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Fourier Transform Infrared Spectroscopy and Raman Spectroscopy as Tools for Identification of Steryl Ferulates

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ABSTRACT: Steryl ferulates are a mixture of minor bioactive compounds, possessing well-established health benefits. However, individual steryl ferulate species show structural differences, which seem to substantially influence their health-promoting potential. In this study, we tested Fourier transform infrared (FTIR) spectroscopy and Raman spectroscopy, as potential tools in the identification of steryl ferulates. On the basis of our spectral data obtained from various individual steryl ferulates and steryl ferulate mixtures extracted from rice (γ-oryzanol), corn bran, and wheat bran, we provide comprehensive peak assignment tables for both FTIR and Raman. With the help of FTIR spectroscopy, structural differences between individual steryl ferulates were possible to identify, such as the presence of the cyclopropane ring and additional differences in the side chain of the sterane skeleton. Data obtained with Raman spectroscopy provided us with a control for FTIR peak assignment and also with some additional information on the samples. However, detecting structural differences between steryl ferulates was not possible with this method. We consider that FTIR spectroscopy alone or combined with Raman provides detailed data on the structures of steryl ferulates. Moreover, thorough peak assignment tables presented in this study could prove to be helpful tools when identifying steryl ferulates, especially as a group, in future studies.

KEYWORDS: *Steryl ferulate, FTIR spectroscopy, Raman spectroscopy*

INTRODUCTION

Steryl ferulates are a mixture of bioactive compounds, in which plant sterols are esterified with ferulic acid. Because of structural differences in sterols, steryl ferulate structures also slightly differ (Figure 1). Previous results show that these small structural differences play a significant role in their health-promoting properties, such as in their antioxidant activity. Yagi and Ohisi[1] found that the oxidation of linoleic acid because of ultraviolet (UV) irradiation was most effectively inhibited by campesteryl ferulate, whereas 24-methylenecycloartanyl ferulate was less effective and cycloartenyl ferulate was the least effective in inhibiting oxidation. Xu and Godber[2] evaluated these three steryl ferulates to be equally potent antioxidants; however, in their later study, Xu and co-workers[3] demonstrated that 24-methylenecycloartanyl ferulate inhibited cholesterol oxidation more effectively than campesteryl ferulate and the latter proved to be better or similarly good an antioxidant as cycloartenyl ferulate. In the report by Kikuzaki and co-workers,[4] cycloartenyl ferulate exhibited stronger antioxidative activity than 24-methylenecycloartanyl ferulate when inhibiting oxidation of methyl linoleate. In addition, Nyström and co-workers[5] found similar radical scavenging activity for cholesteryl ferulate and sitosteryl ferulate.

In light of the above-mentioned differences in their health-promoting potential, it would be of high interest to clearly identify steryl ferulates individually. ^{1}H and ^{13}C nuclear magnetic resonance (NMR)[6] as well as mass spectrometry (MS)[7,8] are usually the methods of choice for steryl ferulate identification. However, identification of individual steryl ferulates is hindered by the usually limited sample amounts and the lack of existing standard compounds, with only cycloartenyl ferulate and γ-oryzanol (a mixture of steryl ferulates extracted from rice) being commercially available. A NMR measurement necessitates approximately 10−50 mg of steryl ferulates, for ^{1}H and ^{13}C NMR, respectively. A MS measurement of steryl ferulates in turn only requires micrograms, although the sample cannot be recuperated after analysis. In a recent study, a mixture of phytosterols was esterified with ferulic acid and identification of the obtained compound was performed with NMR, MS, and also Fourier transform infrared (FTIR) spectroscopy.[9] However, neither a detailed peak assignment table nor identification of individual steryl ferulate molecules was provided for the FTIR method. Raman spectroscopy, usually used as a complementary method for FTIR, has not yet been applied to identify individual steryl ferulate species either. The Raman spectrum of γ-oryzanol has been published; however, authors did not provide detailed peak assignment.[10] Ferulic acid and sterol moieties of steryl ferulates separately have also been studied to some extent with FTIR and Raman spectrometric methods. Sebastian and co-workers[11] gave exhaustive peak description for both FTIR and Raman spectra of ferulic acid. Of the free sterols present in our samples, only the infrared spectra of β-sitosterol has been investigated with the help of quantum chemical methods.[12]

The aim of this study was to evaluate spectrophotometric methods for the identification of steryl ferulates. Considering the limited sample amount (1−2 mg per sample), we opted for attenuated total reflection (ATR) FTIR spectroscopy and Raman microscopy. In this paper, we provide exhaustive peak assignment tables for both the infrared and Raman spectra of six individual steryl ferulates and those of three natural steryl ferulate

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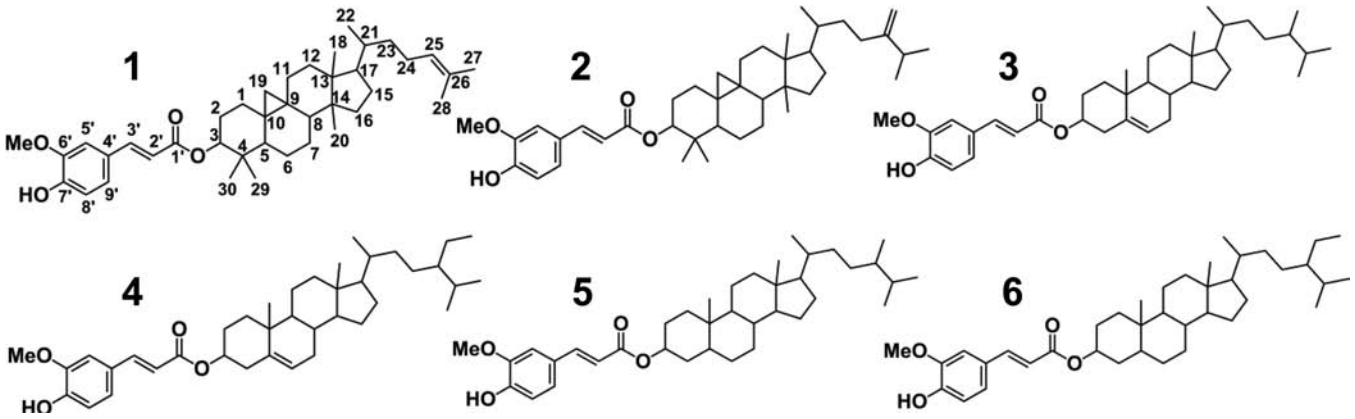

Figure 1. Chemical structures of cycloartenyl ferulate (**1**), 24-methylenecycloartanyl ferulate (**2**), campesteryl ferulate (**3**), sitosteryl ferulate (**4**), campestanyl ferulate (**5**), and sitostanyl ferulate (**6**).

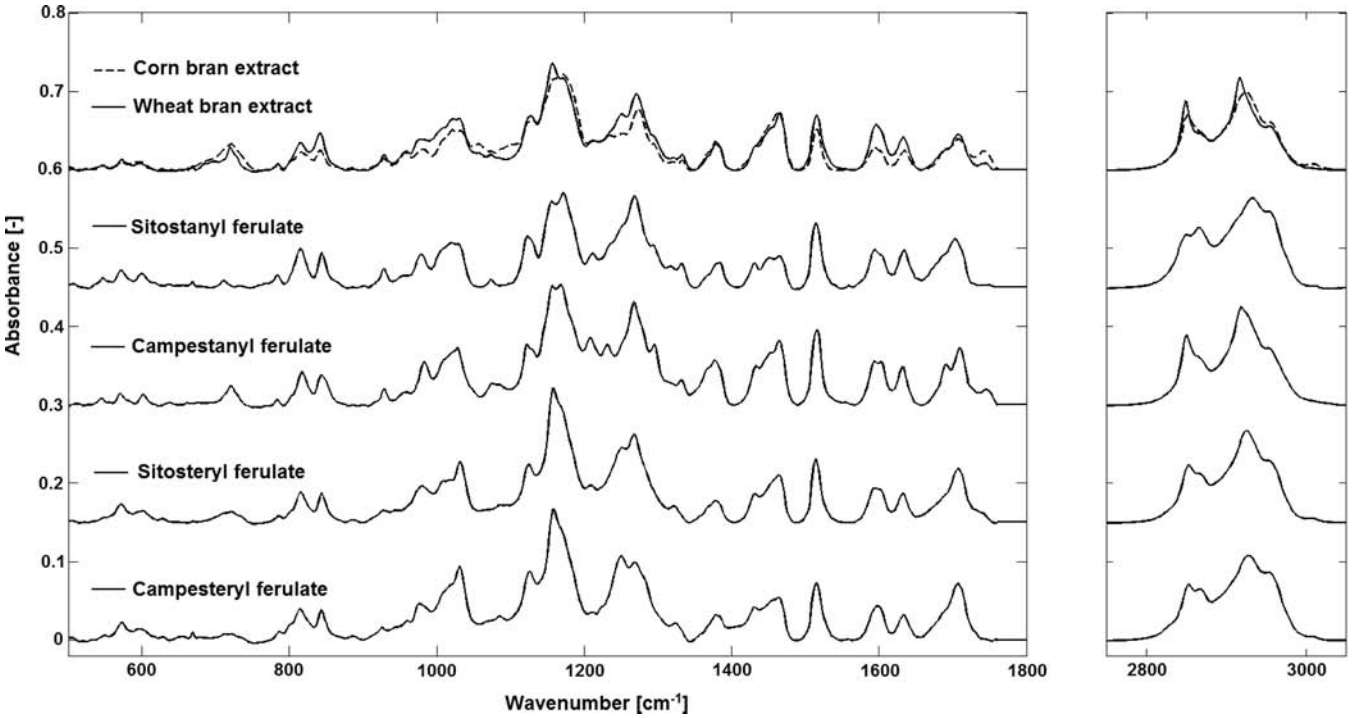

Figure 2. FTIR spectra of campesteryl ferulate, sitosteryl ferulate, campestanyl ferulate, sitostanyl ferulate, and their mixtures extracted from corn bran and wheat bran.

mixtures extracted from rice, corn, and wheat. γ-Oryzanol contains all six individual steryl ferulates analyzed, whereas in corn and wheat, only four of these species (campesteryl ferulate, sitosteryl ferulate, campestanyl ferulate, and sitostanyl ferulate) can be found, in different proportions.[13] In addition, we evaluated the potential of the two analytical methods used to distinguish between different steryl ferulate species. We consider that the comprehensive infrared and Raman peak assignment tables will become helpful tools for the identification of these bioactive compounds in future studies.

MATERIALS AND METHODS

Chemicals and Samples. Acetic acid (Ph Eur) was purchased from Merck, Darmstadt, Germany. Acetone (≥99.9%), acetonitrile (≥99.9%), butanol (≥99.7%), heptane (≥99%), hydrochloric acid (37%), and potassium hydroxide (≥85%) were obtained from Sigma-Aldrich, St. Louis, MO. Cycloartenyl ferulate (≥99%) and γ-oryzanol (≥98%) were purchased from Wako, Osaka, Japan. Corn bran and wheat bran were provided by Swissmill, Switzerland, as milled fractions.

Sample Preparation. From 100 g of corn bran and 100 g of wheat bran, first lipids were extracted with heptane and acetone, respectively.[14] Samples (10 g, 10 times) were extracted 3 times, sequentially, with 40 mL of extraction solvent. Corn bran and wheat bran lipid extracts were collected and combined in round-bottom flasks. Extracts were then evaporated at 50 °C using a rotary evaporator (Büchi R-114, Switzerland). They were transferred into test tubes and were redissolved in 10 mL of methanol. Subsequently, samples were subjected to base–acid cleanup[14] to eliminate neutral lipids. First, the methanol solution was made basic (pH 10) by adding 0.5 mL of saturated potassium hydroxide (KOH). It was partitioned with 2 times 10 mL of heptane, and the heptane phase was discarded. The aqueous portion was then acidified (pH 2) with 1 mL of 6 M HCl and partitioned again with 3 times 3 mL of heptane. The final heptane extract was evaporated under gentle nitrogen stream at 50 °C, and the residue was redissolved in butanol for further purification by preparative high-performance liquid chromatography (HPLC, Merck-Hitachi, Japan). Separation was

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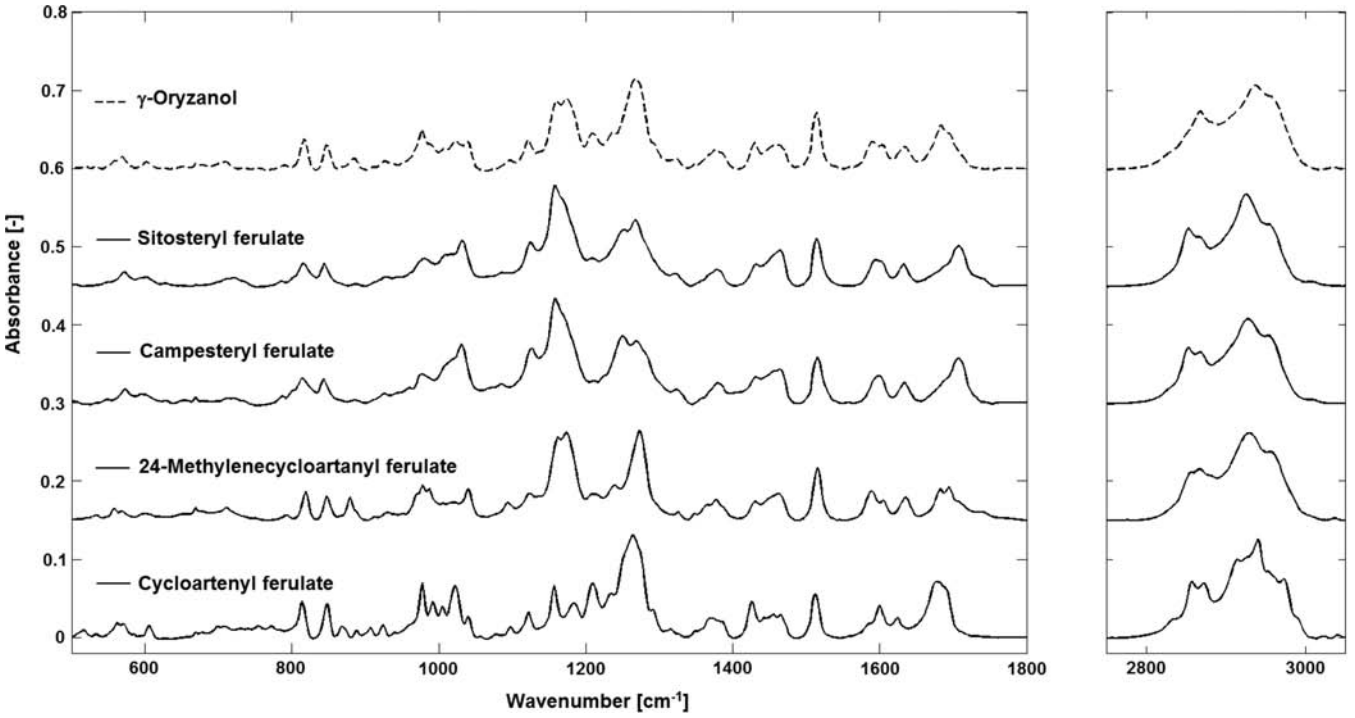

Figure 3. FTIR spectra of cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, sitosteryl ferulate, and their mixtures in γ-oryzanol.

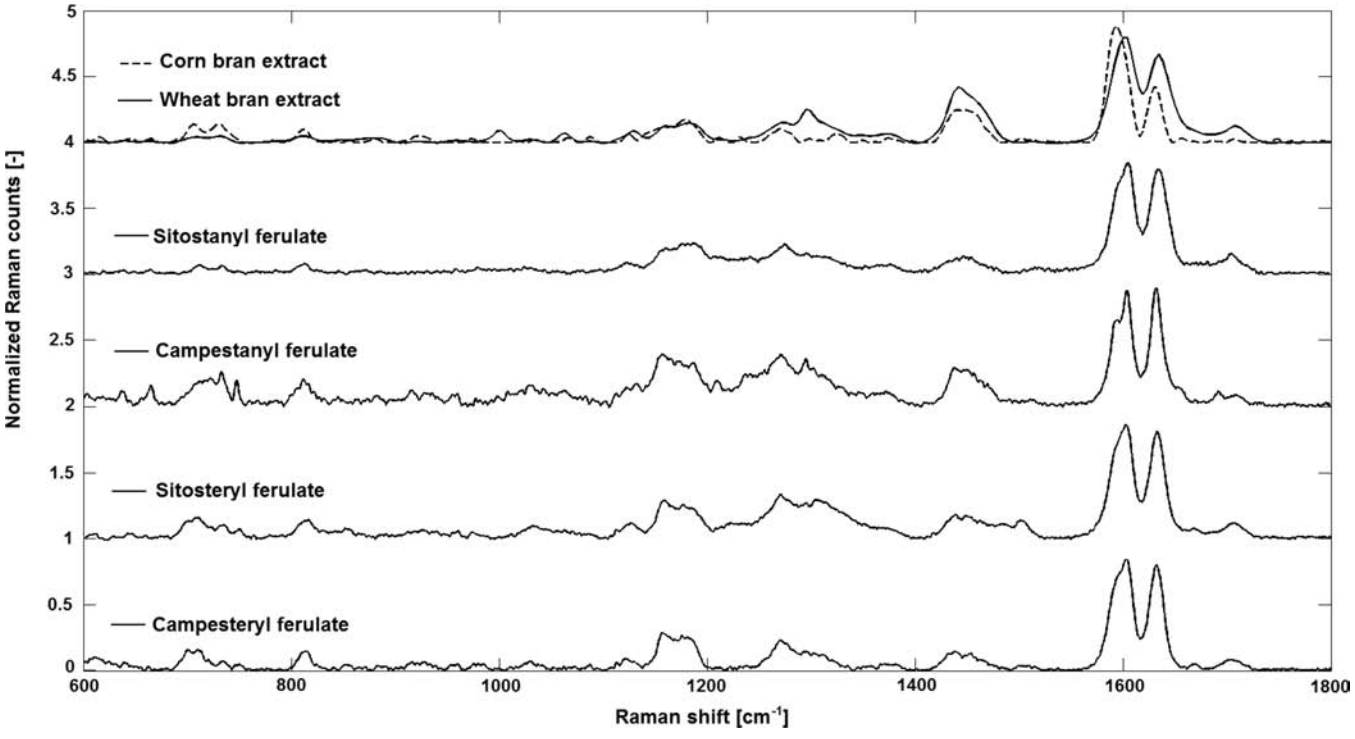

Figure 4. Raman spectra of campesteryl ferulate, sitosteryl ferulate, campestanyl ferulate, sitostanyl ferulate, and their mixtures extracted from corn bran and wheat bran.

achieved with an XBridge Prep Shield RP C18 column (5.0 μm, 10 × 250 mm, Waters, Ireland), using a mixture of acetonitrile/water/butanol/acetic acid (88:6:4:2, v/v/v/v) as an eluent (modified from Norton),[15] with a 6.6 mL/min flow rate at 25 °C. Steryl ferulate mixtures from corn bran and wheat bran and individual steryl ferulate species were collected from HPLC. Campestanyl ferulate (95% purity) was collected from wheat bran (99%), whereas sitostanyl ferulate (98%) was obtained from corn bran (99%). 24-Methylenecycloartanyl ferulate (96%) was obtained from γ-oryzanol. Campesteryl ferulate (96%) and sitosteryl ferulate (97%) were collected from both wheat bran and γ-oryzanol. All samples were collected in round-bottom flasks, from where solvent was evaporated, and the solid powder was transferred into a glass vial for spectroscopic analyses.

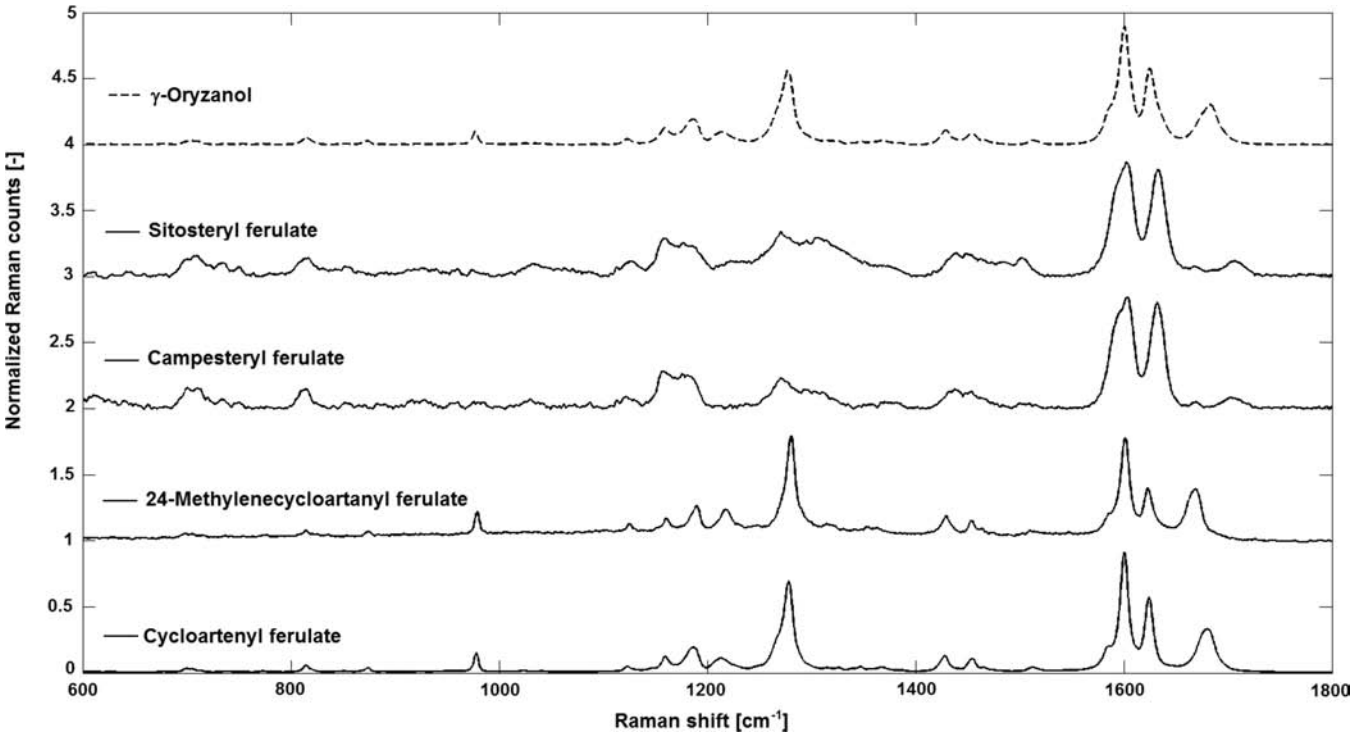

Figure 5. Raman spectra of cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, campesteryl ferulate, sitosteryl ferulate, and their mixtures in γ-oryzanol.

The purity of steryl ferulates was determined with analytical HPLC (Agilent 1200, Switzerland) as area percent. Separation was achieved with an XBridge Shield C18 column (Waters), and detection was performed at 325 nm with a diode array detector (DAD). A mixture of acetonitrile/water/butanol/acetic acid (88:6:4:2, v/v/v/v) was used as an eluent (modified from Norton),[15] with a 1 mL/min flow rate at 25 °C. Identification or tentative identification of individual steryl ferulate species has already been performed in our previous study by mass spectrometric measurements.[13]

FTIR Measurement. Infrared spectra were recorded using a Vertex 70 spectrometer (Bruker Optics, Germany), equipped with a Platinum ATR unit with a diamond ATR window. Spectral data of each sample was collected from 32 scans with a spectral resolution of 4 cm^{-1} in the range of 500−4000 cm^{-1}.

Raman Measurement. Raman spectra were collected using a SENTERRA dispersive Raman microscope (Bruker Optics, Germany) in the range of 400−1800 cm^{-1}. The excitation source was a 785 nm laser with a power of 10 mW. The laser excitation was focused using an Olympus MPLN 50× objective, and spectral resolution was provided by 1200 lines/mm high-resolution grating. Each spectrum represents the mean of 30 × 2 s scans from three distinct 2 × 2 μm areas of the corresponding sample.

Data Treatment. Raw spectroscopic data were obtained from the Bruker OPUS 7.0 software, then further processed, and visualized with Matlab 2012a. Baseline correction was performed on both infrared and Raman data sets using a concave rubber band algorithm. Subsequently, spectra were normalized.

RESULTS AND DISCUSSION

Peak Assignment of FTIR and Raman Spectra of Steryl Ferulates. Exhaustive peak assignment tables for both FTIR spectra (Figures 2 and 3) and Raman spectra (Figures 4 and 5) of steryl ferulates were completed using general tables of spectral data,[16,17] spectral data of compounds with similar molecular structure,[11,18] and available literature on spectral assignments of steryl ferulates.[9] Peak assignments discussed below are based on our own data, and corresponding supporting literature references are given for each of them in the tables (Tables 1 and 2). Although in Raman, less peaks could be observed, peaks obtained with the two methods and their related peak assignments correspond well with each other.

Vibrations in the Ferulic Acid Moiety. Strong and medium intensity peaks in the infrared spectra between 1512 and 1605 cm^{-1} have been assigned to aromatic C=C stretching. Similarly, in Raman, peaks of various intensity between 1502 and 1605 cm^{-1} could be assigned to aromatic C=C stretching,[11] originating from the aromatic ring of ferulic acid. Strong intensity peaks in the infrared spectra at 1169−1184 cm^{-1} have been assigned to the asymmetric stretching of the C−O−C group, when linked to the aromatic ring. Strong intensity peaks in the infrared spectra at 1156−1159 cm^{-1} have been assigned to C−OH stretching, also attached to the aromatic ring. Correspondingly, medium intensity peaks in the Raman spectra at 1155−1160 cm^{-1} could be assigned to aromatic C−OH stretching. To differentiate between the two peaks arising from the vibrations of the two functional groups on the aromatic ring, we used the infrared spectra of anisole and methoxyphenol as references.[18] In the Raman spectra, peaks at 699−711 cm^{-1} have been assigned to out-of-plane bending of the phenolic O−H group. However, because they could not be detected in infrared, we consider this as only a tentative peak assignment. In the infrared spectra, medium intensity peaks at 841−848 cm^{-1} and weak intensity peaks at 596−605 cm^{-1} have been assigned to aromatic C−H out-of-plane bending. The corresponding in-plane bending has been found with Raman, as medium to strong intensity peaks at 1261−1280 cm^{-1} and medium intensity peaks at 1176−1189 cm^{-1}.[11] In the infrared spectra, medium intensity peaks at 814−819 cm^{-1} have been assigned to aromatic ring breathing. This corresponded well with findings in Raman, where weak to medium intensity peaks at 811−820 cm^{-1} could also be assigned to aromatic ring breathing.[11]

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Table 1. Peak Assignment Table for the FTIR Spectra of Cycloartenyl Ferulate (1), 24-Methylenecycloartanyl Ferulate (2), Campesteryl Ferulate (3), Sitosteryl Ferulate (4), Campestanyl Ferulate (5), Sitostanyl Ferulate (6), γ-Oryzanol, and Steryl Ferulate Mixtures Extracted from Corn Bran (CB) and Wheat Bran (WB)[a]

1	2	3	4	5	6	γ-oryzanol	CB extract	WB extract	peak assignment	reference
	3009w	3009w	3009w	3009w	3009w		3009w	3009w	ν =C−H[b]	11 and 17
2941s	2957s	2955s	2955s	2955s	2955s	2955s	2955s	2955s	ν C−H (C15, C16)	17
2914s	2930s	2928s	2926s	2918s	2934s	2936s	2926s	2917s	ν C−H (cyclohexane)	17
2872s	2872s	2868s	2868s	2868s	2868s	2870s	2866s	2868s		
2856s	2856s	2853s	2853s	2851s	2851s	2849s	2851s	2850s	ν CH_3	17
	1740m	1740m	1740m	1740m	1740m		1746m	1746m	ν C=O[b]	17
1690s	1694s	1707s	1707s	1710s	1705s	1695s	1707s	1708s	ν C=O (conjugated ester)	9 and 17
1678s	1682s	1688s	1686s	1692s	1688s	1684s	1690s	1688s		
1624m	1636s	1634s	1634s	1632s	1634s	1634s	1636s	1634s	ν C=C (conjugated ester)	17
1601m	1605s	1599s	1603s	1603s	1603m	1605m	1603s	1603s	ν ar C=C	11 and 17
1585m	1588s	1593s	1593s	1593m	1589m	1595s	1595s			
1512s	1514s	1514s	1514s	1516s	1514s	1514s	1514s	1514s		
1466m	1464s	1464s	1464s	1466s	1466m	1464m	1464s	1466s	δ_{asy} CH_3 and δ CH_2	11 and 17
1456m	1456s	1456s	1458s	1452s	1450m	1456m	1452s	1452s	δ alicyclic $(CH_2)_n$	17
1443m									δ_{asy} CH_3−C=C	17
1425m	1430s	1431s	1431s	1433s	1431m	1429m	1431s	1431s	δ_{asy} CH_3 and δ alicyclic $(CH_2)_n$	17
1387m	1388m	1385m	1385m	1377m	1385m	1387m	1385m	1385m	δ_{sym} CH_3	11 and 17
1369m	1377m	1369m	1369m	1367m	1377m	1375m	1377m	1377m		
1358m	1366m	1358m	1358m	1352m	1366m	1364m	1366m	1366m		
1348w	1348w					1348w			δ_{sym} CH_3−C=C and CH_2−C=C	17
1327w	1327w			1333w	1333w	1325w	1333w	1333w	δ ip =C−H	17 and 18
1315w		1323w	1321w	1317w	1318w		1321w	1321w		
1292w	1294w	1295w	1294w	1296w	1296w	1292w	1296w	1296w	δ_{sym} O−CH_3	17 and 18
1263s	1273s	1269s	1267s	1267s	1269s	1269s	1275s	1271s	ν_{asy} C−O	17 and 18
		1250m	1250m	1254m	1252m		1252m	1252m	δ ip =C−H (ring)[c]	17
1234m	1238m			1232m	1234m	1236m	1234m		δ ip ar O−H	17
1209m	1211m	1211m	1209m	1208m	1211m	1209m	1211m	1211m	δ ip =C−H	17 and 18
1184s	1174s	1171s	1169s	1169s	1173s	1169s	1173s	1173s	ν_{asy} ar C−O−C	17 and 18
1157s	1163s	1157s	1157s	1157s	1156s	1159s	1157s	1157s	ν ar C−OH	17 and 18
1123m	1124m	1126m	1125m	1130m	1130m	1123m	1130m	1126m	δ_r CH_3	16
				1123m	1123m		1124m			
1097w	1094w	1086w	1086w	1086w	1097w	1097w	1097w	1086w	δ C−C (C3)	16
1078w	1072w	1072w	1070w	1074w	1074w	1072w	1072w	1074w		
1040m	1040m					1040m			δ_r CH_2 (C19)	20
1022m	1022m	1030m	1032m	1028m	1030m	1022m	1034m	1032m	ν_{sym} C−O	17 and 18
995w	988w					995w			δ cyclopropane ring	20
978m	978m	976m	980m	984m	980m	978m	981m	978m	*trans* δ oop =C−H	11 and 17
924w	927w	926w	927w	927w	927w	927w	928w	928w	δ C−H (cyclohexane)	16
889w	880w	885w	887w	887w	887w	885w	885w	885w	δ C−H (cyclopentane)	16
848m	847m	843m	844m	845m	845m	847m	843m	841m	δ oop ar C−H	11 and 17
814m	819m	816m	816m	817m	816m	817m	816m	816m	ar ring breathing	11
605w	602w	602w	602w	602w	601w	602w	596w	598w	δ oop ar C−H	17
571m	569m	572m	571m	571m	572m	570m	572m	572m	δ ring (cyclopentane)	16

[a] s, strong; m, medium; w, weak; ar, aromatic; phe, phenolic; ν, stretching; ν_{sym}, symmetric stretching; ν_{asy}, asymmetric stretching; δ, deformation; δ_{sym}, symmetric deformation; δ_{asy}, asymmetric deformation; δ ip, in-plane bending; δ oop, out-of-plane bending; and δ_r, rocking. [b] From free unsaturated fatty acid. [c] Tentative assignment.

In the infrared spectra, medium intensity peaks between 976 and 984 cm^{-1} have been assigned to *trans*-olefinic C−H out-of-plane bending. Weak and strong intensity peaks at 974−980 cm^{-1} in the Raman spectra corresponded well with this assignment and could also be assigned as out-of-plane bending of the same functional groups at C-2′ and C-3′ positions (Figure 1).[11] In the infrared spectra, strong intensity peaks at 1678−1710 cm^{-1} have been assigned to conjugated C=O stretching.[9] This is in line with findings in Raman spectra, where weak to medium intensity peaks arising at 1670−1692 cm^{-1} have also been assigned to conjugated C=O stretching. In addition, strong intensity peaks in infrared spectra, which appear between 1624 and 1636 cm^{-1} have been assigned to conjugated C=C stretching, similar to medium to strong intensity peaks appearing in Raman at 1622−1632 cm^{-1}.[11] Both C=O and C=C stretchings originate from the conjugated ester bond.

Vibrations in the Sterol Moiety. In the infrared spectra, strong intensity peaks at 2941−2957 cm^{-1} have been assigned to C−H stretchings at C-15 and C-16 positions, in the cyclopentane ring (Figure 1). Strong intensity peaks between 2866 and 2936 cm^{-1} have been assigned to C−H stretchings in cyclohexane rings, and strong intensity peaks appearing at 2849−2856 cm^{-1}

Table 2. Peak Assignment Table for the Raman Spectra of Cycloartenyl Ferulate (1), 24-Methylenecycloartanyl Ferulate (2), Campesteryl Ferulate (3), Sitosteryl Ferulate (4), Campestanyl Ferulate (5), Sitostanyl Ferulate (6), γ-Oryzanol, and Steryl Ferulate Mixtures Extracted from Corn Bran (CB) and Wheat Bran (WB)[a]

1	2	3	4	5	6	γ-oryzanol	CB extract	WB extract	peak assignment	reference
		1703w	1707w		1708w		1707w	1705w	ν C=O[b]	17
1680m	1670m	1670w		1692m		1683m	1685w		ν C=O (conjugated ester)	17
1624s	1622m	1632s	1633s	1632s	1633s	1625s	1630m	1631s	ν C=C (conjugated ester)	11 and 17
1601m	1601s	1604s	1603s	1604s	1605s	1601s	1593m	1596s	ν ar C=C	11 and 17
1512w	1508m		1502m			1513w				
1455m	1453m	1454m				1454w			δ_{asy} CH_3 and alicyclic δ_{sc} CH_2	11 and 19
1428m	1430m	1438m	1439m	1438m	1447m	1429m	1438m	1437m	δ_{asy} CH_3	11 and 17
						1368m		1380m	δ_{sym} CH_3	
1278s	1280s	1271m	1271m	1271m	1275m	1278s	1261m		δ ip ar C−H	11 and 17
1213m	1217m			1210m		1214m	1231m		δ ip =C−H	11 and 17
1187m	1189m	1176m			1182m	1186m	1186m		δ ip ar C−H	11 and 17
1160m	1160m	1157m	1159m	1157m		1159m	1160m	1155m	ν ar C−OH	11, 17, and 18
1123m	1125w	1122m	1127m		1123m	1123w	1124w	1124w	δ_r CH_3	11
				1029w				1023w	ν_{sym} C−O	11, 17, and 18
978s	979s	980w	975w	974w	978w	977s	975w		δ oop *trans* =C−H	18 and 19
815w	814m	813m	817m	811m	813w	815w	811w	820w	ar ring breathing	11
699w	704m	710m	711m	699w	712w	704w	702w	710w	δ oop O−H[c]	16

[a]s, strong; m, medium; w, weak; ar, aromatic; ν, stretching; ν_{sym}, symmetric stretching; δ, deformation; δ_{sym}, symmetric deformation; δ ip, in-plane bending; δ oop, out-of-plane bending; δ_r, rocking; and δ_{sc}, scissoring. [b]From free unsaturated fatty acid. [c]Tentative assignment.

have been assigned to C−H stretchings in various methyl groups. Also, in the infrared spectra, medium and strong intensity peaks at 1464−1466 and 1425−1431 cm^{-1} have been assigned to both asymmetric C−H deformation in methyl groups and C−H deformation in methylene groups.[11] Additionally, medium to strong intensity peaks at 1452−1456 cm^{-1} have been assigned to C−H deformation in methylene groups. This corresponded to findings in the Raman spectra, where weak and medium intensity peaks at 1453−1455 cm^{-1} could be assigned to asymmetric C−H deformation in methyl groups and C−H scissoring in methylene groups.[11,19] In addition, medium intensity peaks between 1428 and 1447 cm^{-1} could be assigned to asymmetric C−H deformation in methyl groups.[11] In the infrared spectra, medium intensity peaks between 1352 and 1388 cm^{-1} have been assigned to symmetric C−H deformation in methyl groups.[11] Corresponding medium intensity peaks have been found with Raman at 1368−1380 cm^{-1}. In the infrared spectra, medium intensity peaks at 1123−1130 cm^{-1} have been assigned to C−H rocking in methyl groups.[11] We found the corresponding medium intensity Raman peaks at 1122−1127 cm^{-1}. In the infrared spectra, strong peaks appearing at 1263−1275 cm^{-1} have been assigned to asymmetric C−O stretching, most likely at the C-3 position (Figure 1).[18] Medium intensity peaks, which appear at 1022−1032 cm^{-1} have been assigned to symmetric C−O stretching at the same position.[18] Well corresponding weak intensity Raman peaks were found at 1023−1029 cm^{-1}.

On the basis of the infrared spectra, we successfully identified structural differences between individual steryl ferulate molecules, originating from the sterol moiety. A medium intensity peak at 1443 cm^{-1} could only be detected in cycloartenyl ferulate. This peak has been assigned to C−H asymmetric deformation in a methyl group, when the latter is linked to an olefinic carbon atom. This structure is present only in the side chain of cycloartenyl ferulate (C-27 and C-28 positions; Figure 1). Weak intensity peaks could be detected at 1348 cm^{-1} in cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and γ-oryzanol. The peak has been assigned to C−H symmetric deformation in a methyl or methylene group, in case these groups are linked to an olefinic carbon atom. This structure is present in the side chains of cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and γ-oryzanol, which contains approximately 60−70% of the first two compounds.[13] Medium intensity peaks at 1040 cm^{-1} could also only be seen in cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and γ-oryzanol. The peak has been assigned to CH_2 rocking in the cyclopropane ring at the C-19 position (Figure 1).[20] Weak intensity peaks arising at 988−995 cm^{-1}, present only in cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and γ-oryzanol, have also been assigned to the cyclopropane ring (ring deformation).[20] The cyclopropane ring is present in the above-mentioned three compounds and not in the other steryl ferulates studied; therefore, these findings clearly show differences within steryl ferulate structures.

Additional Findings. In all but two samples, a possible impurity, free unsaturated fatty acid, could be detected. It might have co-eluted with steryl ferulates in the HPLC system. Corn bran oil and wheat flour have been reported to contain 3%[21] and 5−10%[22] of free fatty acids, respectively, of which approximately 80% is unsaturated.[23,24] Considering that free fatty acids behave similarly to steryl ferulates during base−acid wash, a possible contamination might have occurred during sample preparation. The weak intensity peak at 3009 cm^{-1} has been assigned to a non-conjugated olefinic C−H stretching,[11] whereas the medium intensity peak at 1740−1746 cm^{-1} has been assigned to C=O stretching. These two peaks combined might indicate the presence of an unsaturated fatty acid in the samples. The two peaks in question were not present in cycloartenyl ferulate and γ-oryzanol samples, which were commercially purchased; therefore, their purification was performed industrially. Additionally, a weak intensity peak at 1705 cm^{-1}, which could be assigned to C=O stretching, was also detected in the Raman spectra of some samples. The weak intensity of this peak suggests that the impurity was only present in a small amount.

Limitations of the Methods. The combination of Raman spectroscopy and FTIR spectroscopy provided valuable information on the structural characterization of steryl ferulates, but unfortunately, quantification was not possible with these

techniques. In the case of Raman spectroscopy, quantitative analysis of the data was not possible because of the high level of fluorescent background. As for the FTIR, physical phenomena, such as differences in crystal structures, resulting for instances in the formation of hydrogen bonds influenced peak intensities to a high extent; consequently, quantitative analysis could not be carried out. An example for this phenomena is peaks at 1156−1159 cm^{-1}, corresponding to the stretching vibration of the phenolic group in the ferulic acid moiety. Identifying structural differences between steryl ferulates present in wheat and corn was also not possible, given that these differences are only the number of methyl groups and presence or absence of a double bond in the sterane skeleton. Because quantitative analysis was not possible, detecting the presence of one additional methyl group or one additional unsaturated bond (difference between sterols and stanols) could not be achieved. Additionally, localization of the exact atom, which is involved in the vibration, was very limited with both methods. For instance, in the case of the out-of-plane bending of C−H groups in the aromatic ring, we detected two peaks, but we do not know if they correspond to C-5′, C-8′, or C-9′ (Figure 1). Finally, structural differences between individual steryl ferulates could only be detected with FTIR and not with Raman.

In summary, we consider that our thorough infrared and Raman peak assignment tables provide sufficient information for quick and reliable identification of steryl ferulate groups with common structural features in the future. Additionally, with the help of the FTIR peak assignment table, it is also possible to detect some of the structural differences between different steryl ferulates, namely, the presence of the cyclopropane ring and further differences in the side chain of the sterane skeleton. Identifying differences between individual steryl ferulate structures is crucial because of the important variability in their health-promoting potential.

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Notes

The authors declare no competing financial interest.

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