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Determination of Lignans in Edible and Nonedible Parts of Pomegranate (*Punica granatum* L.) and Products Derived Therefrom, Particularly Focusing on the Quantitation of Isolariciresinol Using HPLC-DAD-ESI/MS^n

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ABSTRACT: A method for the characterization and quantitation of phyto-estrogenic lignans from pomegranate (*Punica granatum* L.) fruits and fruit-derived products by HPLC-DAD-MS^n was developed. For this purpose, edible and nonedible parts of pomegranate (aril, peel, mesocarp, seed, and twigs), commercial juices, juices produced on pilot-plant scale, and encapsulated dietary supplements were analyzed. In addition to the peel, mesocarp, and twigs, lignans were detected in two juices obtained from entire fruits, four commercial juices, and three encapsulated pomegranate extracts. Isolariciresinol was the predominant lignan with contents of 5.0, 10.5, and 45.8 mg/kg dry matter in processed pomegranate mesocarp, peel, and twigs, respectively. In contrast, due to their low amounts, quantitation of lignans in pomegranate products was impossible. Therefore, contrary to previous assumptions, lignans were found to be less relevant in pomegranate-derived products. However, the byproduct from pomegranate processing may be used for lignan extraction. The method presented allows one to differentiate between pomegranate-derived products obtained from fruits without peels or by dejuicing applying low pressures, which were devoid of lignans, and those obtained from entire fruits applying high pressures, thus containing lignans. Consequently, this study helps to optimize process technology aiming at the recovery of preparations with well-desired compositions, which may reduce the risk of a wide range of diseases, such as certain types of cancer.

KEYWORDS: *aril, dietary supplements, HPLC-DAD-MS^n, lignans, peel, phytoestrogens, pomegranate (Punica granatum L.)*

INTRODUCTION

Plant-based diets rich in phytochemicals have been associated with a reduced risk of a wide range of diseases, such as certain types of cancer. Therefore, food consumption is increasingly expected to add health benefits beyond nutritional values. In this context, functional foods and so-called superfruits such as pomegranates (*Punica granatum* L.),[1] which are traditionally used for the production of grenadine syrup or consumed as fresh fruit, have gained increasing popularity in recent years. In addition to the preferred consumption of pomegranate juices, extracts are used in numerous products to exploit the specific nutritional and health-related properties of pomegranate phytochemicals.[2] Therefore, there is a steadily increasing interest in dietary supplements ranging between pharmaceuticals and foodstuffs. Consequently, the market for pomegranate products has continuously grown.[3]

In several studies, pomegranate secondary metabolites have been demonstrated to prevent hormone-dependent tumors, e.g., breast and prostate cancers, due to their inhibitory action on 17-β-estradiol based on their competitive binding to estrogen receptors.[2,4,5] Flavonoids have commonly been assumed to cause this effect. However, this has not been conclusively elucidated and recently questioned in a study revealing pomegranate juices derived from different cultivars to be devoid of phyto-estrogenic flavonoids.[6] On the basis of estrogen-like activities observed in unspecific in vivo and in vitro assays, some authors even concluded that pomegranate seeds and peel extracts contain steroid hormones including estrone[7,8] and estradiol.[9] In contrast, the presence of any steroid estrogen could not be confirmed in another study applying chromatographic methods coupled to mass spectrometric detection.[10] Since flavonoids and steroid-hormones are obviously not responsible for the aforementioned effects, the active principles of pomegranate products are still largely unknown. Therefore, lignans displaying estrogenic activity have been claimed to occur in pomegranates, which may be of particular relevance from a health point of view.[11] Lignans are secondary plant metabolites widely distributed in the plant kingdom contributing to plant defense systems. They reveal phyto-estrogenic activity, which also applies to isoflavonoids and coumestans. These compounds display structural similarity to 17-β-estradiol competing for its receptor in mammalian organisms.[12] In the gastrointestinal tract, such steroid-like metabolites are converted to enterodiol and enterolactone, which regulate sexual-specific processes and appear to be involved in the prevention of cancer.[13]

Lignans are the products of specific chemical coupling reactions of two phenylpropanoid units (C6−C3). They have been suggested to induce a wide range of biological activities and therapeutic effects on human health, such as antioxidant, antitumor, antiviral, antibacterial, insecticidal, fungistatic,

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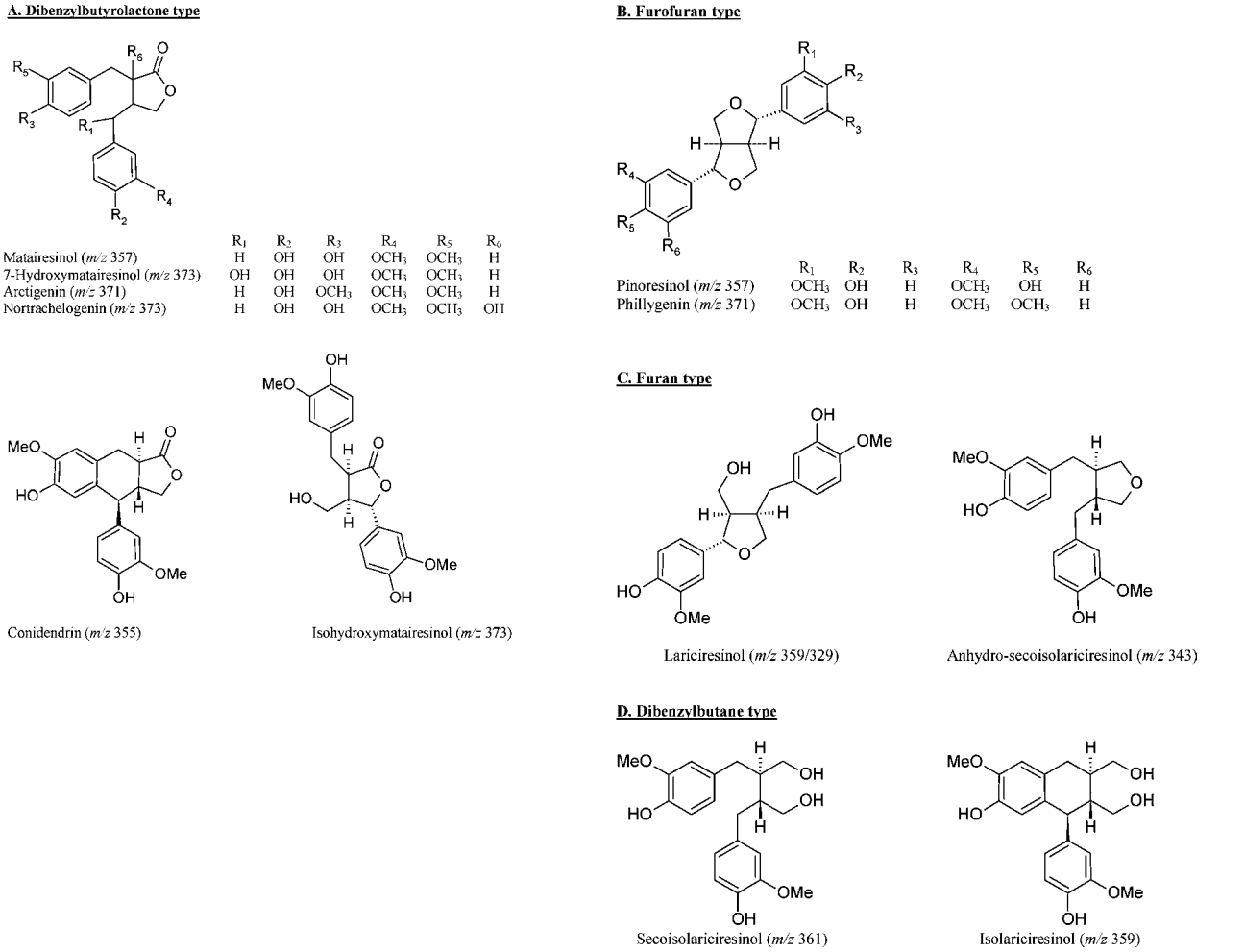

Figure 1. Chemical structures of different lignan types. (A) Dibenzylbutyrolactone type; (B) furofuran type; (C) furan type; (D) dibenzylbutane type.

estrogenic, and antiestrogenic activities, and to protect against coronary heart disease.[14−20] Lignans often occur as diglycosides and also in the form of more complex glycosides. To simplify analytical determination, a number of hydrolysis methods have been described in order to split off ether- and/or ester-bound lignans. Furthermore, enzymatic hydrolysis[15,21] with β-glucuronidases, β-glucosidases, or cellulases is carried out under mild conditions preventing the formation of artifacts. However, cleavage of complex glycosides cannot be achieved under these conditions. In contrast, hydrolysis using hot acidic solutions not only cleaves the glycosidic bonds but also degrades the resulting aglycones into fragments.[15,22] A number of methods for the determination of lignans in different matrices have been reported in the literature[23] also covering pomegranates.[11] However, the GC method applied in the latter study possibly overestimates lignan contents in pomegranate fruits due to the application of a response factor based on only one reference compound to the quantitation of a number of structurally diverse lignans. In contrast, the characterization of lignans in pomegranate fruits and products derived therefrom using liquid chromatography coupled to mass spectrometry has not been reported so far. Therefore, the main objective of the present study was to develop an HPLC method for the quantitative determination of lignans in pomegranate fruits, twigs, and processed pomegranate products. The method should be applicable to the characterization of lignans belonging to the structural subclasses of dibenzylbutyrolactones, dibenzylbutanes, furans, and furofurans (Figure 1) in generative (aril, peel, and mesocarp) and vegetative parts (twigs) of pomegranate as well as juices and dietary supplements.

MATERIALS AND METHODS

The method was optimized with lyophilized peels from pomegranate fruits (*Punica granatum* L.) of an unknown cultivar, cultivated in Iran and Israel. Furthermore, Italian pomegranate fruits of organic production (cultivar unknown) were used for juice production. The twigs (5.0 mm diameter) from the same Italian pomegranate trees were also used for lignan analysis after lyophilization. Commercial pomegranate juices and dietary supplements were obtained from health or whole-food stores as well as pharmacies. According to their product specification, juices 8, 10, and 13 contained added flavors, spices, pectin, agave concentrate, and extracts obtained from other fruits, whereas all other juices were produced without any additives (Table 1).

Solvents and Reagents. All reagents and solvents of analytical or HPLC grade were purchased from VWR (Darmstadt, Germany) unless otherwise stated. The following standards were used for identification and quantitation purposes with HPLC-MS and HPLC-DAD, respectively: isolariciresinol, lariciresinol, secoisolariciresinol, and matairesinol (ArboNova, Turku, Finland). *Helix pomatia* β-glucuronidase/sulfatase (>300 U/mg) was from Sigma Aldrich (Steinheim, Germany). Deionized water was used throughout.

Juice Production on Pilot-Plant Scale. For the production of pomegranate juice, amounts of 20 to 30 kg of fresh fruits were used

Table 1. Commercial Juices and Dietary Supplements Analyzed in the Present Study

no.	manufacturer specifications
	Juices
1	pomegranate (100%)
2	pomegranate (100%), only from arils
3	pomegranate (100%)
4	pomegranate (100%)
5	pomegranate (100%)
6	pomegranate (100%)
7	pomegranate (100%), from concentrate
8	pomegranate (97% juice and pulp concentrate, partly fermented), lemon juice concentrate, aroma, vanilla extract, pectin, spices (0.1%)
9	pomegranate pulp (100%)
10	27.5% fruit content, white tea, agave concentrate, 6% fruits (aronia, pear)
11	pomegranate (100%)
12	pomegranate (100%)
13	pomegranate concentrate (90% partly fermented), elderberry concentrate, pectin
14	–
15	pomegranate (100%)
16	pomegranate (100%)
17	pomegranate (100%)
18	pomegranate (100%)
	Encapsulated Dietary Supplements
19	with fermented pomegranate juice
20	77% pomegranate extract
21	–
22	pomegranate juice extract with lycopene and selenium
23	400 mg pomegranate extract per capsule

per variant. Whole pomegranate fruits were washed, steamed (8 min) to inactivate natural enzymes, and subsequently pressed with a rack and cloth press (Wahler, Stuttgart, Germany) with pressures of 10, 50, and 150 bar to obtain juice variants 1a–c.

For the production of juice variant 2, pomegranate fruits without previous steaming were directly dejuiced in a rack and cloth press in the same way as that described above. For the production of juice variants 3 and 4, the leathery peel (exocarp) and most of the fleshy white mesocarp were manually removed from pomegranate fruits. Subsequently, dejuicing of the seed-containing arils was carried out with a rack and cloth press as described for the treatment of the entire fruit (variant 2). Alternatively, peeled fruits were passed through a type PAP 0533 finisher (Alberto Bertuzzi, Brugherio, Italy) with a mesh size of 5.0 mm (800 rpm) in a first step and 1.5 mm (900 rpm) in a second step to obtain juice variant 4. For complete separation of mesocarp fibres and seeds, a mesh size of 0.4 mm and speed of 700 rpm were applied. Both juice variants were produced using Fructozym color (0.059 g/kg; Erbslöh, Geisenheim, Germany) over a period of 2 h at 25 °C, to remove pectic-like material,[24] filtered with a chamber filter system (Pall Seitz Schenk Filtersystems, Waldstetten, Germany) equipped with T 5500 filter sheets (Pall Seitz Schenk Filtersystems, Bad Kreuznach, Germany), and a second filtration was performed using a pilot-plant scale microfiltration unit (Pall Seitz Schenk Filtersystems, Waldstetten, Germany) fitted with a 0.2 mm Membralox P19-60 ceramic membrane (Waldstetten, Germany). Juice variants 1a and 2–4 were pasteurized in an Actijoule HTST system (T = 92 °C, flow rate = 80 L/h; Ruland Engineering and Consulting, Neustadt, Germany), bottled in 0.5 L wide-necked bottles under steam injection and cooled to ambient temperature.

Additionally, the fruits were manually separated into leathery peel (exocarp), fleshy mesocarp, and seeds with adherent arils, and juice variant 5 was prepared solely from the isolated arils coating the seeds using a Hafico tincture press (Fischer Maschinenfabrik, Neuss, Germany) at 250 bar. Peel, mesocarp, seeds, and twigs were separately lyophilized and ground with a blender (Waring Products Division, Torrington, CT, USA).

Sample Preparation for HPLC-DAD Analysis. *Method Optimization.* Different extraction methods were performed with lyophilized pomegranate peel powder (5 g) according to previous reports[25,26] with adaptions and modifications. Soxhlet extraction and extraction under stirring with different solvents were used to recover the lignans from pomegranate samples as specified in Table 2. Hydrolysis of the compounds and extraction of lignan aglycones from the hydrolysates was performed as described below for final sample pretreatment. Furthermore, hydrolysis conditions were varied following four different extraction methods (Table 2).

Enzymatic Hydrolysis. In accordance with Smeds et al.,[26] the extracts were evaporated to dryness and redissolved in 5 mL of acetate buffer (0.01 M, pH 5.0). An aliquot of 1 mL was hydrolyzed with *H. pomatia* β-glucuronidase/sulfatase (1.6 mg in 1 mL of acetate buffer, pH 5.0) for a period of 19 h at 37 °C.

Acid Hydrolysis. Acid hydrolysis applying different HCl concentrations, i.e., 0.7, 0.3, 0.1, and 0.07 M, was performed using the extracts of method 9. Furthermore, the extracts were analyzed according to Smeds et al.[26] without previous hydrolysis.

Final Sample Pretreatment. The lyophilized and homogenized powder of pomegranate peels, mesocarp, and twigs (5 g) was extracted with acidified acetone (80%, v/v, 0.01 M HCl; 150 mL) for 1 h at 60 °C in a W 350 T water bath (Memmert, Schwabach, Germany). After centrifugation (15 min, 3315*g*, Varifuge 3.0, Heraeus Sepatech, Osterode, Germany), the extracts were evaporated in vacuo at 25 °C to remove the organic solvent. The aqueous residues were hydrolyzed according to Sicilia et al.[27] by adding 4 mL of HCl (6 N, final concentration of 0.7 M) and heating for 1 h at 95 °C in a water bath (Memmert, Schwabach, Germany). Pomegranate seeds were defatted prior to extraction with *n*-hexane (100 mL, 30 min). Juices (30 mL) were directly used for hydrolysis. The solutions of hydrolyzed samples were extracted three times with ethyl acetate/*n*-hexane (1:1, v/v, 50 mL), and the combined extracts were evaporated to dryness, dissolved in methanol, membrane-filtered (0.45 μm), and used for LC analysis.

Recovery rate was determined by spiking lyophilized pomegranate mesocarp with 50 μL of isolariciresinol standard solution (1000 mg/L) prior to sample preparation.

Hydrolysis of Standard Compounds. Lignan standard solutions (100 mg/L) of matairesinol, lariciresinol, secoisolariciresinol, and isolariciresinol were hydrolyzed under the same conditions as the samples by heating 2 mL of standard solution after adding 0.27 mL of HCl in pyrex culture tubes (100 × 14 mm i.d., Bibby Sterilin, Stone, Staffordshire, U.K.). After cooling in an icebath, potassium hydroxide solution (440 μL, 20%; 130 μL, 2%) was added for neutralization. The resulting solution was membrane-filtered (Chromafil RC-45/15 MS, 0.45 μm, Macherey-Nagel, Düren, Germany) and subsequently used for HPLC-DAD and HPLC-DAD-MS^n analysis.

HPLC-DAD and LC-MS Analysis. Lignan analysis was performed using a Merck-Hitachi LaChrom Elite HPLC system (Merck, Darmstadt, Germany) equipped with an L-2200 autosampler, an L-2130 pump, a Jetstream column oven, and an L-2450 diode array detector.

The column used was a 150 × 4.6 mm i.d., 3 μm, Hypersil Hydro-RP 18 with a 4 mm × 3 mm i.d. guard column of the same material (Phenomenex, Torrance, CA, USA) at a flow rate of 0.4 mL/min and a constant temperature of 30 °C. The diode array detector was set at an acquisition wavelength of 280 nm.

The mobile phase consisted of 0.1% (v/v) acetic acid in water (eluent A) as well as of methanol, water, and isopropanol (69/30/1, v/v/v; eluent B). The flow rate was 0.4 mL/min, and the gradient program was optimized as follows: 10–60% B (50 min), 60% B isocratic (20 min), 60–100% B (7 min), 100% B isocratic (8 min), 100–10% B (3 min), and 10% B isocratic (5 min). Total run time was 93 min. The injection volume for all samples ranged from 15 to 30 μL.

LC-MS analyses were carried out with the aforementioned column and elution program using an Agilent HPLC series 1100 (Agilent, Waldbronn, Germany) equipped with ChemStation software, a model G1379A degasser, a model G1312A binary gradient pump, a model

Table 2. Parameters of the Lignan Extraction Methods and Isolariciresinol Extraction Yields[a]

no.	extraction solvent	volume [mL]	time [h]	temperature/conditions	isolariciresinol yield [mg/kg DM]
		Soxhlet-Extraction			
1[b]	*n*-hexane[c]	200	1	reflux	5.7 ± 0.0 B,C,D
	acetone (100%)	150	1		
	acetone (80%, v/v)	150	1		
2	acetone (100%)	150	1	reflux	1.6 ± 0.0 E
	acetone (70%, v/v)	100	1		
3	*n*-hexane[c]	150	1	reflux	n.d.
	acetone (100%)	150	1		
	acetone (80%, v/v, 0.3 M NaOH)	150	1		
4	*n*-hexane[c]	200	1	reflux	3.1 ± 0.3 D,E
	acetone (100%)	150	1		
	acetone (80%, v/v, 0.01 M HCl)	150	1		
	pH adjustment to 5−6 after extraction				
5	*n*-hexane[c]	200	1	reflux	4.4 ± 0.2 C,D,E
	acetone (80%, v/v, 0.01 M HCl)	150	1		
	pH adjustment to 5−6 after extraction				
		Extraction under Stirring			
6	acetone	100	1	ambient	1.7 ± 0.3 E
	acetone (70%, v/v)	100	1		
7[b]	methanol (70%, v/v, 0.3 M NaOH)	120	1	60 °C	n.d.
	pH adjustment to 5−6 after extraction				
8[b]	methanol (70%, v/v, 0.01 M HCl)	120	1	60 °C	2.0 ± 0.2 E
	pH adjustment to 5−6 after extraction				
9	acetone (80%, v/v, 0.01 M HCl)	150	1	60 °C	9.1 ± 0.3 A
	pH adjustment to 5−6 after extraction				

[a]Abbreviations: n.d., not detected. Values expressed as the means ± standard error of determinations performed in duplicate. Significant differences between values in the same column are indicated by different letters A−E ($P < 0.05$). [b]Optimization of hydrolysis conditions (enzymatic, acid hydrolysis, and without hydrolysis). [c]Removal of lipophilic compounds, *n*-hexane fractions were discarded.

G1313A autosampler, a model G1316A column oven, and a model G1315A diode array detection system. The HPLC system was connected in series with a Bruker (Bremen, Germany) model Esquire 3000+ ion trap mass spectrometer fitted with an ESI source. Data acquisition and processing were performed using Esquire Control software (ver. 5.1). Negative ion mass spectra of the column eluate were recorded in the range m/z 50−520 at a scan speed of 13000 m/z per s. Nitrogen was used as drying gas at a flow rate of 9.0 L/min and nebulizing gas at a pressure of 40.0 psi. The nebulizer temperature was set at 365 °C. Helium was used as collision gas at a pressure of 4×10^{-6} mbar. HPLC-DAD-MS^n conditions were previously optimized to achieve maximum lignan sensitivity.

Quantitation of Individual Lignans by HPLC-DAD. Individual lignans were assigned based on their retention times and UV as well as mass spectra. Isolariciresinol was quantitated using a calibration curve of the reference compound. For this purpose, a stock solution (1.000 mg/L) was diluted to obtain concentrations of 5−500 mg/L.

Data Analysis. Tukey's studentized range (HSD) test, using SAS software (ver. 9.1., SAS Institute, Cary, NC, U.S.A.) was performed to determine significant differences. The significance was determined using least significant difference (LSD) ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Sample Pretreatment. *Extraction.* Preliminary tests revealed highest lignan contents for pomegranate peel and mesocarp compared to those of the arils. Therefore, peels were used for method optimization. Individual compounds of the pomegranate peel extracts were characterized by comparison of their UV/vis spectra, retention times, and mass spectra with those of the reference substances. Isolariciresinol was found to be the predominant lignan in pomegranate samples. Consequently, all extraction methods were compared based on the isolariciresinol contents of the resulting extracts. Table 2 provides an overview of all methods also specifying the isolariciresinol yields when using pomegranate peels for extraction. Figure 2 illustrates a typical HPLC chromatogram of a pomegranate mesocarp extract.

Extraction methods 3 and 7 (Table 2) applying alkaline solvents were characterized by high amounts of coextracted matrix components and concomitant inferior chromatographic resolution. Accordingly, the low amounts of isolariciresinol could not be quantitated because the predominance of matrix components made unambiguous identification of individual lignans impossible. In contrast, Milder et al.[25] observed optimal lignan yields upon broccoli and bread extraction under similar alkaline conditions, which is probably due to lower amounts of extractable matrix compounds. In the present study, highest isolariciresinol contents (9.1 mg/kg DM) were determined applying variant 9, i.e., extraction under stirring with acidified aqueous acetone (80%, v/v; 0.01 M HCl). Consequently, this extraction variant was used for further sample pretreatment.

Hydrolysis. In addition to acid hydrolysis, which was performed following extraction, enzymatic hydrolysis with *H. pomatia* β-glucuronidase/sulfatase was carried out in combination with three selected extraction methods (numbers 1, 7, and 8; Table 2) to determine optimum hydrolysis conditions. Enzymatic hydrolysis was used in previous studies for the pretreatment of human plasma, flaxseed, and pumpkin seed extracts prior to lignan analysis.[27,28] However, lower efficiency of enzymatic lignan hydrolysis compared to acid hydrolysis was observed in other studies investigating flaxseed meal.[29,30] In contrast, a combined process consisting of methanolic extraction and alkaline hydrolysis followed by enzymatic cleavage with *H. pomatia* β-glucuronidase/sulfatase was shown to be

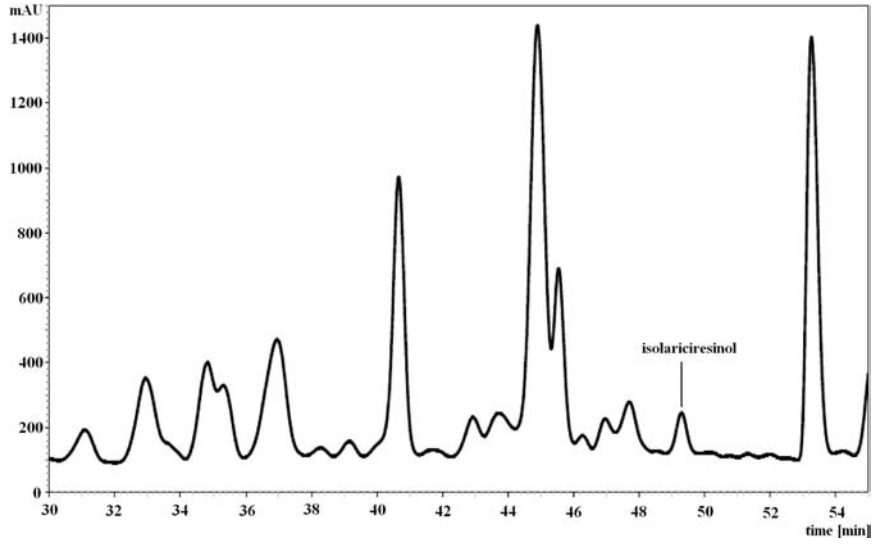

Figure 2. Separation of lignans of a pomegranate mesocarp extract by HPLC (280 nm).

efficient for the release of lignan aglycones from bread, broccoli, flaxseed, and black tea,[25] again demonstrating the large dependency of extraction yields on the composition of the food matrix. The extracts obtained in the present study were also analyzed prior to hydrolysis to verify the necessity of an additional sample pretreatment. Since neither prior to hydrolysis nor after enzymatic treatment lignans could be identified, both methods were found to be unsuitable for lignan analysis in pomegranate fruits.

On the basis of these results, acid hydrolysis was further applied for sample preparation. To select optimum acid concentrations for hydrolysis, extracts obtained following extraction variant 9 were hydrolyzed with HCl concentrations of 0.3 M, 0.1 and 0.07 M HCl in addition to 0.7 M. Differences in isolariciresinol contents after hydrolysis with 0.7 M HCl were insignificant compared to those in the application of lower acid concentrations (data not shown).

Acid hydrolysis with an HCl concentration of 1 M was also used for the treatment of flaxseed meal lignans after extraction with 80% methanol in a water bath.[29] Smeds et al.[26] compared different methods such as accelerated solvent extraction (ASE) and alkaline and acid extraction to isolate lignans from cereals, oilseeds, and nuts and observed matrix-dependent differences in lignan extraction. This corroborates previous observations regarding the variability of lignan extraction yields depending on the food matrix, thus emphasizing the necessity of a systematic method optimization for the analysis of lignans in pomegranate fruits and pomegranate-derived products. Furthermore, the mass spectra of all samples were screened for mono-, di-, and triglycosides of the predominant lignans secoisolariciresinol, lariciresinol, and isolariciresinol. According to these results, incomplete hydrolysis of the lignans could be excluded since glycosidic lignans were absent.

Accuracy of the method was evaluated by the standard addition method. The recovery rate of isolariciresinol amounted to 86%, thus being in a range similar to that in the findings of Peñalvo et al.[28] determining recoveries for lignans in the range of 77% to 94% in plasma samples, with 85% for isolariciresinol after enzymatic hydrolysis. Milder et al.[25] observed recovery rates ranging from 51% to 123% depending on lignan structures and food matrices after alkaline extraction and enzymatic hydrolysis.

Characterization of Lignans by LC-MS^n. Pomegranate lignans belonging to the structural subclasses of dibenzylbutyrolactones, dibenzylbutanes, furans, and furofurans were characterized by comparison of their UV/vis spectra, retention times, and mass spectra with those of reference substances. Figure 3 illustrates the extracted ion chromatograms of selected lignans of a pomegranate peel extract. The retention times, UV/vis and mass spectrometric characteristics as well as peak assignments of all compounds are specified in Table 3, and individual lignans are discussed below.

Furofuran Lignans. Peak 4 revealed an $[M - H]^-$ ion at m/z 371 and a loss of 15 Da in the MS^2 experiment resulting in a fragment ion at m/z 356, which indicates the release of a methyl moiety. The MS data were in agreement with those previously reported for phylligenin (Figure 1b).[31] This lignan was only detected in the fruit peel.

Dibenzylbutane Lignans. The loss of 48 Da in the MS^2 experiment was the most generally observed fragmentation pattern for the butandiol lignans such as secoisolariciresinol and isolariciresinol (Figure 1d). Secoisolariciresinol (peak 3) exhibited an $[M - H]^-$ ion at m/z 361 with further characteristic fragment ions at m/z 313, 165, and 179 (Figure 4 and Table 3). The aforementioned loss of 48 Da may be explained by the combined loss of formaldehyde and water from the diol structure. The latter two fragments may be produced by cleavage in the β-position, according to Eklund et al.[31] In addition, the ions at m/z 346 and 331 were attributed to fragments formed upon the loss of a methyl group and formaldehyde, respectively (Figure 4 and Table 3).

Among the dibenzylbutane lignans, the characteristic isolariciresinol (peak 2), which revealed an $[M - H]^-$ ion at m/z 359, was detected. The primary product ion at m/z 344 was produced by the loss of a methyl radical (15 Da) in the MS^2 experiment. The loss of 46 Da producing a fragment at m/z 313 may be attributed to a combined loss of a methoxyl radical (31 Da) and a methyl radical (15 Da) or to the loss of a CH_2OH moiety and a methyl radical. The loss of a methyl radical was the major fragmentation reaction of this lignan type, which is due to the stabilization of the resulting radical by the aromatic moiety. Both peaks exhibiting different retention times corresponded to isomeric structures, also differing in their fragmentation patterns. These patterns and the UV/vis

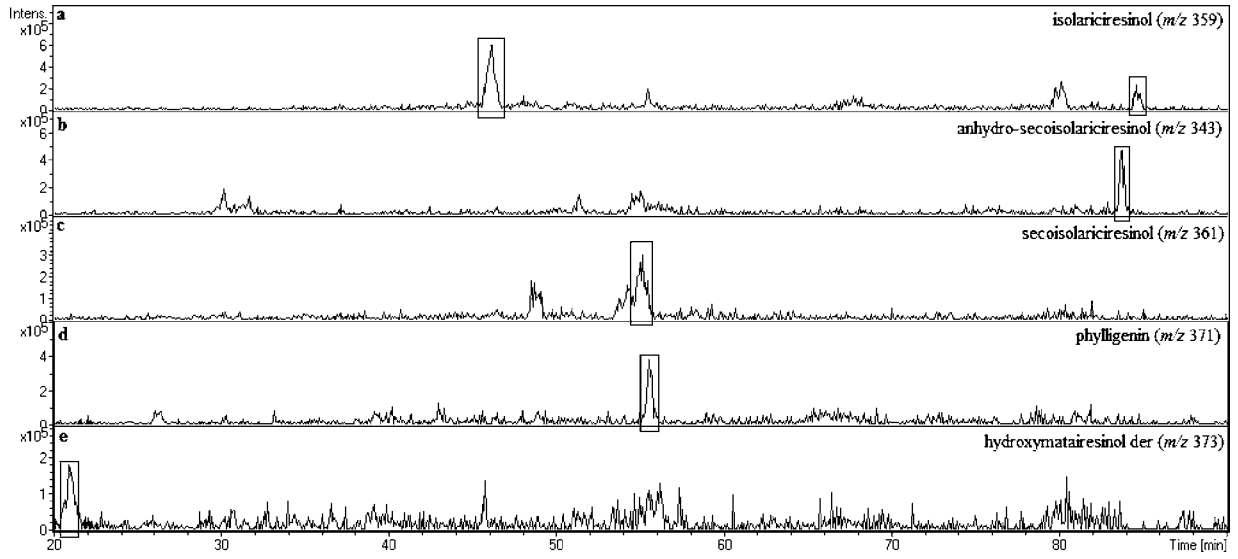

Figure 3. HPLC-DAD-MS^n analysis of a pomegranate peel extract. Extracted ion chromatograms: the occurrence of selected ions at m/z 359 (a), 343 (b), 361(c), 371 (d), and 373 (e) has been highlighted.

absorption spectra were consistent with the standard components of secoisolariciresinol and isolariciresinol. Both peaks were detected in pomegranate peels, mesocarp, and twigs, while the latter peak was also identified in pomegranate juice variants 1 and 2 produced on a pilot-plant scale as well as three commercial products (numbers 13, 20, and 23). Furthermore, secoisolariciresinol was detected in one commercial juice (no. 10) produced from peeled pomegranates and one encapsulated dietary supplement (no. 23).

Isolariciresinol is formed from the furan lignan lariciresinol (Figure 1c) under acidic conditions.[26] This is in accordance with the results obtained in the standard hydrolysis experiment performed with lariciresinol. In the present study, this labile lignan could not be detected in pomegranate samples, even under mild conditions of hydrolysis. In contrast, isolariciresinol was stable during acid hydrolysis not being converted into other lignans or degradation products.

Dibenzylbutyrolactone Lignans. Peak 5 produced an $[M - H]^-$ ion at m/z 373 and a fragment ion at m/z 355, resulting from the characteristic loss of water (18 Da). Thus, this peak may be assigned either to 7-hydroxymatairesinol, nortrachelogenin, or iso-hydroxymatairesinol (Figure 1a). Eklund et al.[31] observed a fragment at m/z 327 for nortrachelogenin due to the loss of CO_2 and H_2 or CO and H_2O with almost the same intensity as that of the fragment at m/z 355. In the present study, a fragment ion at m/z 327 was not formed from the $[M - H]^-$ ion at m/z 373. Therefore, peak 5 was tentatively assigned to 7-hydroxymatairesinol or iso-hydroxymatairesinol, described in the following as the hydroxymatairesinol isomer. Peak 5 occurred in two isomeric forms as can be deduced from the retention times specified in Table 3. Furthermore, the isomers differed slightly in their fragmentation pathways. It may be assumed that hydroxymatairesinol was degraded under alkaline and acid conditions or was converted into derivatives with similar molecular weight, such as α-conidendrin and iso-hydroxymatairesinol.[26] In the present study, a matairesinol standard solution was hydrolyzed under the aforementioned conditions to assess the stability of dibenzylbutyrolactone lignans, showing that matairesinol was not degraded under acid conditions. The hydroxymatairesinol isomer was detected in pomegranate peels, mesocarp, and twigs.

Among the dibenzylbutyrolactone lignans, peak 6 showed an $[M - H]^-$ ion at m/z 355 with the characteristic primary product ion $[M - H\text{-}15]^-$ resulting from the loss of a methyl moiety yielding a fragment at m/z 340. The additional loss of CO_2 and methyl moieties in the MS^3 experiment yielded fragments at m/z 296 and 325, respectively. Accordingly, this peak was identified as conidendrin (Figure 1a), which was in accordance with the findings of Eklund et al.[31] However, this dibenzylbutyrolactone lignan was only detected in commercial juice no. 14.

Furan Lignans. Peak 1 was identified as the dibenzyltetrahydrofurano lignan anhydro-secoisolariciresinol (Figure 1c). Its $[M - H]^-$ ion at m/z 343 released fragments at m/z 328 and 313, resulting from the loss of one or both methyl moieties. Anhydro-secoisolariciresinol may be regarded as the dehydrated product of secoisolariciresinol, which is formed by acid hydrolysis.[32] During hydrolysis of a standard solution, secoisolariciresinol was dehydrated yielding almost equal amounts of both corresponding lignans. In contrast, Smeds et al.[26] did not observe the conversion of secoisolariciresinol during acidic sample pretreatment, maybe due to the considerably milder acid hydrolysis conditions (0.01 M HCl) used for cereals, oilseeds, and nuts. In accordance with our results of the experiments performed with the isolated standard compound, the formation of anhydro-secoisolariciresinol was observed in extracts of peel, mesocarp, twigs, and juice variant 2 produced on pilot-plant scale as well as in one of the commercial products (no. 19).

The peel extract revealed the greatest diversity of lignans comprising five components, followed by the mesocarp and twigs, which showed the presence of four different lignans. Some of the peaks detected in the present study revealed $[M - H]^-$ ions and fragments in the MS experiments (Figures 3 and 4) similar to those described in the literature for lignans.[31] However, the identification of lignans in pomegranate fruits and juices without reference compounds proved to be difficult. In a previous study, six lignans were identified and quantitated in pomegranate fruits by GC-MS. However, their identification

Table 3. Retention Times, UV/Vis Spectra, and Mass Spectrometric Behavior of Lignans in Pomegranate Fruits and Pomegranate-Derived Products[a]

						occurrence																	
										juice variant[b]							commercial pomegranate products[c]						
peak. no	assignment	rt [min]	λ_{max} [nm]	$[M-H]^-$ m/z	HPLC-ESI(−)-MS^n-experiment m/z [% base peak]	p	m	s	t	1a	1b	1c	2	3	4	5	1	10	13	14	19	20	23
1	anhydro-seco-isolariciresinol	45.6	–	343	MS^2 [343]: 328 (100), 311 (28), 329 (21), 312 (15)	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	+	–	–
					MS^3 [343→328]: 284 (100), 313 (67), 160 (60)																		
		83.8	287		MS^2 [343]: 328 (100), 329 (16), 325 (15), 245 (5), 326 (4), 313 (3)	+	+	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–
					MS^3 [343→328]: 313 (100), 314 (25)																		
					MS^3 [357→151]: 136 (100)																		
2	isolariciresinol (cyclolariciresinol)	46.2	284	359	MS^2 [359]: 344 (100), 345 (18), 313 (14)	+	+	–	+	+	–	–	+	–	–	–	–	–	+	–	–	+	+
					MS^3 [359→344]: 313 (100), 159 (27), 109 (16), 255 (12), 329 (10)																		
		84.8	–-		MS^2 [359]: 344 (100), 345 (13), 329 (10)	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
					MS^3 [359→344]: 329 (100), 330 (24)																		
3	secoisolariciresinol	55.2	281	361	MS^2 [361]: 165 (100), 179 (48), 331 (48), 313 (39), 346 (39)	+	+	–	+	–	–	–	–	–	–	–	–	+	–	–	–	–	+
					MS^3 [361→165]: 147 (100), 129 (38)																		
4	phylligenin	55.8	329,	371	MS^2 [371]: 356 (100), 341 (45), 359 (41), 325 (31), 326 (25), 136 (6)	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
			276		MS^3 [371→356]: 341 (100), 160 (9), 136 (58)																		
5	hydroxymatai-resinol isomer	21.7	280	373	MS^2 [373]: 355 (100), 343 (36), 283 (27), 271 (13), 311 (6)	+	+	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
					MS^3 [373→355]: 340 (100), 283 (76), 268 (30)																		
		30.4	285		MS^2 [373]: 355 (100), 343 (31), 261 (30), 329 (21)	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
					MS^3 [373→355]: 340 (100), 268 (99), 283 (59)																		
6	conidendrin	43.5	–	355	MS^2 [355]: 340 (100), 296 (70)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–
					MS^3 [355→340]: 296 (100), 325 (35), 285 (15), 189 (16)																		

[a]Abbreviations: t, twigs; der, derivative; m, mesocarp; p, peel; rt, retention time, s, seeds. [b]Sample assignment: see the Materials and Methods section. [c]Sample assignment: see Table 1.

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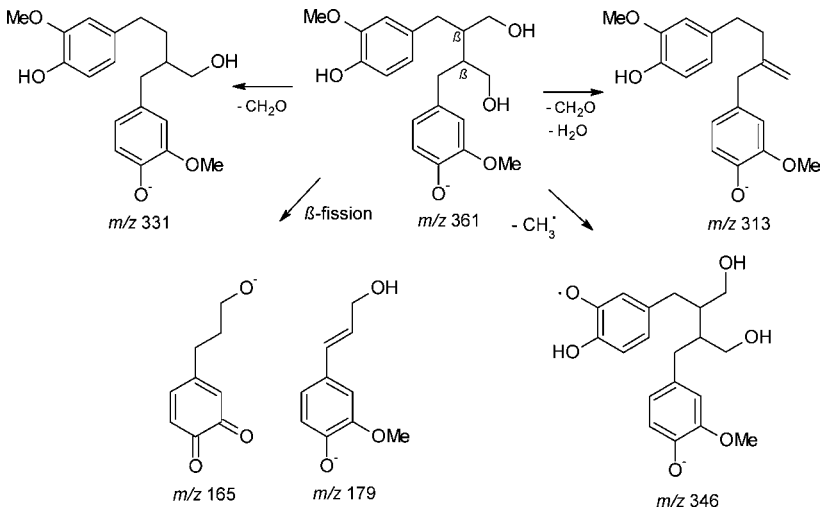

Figure 4. Proposed fragmentation pathway of secoisolariciresinol in collision induced dissociation experiments, modified from Eklund et al.[31]

was based on literature data and the comparison with one single standard compound, while detailed comparison of the data with those of individual reference substances is lacking.[11]

Quantitation of Lignans by HPLC-DAD. Because of the low lignan contents in pomegranate products and seeds, quantitation of the predominant lignan isolariciresinol was only possible in the peel and mesocarp as well as in twigs. The highest lignan content of 45.8 mg/kg dry matter was determined in the twigs followed by the peel (10.5 mg/kg DM) and mesocarp (5.0 mg/kg DM). Highest lignan contents were expected in the twigs of the pomegranate tree and also in the protecting endocarp of the fruit. In contrast, selective accumulation of lignans in seeds, such as sesame and linseed, seed-like fruits as caryopses and brans of rye and wheat, respectively, as previously reported,[33] could not be confirmed for pomegranate in the present study.

Lower total lignan contents in pomegranate endocarp, ranging from 1.4 to 3.3 mg/kg as well as in pomegranate twigs (17.8 mg/kg), were estimated in another study using only one standard component. In contrast, higher amounts were determined in the seeds (15.8−36.1 mg/kg) and pulp (11.2 mg/kg) of pomegranate fruits.[11] The differences between the fruit and twig compositions of the Italian samples of the present study and the published data may be due to different cultivation areas, varieties, or maturity degrees of the samples but may also be due to methodological deficiencies of the aforementioned study since the response factor for quantitating individual lignans by GC was only calculated for one reference compound but applied to the quantitation of all components.

Compared to other foods rich in lignans such as flaxseed or sesame with contents of ∼3000 mg/kg and ∼400 mg/kg, respectively, pomegranate fruits are of minor relevance with regard to dietary lignan uptake. However, other fruits, such as nectarines, strawberries, and apricots, exhibit even lower lignan levels ranging from 1.9 to 4.5 mg/kg.[33] Nevertheless, the byproduct of pomegranate processing may serve as an interesting source for the recovery of health-promoting extracts comprising both lignans and further valuable phenolics.

The presence of lignans could be confirmed in pomegranate fruits and processed products, however, only at low levels, especially in the juices and encapsulated products. In contrast, lignans were not detected in arils and seeds. Only the peels and mesocarp of pomegranate fruits revealed quantifiable amounts of lignans. These results were also confirmed by the different juice variants. Lignans were only detected in juice variants 1a and 2, obtained by pressing the entire fruit at maximum pressure, whereas juices obtained by pressing at 10 and 50 bar (variants 1b and c) were devoid of lignans. Partial (juice variants 3 and 4) and entire (juice variant 5) removal of the peel excluded lignans from the liquid products. The commercial products also contained only few lignans at low amounts. Thus, to increase lignan contents of pomegranate products, enhanced extraction of peel and mesocarp is required, and the so-obtained extracts may be used in the manufacture of encapsulated dietary products.

Furthermore, the identification and quantitation of lignans in pomegranate juices may be used together with their total phenolic contents[24,34] to differentiate between aril-based products or juices obtained at low pressure and those which have been produced including peels and mesocarp, thus allowing process technology evaluation based on the composition of the juices.

Only one encapsulated dietary supplement was shown to contain lignans. This demonstrates the necessity of an exhaustive selection of pomegranate fruits or raw material and the application of appropriate process technology to optimize the production of encapsulated dietary supplements based on the contents of their active principles.

The influence of pomegranate variety and growing conditions on the lignan profile and contents in pomegranate fruits are still unknown but of particular importance for the production of health-promoting, enriched pomegranate products. However, the lignan dosages that need to be ingested to profit from their biological activities have not yet been established. Most probably, the health-promoting effects, in particular anticancer activities of pomegranate products, may not be attributed to individual compounds. Instead, this is more likely resulting from a variety of different compounds, mainly phenolic components including lignans, presumably exerting synergistic effects. Moreover, ellagitannins, the predominant bioactive compounds in pomegranate fruits, are hydrolyzed to ellagic acid and metabolized by the intestinal microbiota to urolithin and further derivatives. These metabolites may be enriched in different tissues and thus may be responsible for the inhibition of prostate cancer cell growth due to their reported estrogenic activity.[35] Furthermore, contrary to previous assumptions,[7,8] estrone was not found in pomegranate fruit, twigs, juices, and

encapsulated dietary supplements emphasizing once more that ellagitannins are presumably responsible for the discussed activities.

To the best of our knowledge, the present study describes for the first time an LC-MS based method for the identification and quantitation of lignans in individual pomegranate fruit parts, differently produced juices, and commercial juices as well as encapsulated dietary supplements. The optimized method is appropriate for the characterization and quantitation of lignans in pomegranates and pomegranate-derived products. Furthermore, byproducts of pomegranate processing have been demonstrated to be a promising source of lignans that may be used as functional food ingredients, thus valorizing these side-streams of food processing.

The present study revealed pomegranate-derived products to be devoid of estrone, and only few products contained lignans. Additionally, the pilot-plant experiments allow a classification of pomegranate-derived products based on their lignan contents. The aforementioned commercial products were presumably obtained from peeled fruits and thus are strictly aril-based or were dejuiced at low pressures. Consequently, these products did not contain lignans. A second product category is characterized by marked amounts of lignans. These are recovered from the entire fruit and using high pressures, which allows the enhanced transfer of secondary metabolites into the extracts and juices. The effects of the latter products as human health promoters need to be thoroughly studied in the future.

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