA	system IB	system II
simmondsin 3′-*trans*-ferulate (I)	0.80	violet	blue	4.01	3.81	5.50
simmondsin 3′-*cis*-ferulate (I)	0.80	violet	blue	3.89	3.81	5.50
simmondsin 2′-*trans*-ferulate (II)	0.64	violet	blue	6.52	4.78	12.89
simmondsin 2′-*cis*-ferulate (I)	0.64	violet	blue	5.63	4.78	22.21
5-demethylsimmondsin 2′-*trans*-ferulate (III)	0.55	violet	blue	7.01	6.19	6.57
5-demethylsimmondsin 2′-*cis* -ferulate (III)	0.55	violet	blue	5.95	6.19	10.18
4-demethylsimmondsin 2′-*trans*-ferulate (IV)	0.50	violet	blue	7.66	7.23	5.81
4-demethylsimmondsin 2′-*cis*-ferulate	0.50	violet	blue	5.59	7.23	10.18
simmondsin	0.44	violet		17.18	8.74	1.49
4-demethylsimmondsin	0.30	violet		22.63	12.94	front
4,5-didemethylsimmondsin	0.16	violet		32.26	18.74	front

tubes (method B). Jojoba meal samples (1.0 g) or feed samples (5.0 g) were placed in 100-mL extraction columns or in extraction tubes. For the column extraction method, 50-mL solvent fractions were collected to study the total quantity of solvent needed for the complete extraction of the different simmondsins and simmondsin ferulates. In the extraction tubes procedure, 1.0-g samples were extracted with 50.0 mL of solvent. After rotating the tubes for 30 min with the rotary mixer, the solvent was separated from the meal or feed by centrifugation. Prior to the HPLC analysis, the obtained fractions were diluted with HPLC eluent; the first 50-mL fractions were diluted 100 times, the second 50.0-mL fractions were diluted 20 times, the third 50.0-mL fractions were diluted 10 times, and the 50-mL fourth fractions were diluted five times. The feed sample extracts were five times less diluted than jojoba meal samples. The extracts obtained with the tube extraction method were diluted 100 times prior to the HPLC analysis. The samples were filtered through nylon filters, and 20-μL aliquots were injected onto the chromatograph. For statistical studies, the extraction procedures were repeated three times, and each sample was injected three times.

Different solvents and solvent mixtures were examined to obtain a quantitative extraction of simmondsins and the simmondsin-ferulates and also to get chromatograms with as little interference from coextracted jojoba compounds as possible. Pure methanol, acetonitrile, and 1-propanol were tried as extraction solvents, as well as mixtures of methanol and water or acetonitrile and water. The extractions were performed in white fluorescent light, and the extracts were kept in bottles protected from light with aluminum tape to prevent any possible isomerization. Finally, the results with the two different methods were compared and evaluated.

Extraction Recovery. Recovery of the ferulates was 100% when no more simmondsins and simmondsin ferulates were detected by the HPLC procedure in 50-mL fractions obtained from the column extraction system. The recoveries with the different solvents in the two extraction systems were measured with HPLC system IA and calculated by comparing the sum of the extracted simmondsins and simmondsin ferulates with the aforementioned 100% recovery.

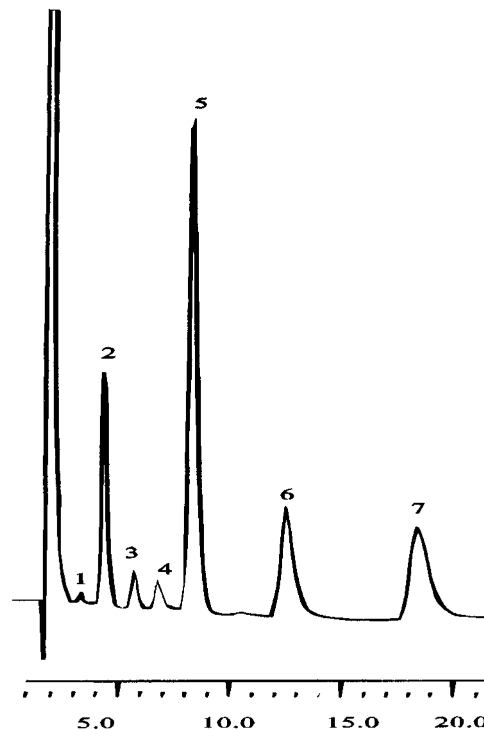

Figure 2. Chromatogram of a jojoba meal extract obtained with system IB. Peak identification: (**1**) simmondsin 3′-ferulate, (**2**) simmondsin 2′-ferulate, (**3**) 5-demethylsimmondsin 2′-ferulate, (**4**) 4-demethylsimmondsin 2′-ferulate, (**5**) simmondsin, (**6**) 4-demethylsimmondsin, and (**7**) 4,5-didemethylsimmondsin.

RESULTS AND DISCUSSION

TLC. The TLC system used separated the different simmondsins and simmondsin *trans*-ferulates; however, the *cis* and *trans* isomers of the different ferulates were not separated (Table 1). Simmondsin ferulates were localized as blue fluorescent spots under long-wavelength UV irradiation (365 nm) or as violet spots following the application of the naphtol reagent; the simmondsins also appeared as violet spots with the naphtol reagent.

HPLC. As shown in Table 1, HPLC systems IA and II resulted in a good separation of the different *trans*-ferulates and their *cis* isomers. Nevertheless, overlappings between some isomers were observed in both systems. The presence of *cis* isomer artifacts in the chromatograms was detected by both methods; in these cases, the procedure must be repeated under better circumstances to avoid the *trans*-to-*cis* isomerization. The isocratic system is not well suited for the simultaneous determination of simmondsins and simmondsin 2′-ferulates with system II. In this system, demethylsimmondsin and didemethyl-simmondsin elute in the front of the chromatogram. However, the method can be adapted by starting the analysis with 10% methanol and programming to 40% methanol to include the simmondsin analogues in the analysis. This protocol requires rather important equilibration times between two analyses. No separation is observed between the *cis*- and *trans*-simmondsin ferulates with method IB. There is no direct need to separate the *cis* and *trans* isomers for the determination of the activity of jojoba specimens, so this method enables a quick and easy analysis of the composition of jojoba samples. The optimal detection wavelength of this system to minimize the absorption of 1,2 dichloroethane was 225 nm. A

Table 2. Precision Data for the Extraction Yields[a] of Simmondsin 2′-Ferulate and Simmondsin from Jojoba Meal Determined with System IA

extraction solvent	simmondsin 2′-ferulate [column (100 mL)	tube (50 mL)]	simmondsin [column (100 mL)	tube (50 mL)]
methanol (100%)	93.8 (5.7)	99.9 (2.1)	96.4 (6.1)	100.1 (1.6)
1-propanol (100%)	26.2 (8.4)	36.2 (4.2)	8.9 (8.6)	21.4 (3.1)
CH_3OH/CH_3CN (50/50)	90.0 (6.1)	92.5 (2.1)	94.6 (7.1)	94.6 (2.8)
CH_3CN/H_2O (90/10)	67.5 (7.3)	82.5 (3.6)	91.1 (6.4)	92.8 (3.2)
CH_3CN/H_2O (80/20)	100.1 (3.4)	99.9 (1.6)	98.9 (4.3)	100.3 (1.4)

[a] Values are expressed as mean (%) and the RSD (%) is given in parentheses.

typical chromatogram obtained with the described system is represented in Figure 2. In systems IA and II, we used the absorption maximum (218 nm) of both simmondsins and simmondsin ferulates as the detection wavelength. The retention times of the different *trans*- and *cis*-ferulates are summarized in Table 1.

Linearity and Precision. The relationships between the peak areas and the amount of the seven references were always linear, with a correlation coefficient invariably exceeding 0.99. To evaluate the precision of the method, three-tube extract samples from jojoba meal and feed samples containing 3% jojoba meal were injected three times, on three different days. To estimate the overall precision, the raw data were subjected to a variance analysis. The results for the seven studied products as well as for jojoba meal and feed samples, were very similar. Intraday repeatability was good, with a mean relative standard deviation (RSD) of <3%. Mean RSD values for interday determinations were <4%. Concentrations as low as 0.001% for the simmondsin ferulates and 0.003% for the simmondsins in jojoba meal could be determined (i.e., in the mentioned feed mixtures as well as in jojoba meal detoxified by continuous acetone extraction) without any interference from other jojoba components or animal feed components.

Extraction Recovery. The recoveries obtained with the different solvents are shown in Table 2. Complete recoveries can be obtained either with pure methanol or with mixtures of acetonitrile and water (80/20). The chromatograms obtained with both solvents were not significantly different concerning coextracted impurities from the jojoba meal. The recoveries obtained with the column system were, in general, lower than those obtained with the extraction tubes. The extraction with the rotatory mixer obviously allows better contact between the solvent and the meal. For that reason, we prefer the extraction tube method for quantitative analysis.

***trans*−*cis* Isomerization.** The *trans* isomers are solutions transformed into an equilibrium mixture of *cis* and *trans* isomers after 3 h of exposure to daylight. The composition of the mixtures was 53% (±1%) *trans* isomer and 47% (±1%) *cis* isomer, calculated on the ratio of the areas of the two peaks. The same composition was obtained following 15 min of exposure to long-wavelength (365 nm) UV radiation. The chromatograms obtained from jojoba meal or feed, analyzed with system IA or II, became complicated if the precautions necessary to prevent *trans*-to-*cis* isomerization were not observed. Therefore, the method may give rise to important mistakes in calculating the concentrations of the different ferulates when using calibration graphs with the pure *trans* isomers.

Isolation of Simmondsin 2′-*cis*-Ferulate. Different HPLC injections of the mixture isomerized under long-wavelength UV radiation resulted in the isolation of the pure *cis* compound, following evaporation of the solvent under a stream of nitrogen. The structure was confirmed by L-SIMS. As shown in Table 3, the mass spectra of the newly isolated compound and the pure *trans* isomer were very similar.

Table 3. Intensities (%) of the Parent Ions and Major Fragment Ions from the *cis* and *trans* Isomer of Simmondsin 2′-Ferulate

ion	*cis*	*trans*
552 (M + H)	1.1	0.6
339	38.9	34.0
321	2.5	3.5
214	3.3	2.3
194	10.8	9.2
177	100.0	100.0
145	9.5	12.0

Conclusions. Simmondsins and simmondsin ferulates can quantitatively be extracted from jojoba meal or from feed by methanol/water or acetonitrile/water (80/20) mixtures. The HPLC methods described herein enable the quantitative determination of simmondsin and the different simmondsin ferulates in untreated jojoba meal, "detoxified" meal, as well as in feed. The HPLC systems on silica gel allow simultaneous isocratic determination of the described compounds. With silica gel as the stationary phase and acetonitrile/water (90/10) as an eluent, *cis* and *trans* isomers of simmondsin ferulates are separated. The reversed-phase method is more appropriate for the separation of the *cis*- and *trans*-simmondsin ferulates (Table 1). Because the meal contains only the *trans* isomers, which can easily be transformed to the *cis* isomers by UV light, the extraction of jojoba meal should be performed in "white" fluorescent light or in tubes protected from UV light to prevent any *trans*-to-*cis* isomerization. If the separation of *cis* and *trans* isomers is not necessary, the HPLC method with a silica gel column and with 1,2-dichloroethane/methanol (80/20) as eluent is a quick and reliable method for the quantitative determination of simmondsin and simmondsin ferulates in all kinds of jojoba meal as well as in feed mixtures.

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Received for review December 4, 1995. Accepted May 29, 1996.[⊗] This work was funded by the Research Board of the Katholieke Universiteit Leuven (Project OT/95/29).

JF950801Z

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1996.