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Rapid dereplication of estrogenic compounds in pomegranate (*Punica granatum*) using on-line biochemical detection coupled to mass spectrometry

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Abstract

During recent years, phytoestrogens have been receiving an increasing amount of interest, as several lines of evidence suggest a possible role in preventing a range of diseases, including the hormonally dependent cancers. In this context, various parts of the pomegranate fruit (*Punica granatum*; Punicaceae), e.g. seed oil, juice, fermented juice and peel extract, have been shown to exert suppressive effects on human breast cancer cells in vitro. On-line biochemical detection coupled to mass spectrometry (LC-BCD–MS) was applied to rapidly profile the estrogenic activity in the pomegranate peel extract. The crude mixture was separated by HPLC, after which the presence of biologically active compounds, known or unknown, was detected by means of an on-line β-estrogen receptor (ER) bioassay. Chemical information, such as molecular weight and MS/MS fingerprint, was obtained in real time by directing part of the HPLC effluent towards a mass spectrometer. Using this approach in total three estrogenic compounds, i.e. luteolin, quercetin and kaempferol, were detected and identified by comparing the obtained molecular weights and negative ion APCI MS/MS spectra with the data of an estrogenic compound library. Although well known in literature and widely distributed in nature, the presence of these phytoestrogenic compounds in pomegranate peel extract was not reported previously. Compared to traditional screening approaches of complex mixtures, often characterized by a repeating cycle of HPLC fractionation and biological screening, LC-BCD–MS was shown to profoundly accelerate the time required for compound description and identification.

Keywords: Pomegranate; *Punica granatum*; On-line biochemical detection–mass spectrometry; Phytoestrogens

1. Introduction

During the last decade, interest in flavonoids and their possible role in preventing cardiovascular disease (Larkin et al., 2000; Hertog et al., 1993, 1995; Knekt et al., 1996) and certain human cancers like the hormonally dependent breast and prostate cancers (Rosenberg Zand et al., 2000; Ranelletti et al., 1992; Scambia, 1990; Yoshida et al., 1990; Messina et al., 1994), has increased dramatically. Flavonoids comprise a class of polyphenolic compounds, most of which are commonly found in fruits, herbs, leguminous plants, red wine and tea (Justesen, 2000: Zin et al.; 2000; de Pascual-Teresa et al., 2000; Lommen et al., 2000; Kiehne and Engelhardt, 1996; Miketova et al., 2000; Zeeb et al., 2000). Several of these compounds are structurally similar to some mammalian estrogens and have been shown to exert weak estrogenic behaviour (Kuiper et al., 1998). These food components, often called phytoestrogens, are consumed in high quantities in cultures that show a lower incidence of hormonally dependent cancers, suggesting a possible role as chemopreventive agents (Setchell and Cassidy, 1999; Kurzer and Xu, 1997). In vitro, flavonoids have been shown to act as antioxidants

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(Williamson et al., 1996), inhibit topoisomerase II (Constantinou et al., 1998), inhibit platelet aggregation (Tzeng et al., 1991), induce phase II detoxification enzymes (Uda et al., 1997) and inhibit growth of cancer cells from the colon (Ranelletti et al., 1992), ovary (Scambia, 1990) and gastrointestinal tract (Yoshida et al., 1990). In vivo, soy isoflavones, like daidzin and genistein, have been demonstrated to influence a range of biochemical processes, most of which are thought to be associated with reduced risks of breast cancer (Cassidy et al., 1994; Xu et al., 1998).

Over the years, the pharmacological properties of various parts of the pomegranate tree (*Punica granatum*) have been described extensively. Flower extracts reduced blood sugar levels in rodents (Jafri et al., 2000), fresh juice was shown to inhibit LDL oxidation and the formation of atheromatous plaque in rodents and humans (Aviram et al., 2000). Strong antioxidant properties of the fermented juice have been reported, while oil polyphenols were found to inhibit the eicosanoid enzymes cyclooxygenase and lipoxygenase (Schubert et al., 1999). So far, a range of estrogenic compounds has been identified in various parts of the fruit. Pomegranate was demonstrated to contain the highest botanical concentration of the steroid estrone at 17 mg/kg dried seed (Heftmann et al., 1966). Campesterol and 17α-estradiol have been detected in seed oil (Kim et al., 2002), whereas coumestrol (Moneam et al., 1988), estriol and testosterone (Abd El Wahab et al., 1998) were found in pomegranate seeds.

Recently, fractions of the pomegranate, i.e. crude seed oil, crude fermented and unfermented juice and peel extract, were shown to exert anti-proliferative effects on human breast cancer cells in vitro (Kim et al., 2002).

With the increasing interest in flavonoids, a multitude of analytical methods has been developed in order to determine their concentration in a range of matrices. Both gas chromatography/mass spectrometry (GC/MS) (Mazur et al., 1996, 1998) and high performance liquid chromatography coupled to a variety of analysis methods including mass spectrometry (Barnes et al., 1998), diode array (Vuorinen et al., 2000) and electrochemical (Franke and Custer, 1994) detection have been used frequently. With the exception of electrochemical detection and post-column reaction detection systems for the measurement of antioxidant activity, typically no direct correlation between the analyte and its biological activity in relation to a specific protein target is obtained. Currently, several types of microtiter plate based biological assays, including competitive binding (Song et al., 1999), reporter gene (Ikeda et al., 2002) and cell proliferation (Schmitt et al., 2001) assays, have been used as methods to assess the estrogenic/antiestrogenic potential of pure compounds or complex mixtures. Although several of these methods are employed in drug discovery programs today, rapid dereplication of known or undesirable compounds in complex samples, such as natural product extracts, has proven to be a time consuming task. Typically, a repeating process of HPLC fractionation and biological activity screening is employed in order to deconvolute the complex mixture and identify the bioactive compounds. Recently however, an on-line biochemical detection (LC-BCD) system combined with complementary chemical analysis techniques such as mass spectrometry (LC-BCD–MS) was used to characterize estrogenic compounds in a large functional food library (Schobel et al., 2001). Crude extracts were separated by HPLC, after which the presence of biologically active compounds was detected by means of an on-line β-ER bioassay (Fig. 1). In contrast to the traditional microtiter-plate based type of bioassays, the number of bioactive compounds and their contribution to the total bioactivity of the sample was obtained within a single run. Moreover, chemical information, which plays a crucial role in the characterization and identification of bioactive compounds, was obtained in real time by directing part of the HPLC effluent towards a mass spectrometer. In this way, the responses measured in the biochemical assay were rapidly correlated to their molecular weights and MS/MS fingerprints.

Using LC-BCD–MS, the presence of estrogenic compounds in pomegranate peel extract, known or unknown, was determined. This paper presents the results of the bioactivity profiling and the characterization of the bioactive compounds. Moreover, it demonstrates the advantage of LC-BCD–MS over traditional screening methods in terms of complex mixture screening and dereplication speed.

2. Results and discussion

2.1. On-line characterization screening

2.1.1. LC-BCD–MS detection of phytoestrogenic compounds

In general, the coupling of continuous-flow biochemical detection to HPLC is characterized by a loss in sensitivity compared to flow injection setups. Frequently used organic modifiers in HPLC, such as methanol and acetonitrile, have been demonstrated to decrease bioassay sensitivity significantly, especially at high concentrations and prolonged reaction times. The influence of organic solvents however, is minimized, by diluting the HPLC effluent continuously. A counter gradient, referred to here as BioGradient, effectively reduces the concentration of organic modifier to a constant, bioassay compatible level, while maintaining a constant output flow rate. In this manner, a stable, biochemical readout is obtained throughout the entire chromatographic run. The dilution caused by the coun-

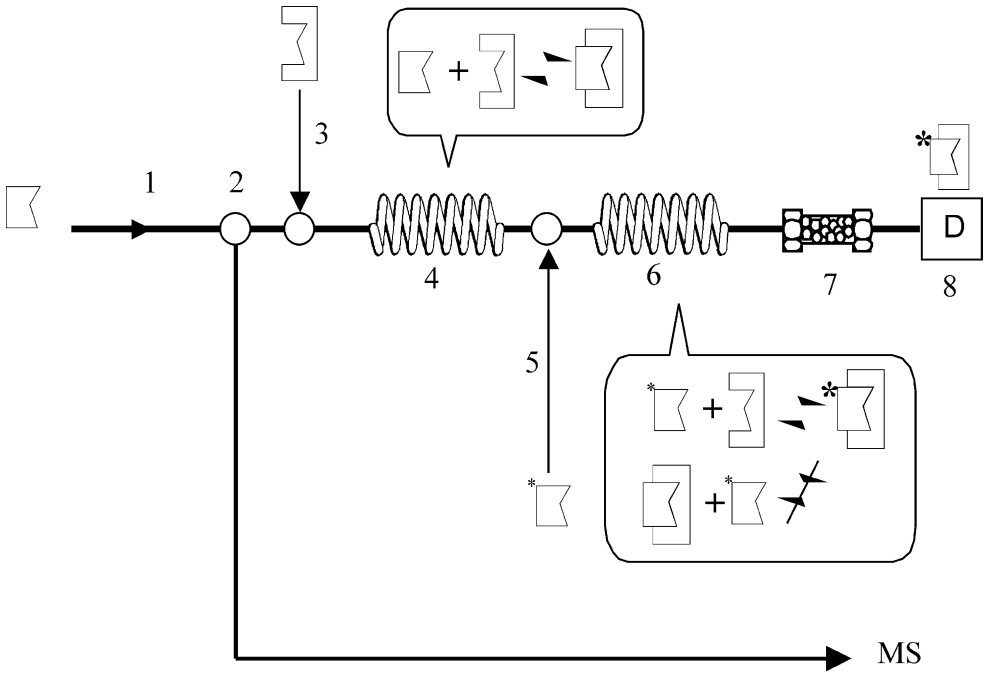

Fig. 1. Configuration of the on-line β-ER bioassay. (1) BioGradient effluent; (2) flow splitting to biochemical assay and MS; (3) reagent pump for β-ER solution; (4) reaction coil; (5) reagent pump for coumestrol solution; (6) reaction coil; (7) restricted access column (2×5 mm, C18 ADS); (8) fluorescence detector (D).

ter gradient, typically between a factor of 5 and 10, is compensated by an increased injection volume applied.

Though the correlation between assay sensitivity and organic modifier is quite clear, the tolerable concentration of organic solvent in the HPLC effluent strongly depends on the properties of the target protein used. Recombinant human β-ER, used in the assay format described herein, was hardly affected by acetonitrile concentrations below 12% (Fig. 2). Above this level however, the dynamic range of the bioassay was rapidly reduced. In order to maintain a high dynamic range, while keeping dilution of the HPLC effluent as low as possible, the acetonitrile concentration was continuously adjusted to 10% during LC-BCD–MS measurements. Bandbroadening caused by the post-column reaction system approximated a factor of 5. Reaction times applied, 80 s in total, were relatively short in order to minimize bandbroadening and reduce further dilution. Under these conditions, quercetin, a compound possessing only weak estrogenic properties (Kuiper et al., 1998), could be detected at a concentration level of 120 μM. Other widespread phytoestrogenic compounds like genistein, daidzein, kaempferol and luteolin showed markedly lower LOD's of respectively 1.1, 2.1, 44 and 18 μM. Although these detection limits are significantly higher compared to the flow injection approach described previously (Schobel et al., 2001), the on-line β-ER biochemical assay still provides sufficient sensitivity to enable the detection of phytoestrogenic compounds in highly concentrated pomegranate extracts.

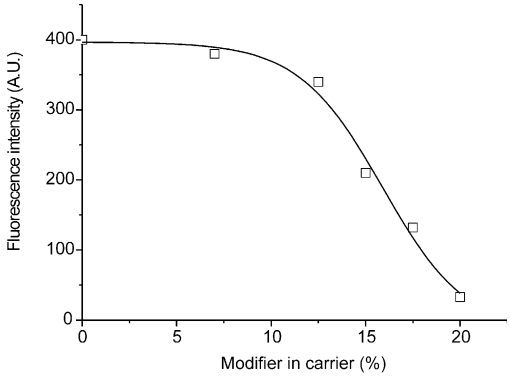

Fig. 2. Influence of organic modifier concentration on bioassay sensitivity. The modifier concentration displayed, represents the percentage acetonitrile present in the BioGradient effluent.

2.1.2. High resolution screening–mass spectrometric (HRS–MS) analysis of acid hydrolyzed pomegranate extract

Phytoestrogenic compounds are widespread in nature, occurring predominantly as *O*-glycosidic conjugates: glucose, galactose, rhamnose, arabinose, xylose and rutinose (Justesen et al., 1998). In general, the metabolic conversion of phytoestrogenic compounds to more polar conjugates, as is commonly observed in most plants, affects biological activity dramatically. In order to assess the full estrogenic potential of the pomegranate extract, glycosidic moieties were removed from the conjugated phytoestrogenic compounds by means of acidic hydrolysis.

As a first step towards detection and identification of phytoestrogenic compounds in the pomegranate peel extract, a general bioactivity profile of a 50-fold concentrated sample was recorded. The bioactivity profile, generated by applying a 5–95% acetonitrile gradient in 15 min, provided crucial information on the obtained resolution and that required for accurate correlation of biochemical and MS data. Although the bioactivity chromatogram of the pomegranate peel extract showed the presence of two separate bioactive regions, MS data indicated that the applied chromatographic resolution was insufficient to separate co-eluting compounds. Consequently, in order to increase resolution and facilitate the correlation between biochemical and chemical data, an additional bioactivity profile was recorded under different HPLC conditions: 20–35% acetonitrile in 8 min, followed by a 7 min post gradient. The total ion current, recorded by the MS detector (Fig. 3A), shows a relatively clean chromatogram, with most of the interfering compounds eluting within the first 5 min. In addition, the bioactivity chromatogram (Fig. 3B) clearly shows two biologically active areas at $t_R = 7.8$ and at $t_R = 11.0$ min, which were previously detected by LC-BCD–MS under standard gradient conditions. The implementation of a small, restricted access column (RA-column) just before the fluorescence detector proved to be crucial for detecting estrogenic activity in the pomegranate peel extract. The autofluorescence background, which was substantially higher than the dynamic range of the β-ER assay, masked the presence of estrogenic compounds in the absence of the RA-column. Subsequent implementation however, reduced the background efficiently and dramatically increased assay sensitivity.

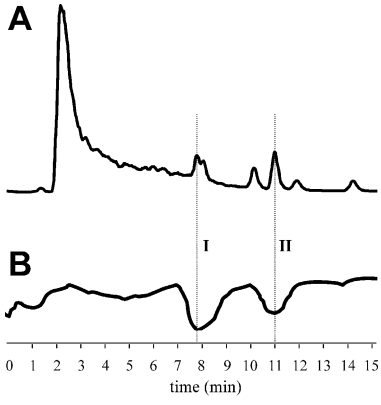

Fig. 3. LC-BCD–MS analysis of acid hydrolyzed pomegranate extract. (A) Full scan total ion current. (B) Biochemical detection readout. (I) First biologically active area. (II) Second biologically active area. Chromatography: 20–35% acetonitrile/2 mM ammonium acetate in 8 minutes followed by a 7 min postgradient. Maximum flow rate 0.5 ml/min. Analytical column: Prodigy ODS(3) 5 μm, 100×3.2 mm.

2.1.3. Dereplication of bioactive compounds

Although biochemical and mass spectrometric data are collected in parallel during LC-BCD–MS measurements, the bioactive molecules are not detected at exactly the same retention time. After chromatographic separation, part of the effluent is introduced almost directly into the mass spectrometer, whereas the remaining part first interacts with several biochemical reagents for a certain period of time. The difference in these retention times was determined by analyzing a reference solution of the phytoestrogenic compound, genistein and was found to be 1.4 +/−0.2 min ($n = 3$). In order to enable accurate correlation of biochemical and chemical data, the retention times of the biochemical responses were corrected for the determined time difference.

The MS spectrum of the first bioactive peak showed the presence of two dominant ions, respectively m/z 285.5 (Fig. 4A) and m/z 301.5 (Fig. 4B). As the total ion current (TIC) trace already indicated (Fig. 3A), reconstructed ion currents (RIC) of the two masses confirmed the presence of two co-eluting compounds, separated only by 0.3 min: m/z 285.5 at $t_R = 7.7$ min and m/z 301.5 at $t_R = 8.0$ min. Based on the corrected retention time of the first bioactive peak ($t_R = 7.8$ min) however, m/z 285.5 was considered to be largely responsible for the detected biological activity. As a first step towards identification of the biologically active compound(s), the molecular ions ($[M–H]^-$), m/z 285.5 and 301.5, as well as their negative ion APCI MS/MS spectra (Fig. 4A and B), were used as a search query in the estrogen database. The molecular ions were found to match multiple well-known phytoestrogenic compounds. M/z

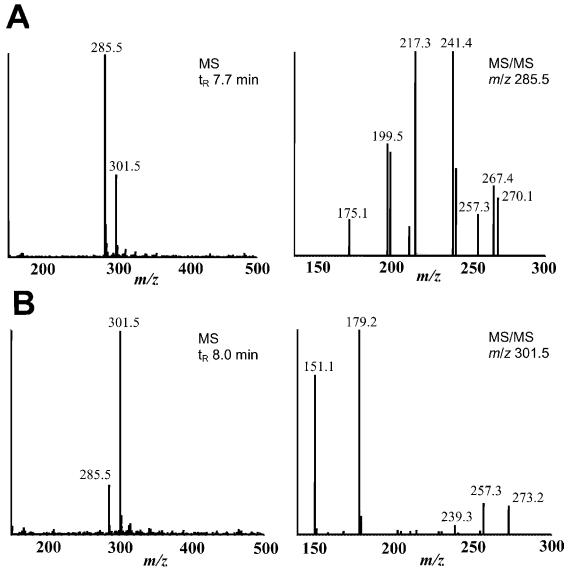

Fig. 4. MS and MS/MS spectra corresponding to the first biologically active area in the biochemical assay. A: MS and MS/MS (m/z 285.5) spectra at t_R 7.7 min. B: MS and MS/MS (m/z 301.5) spectra at t_R 8.0 min.

285.5 corresponded to the estrogenic isomers luteolin and kaempferol, whereas m/z 301.5 matched with the structurally related compounds morin, hesperetin and quercetin.

The MS/MS spectra, obtained during LC-BCD–MS measurements, allowed unambiguous identification by providing highly specific fragmentation patterns, in terms of ion fragments and ion intensities. The MS/MS fingerprint of m/z 285.5 showed intense fragments at m/z 217 and 241 and was found to correspond to luteolin after database searching. The MS/MS spectrum of m/z 301.5 showed characteristic intense ions at m/z 151 and 179, which correlated well with the reference spectrum of quercetin.

Similarly, the second bioactive peak in the bioactivity chromatogram ($t_R = 10.9$ min) was correlated to the corresponding MS and MS/MS data (Fig. 5). The MS spectrum showed the presence of an intense ion at m/z 285.5, which accurately matched the retention time of the bioactive peak. Database searching showed that the MS/MS spectrum of the bioactive compound matched perfectly with that of the phytoestrogenic compound, kaempferol.

The identity of the detected phytoestrogenic compounds was confirmed by LC-BCD–MS analysis of a reference solution, containing luteolin, quercetin and kaempferol (Fig. 6). The retention times of the phytoestrogenic compounds, detected in the pomegranate peel extract (Fig. 6A), were found to be in good agreement with those observed in the reference sample (Fig. 6B). In addition, the bioactivity profile of the reference sample was found to be similar to that of the pomegranate extract (Fig. 6C). Based on the matching MS/MS data, the retention times observed during LC–MS analysis and similar bioactivity profiles, the identities of luteolin, quercetin and kaempferol were confirmed. The presence of these compounds in pomegranate peel extract was not reported previously in literature.

The concentrations of the identified phytoestrogenic compounds in the 50-fold concentrated pomegranate peel extract, were determined via standard addition. Luteolin, quercetin and kaempferol were found to be present at concentration levels of approximately 129, 140 and 230 μM, respectively. Based on the detection limits of luteolin and quercetin (18 and 120 μM), the individual contribution to the response of the first bioactive peak was estimated to be 86 and 14%. The estrogenic activity of luteolin and quercetin relative to genistein has been reported to equal 58 and 10% (Rosenberg Zand et al., 2000). The ratio in estrogenic activity between luteolin and quercetin is reflected well by the detection limits found in the bioassay. In addition, the estrogenic activity of kaempferol has been reported to be in between that of luteolin and quercetin (Kuiper et al., 1998; Zava and Duwe, 1997), which is consistent with the biochemical responses found in the pomegranate pericarp extract.

The dereplication procedure described here differs significantly from those reported previously. Identification of active compounds in crude natural extracts requires some sort of feedback from a biological assay, regardless of the separation techniques and analytical detection methods used. For years, the most commonly used strategy has been to fractionate the crude extract by HPLC, followed by the biological assaying of each individual fraction. This approach is characterized by some very time consuming and labor intensive steps. LC-BCD–MS however, continuously monitors the HPLC effluent for biological activity and consequently eliminates most of the disadvantages of the fraction collection strategy. Besides biological activity information, chemical data such as retention time, molecular

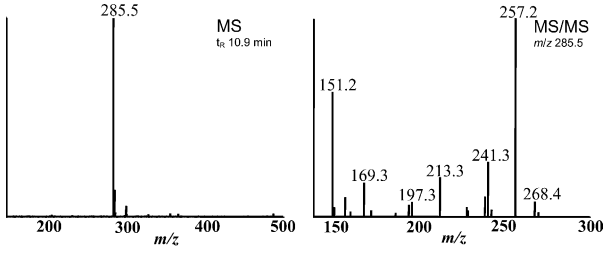

Fig. 5. MS and MS/MS spectra corresponding to the second biologically active area in the biochemical assay. MS and MS/MS (m/z 285.5) spectra at t_R 10.9 min.

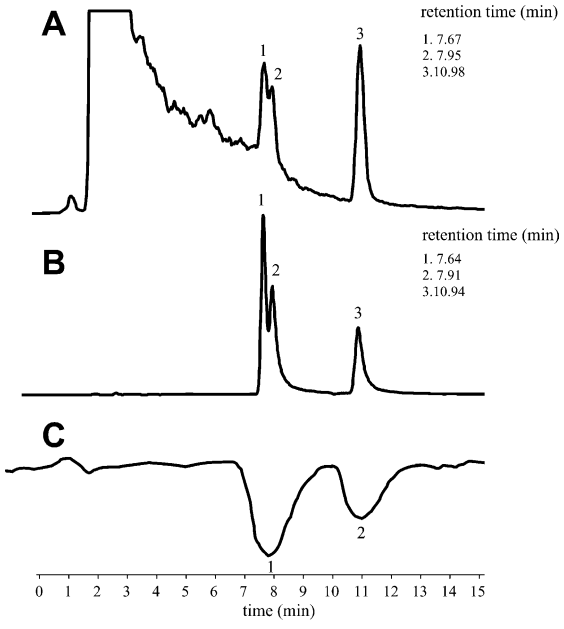

Fig. 6. Identity confirmation of phytoestrogenic compounds found in pomegranate extract. 1. luteolin, 2. quercetin, 3. kaempferol. A: RIC of acid hydrolyzed extract—m/z 285.5 and 301.5. B: RIC of reference solution containing luteolin (260 μM), quercetin (280 μM) and kaempferol (240 μM). C: Bioactivity profile of reference solution.

weight, MS/MS fingerprint and UV spectrum are obtained in real-time by splitting the HPLC effluent to a range of analytical detectors. Depending on the complexity of the sample, bioactive compounds are typically correlated to the corresponding chemical data within one or two HPLC runs. Subsequently, as was shown for the estrogenic compounds in the pomegranate peel extract, the chemical parameters can be used as search queries in databases for rapid identification of the biologically active compounds. Compared to the traditionally used dereplication approaches, LC-BCD–MS speeds up compound characterization and identification dramatically.

2.1.4. LC-BCD–MS analysis of unhydrolyzed pomegranate extract

The influence of conjugation on estrogenic activity was demonstrated by analyzing a 50-fold concentrated, unhydrolyzed pomegranate peel extract (Fig. 7A). In contrast to the hydrolyzed sample, no biological activity was detected as concentrations of the unconjugated phytoestrogens were found to be well below the detection limits of the β-ER assay. Although predominantly present in glycosylated form, a high percentage of the conjugated phytoestrogenic compounds was detected by APCI–MS as a free aglycone. In source fragmentation of the conjugates resulted in the appearance of characteristic neutral mass losses and molecular ions of the phytoestrogenic compounds. For example, MS/MS analysis showed that molecular ions of kaempferol eluted at t_R 6.74 and 7.19 min (Fig. 7A), which indicated the presence of two conjugated species. Subsequent RIC analysis of all mass traces present in the MS spectra at t_R 6.74 and 7.19, showed that only m/z 447.2 and 593.2 exhibited an elution profile identical to that of the free aglycone, kaempferol. At t_R 6.74 min, a neutral loss of 162 Da, from m/z 447.2 to 285.5 (Fig. 7B), indicated the loss of glycoside. A characteristic neutral mass loss of 308 Da, from m/z 593.2 to 285.5 (Fig. 7C), at t_R 7.19 min correlated to the loss of rhamnoglycosidic moiety. Similarly, luteolin and quercetin were found to be predominantly present as just a single glycosylate. The LC-BCD–MS measurements shown here demonstrate the effect of conjugation on the estrogenic potency of the pomegranate peel extract. Similar findings have been reported previously for pomegranate juice. The estrogenic potency of fermented juice was found to be significantly higher, compared to that of fresh juice. During fermentation however, the ether bonds between the aglycone and the glycoside are largely disrupted, resulting in an increase in free phytoestrogen concentration. So far, knowledge regarding the uptake and metabolism of glycosylated phytoestrogens as well as their biological activity in the human body is limited. Hydrolysis by microflora in the intestines as well as endogeneous enzymes in the colon, have been proposed as the key mechanisms in human uptake (Day et al., 2000).

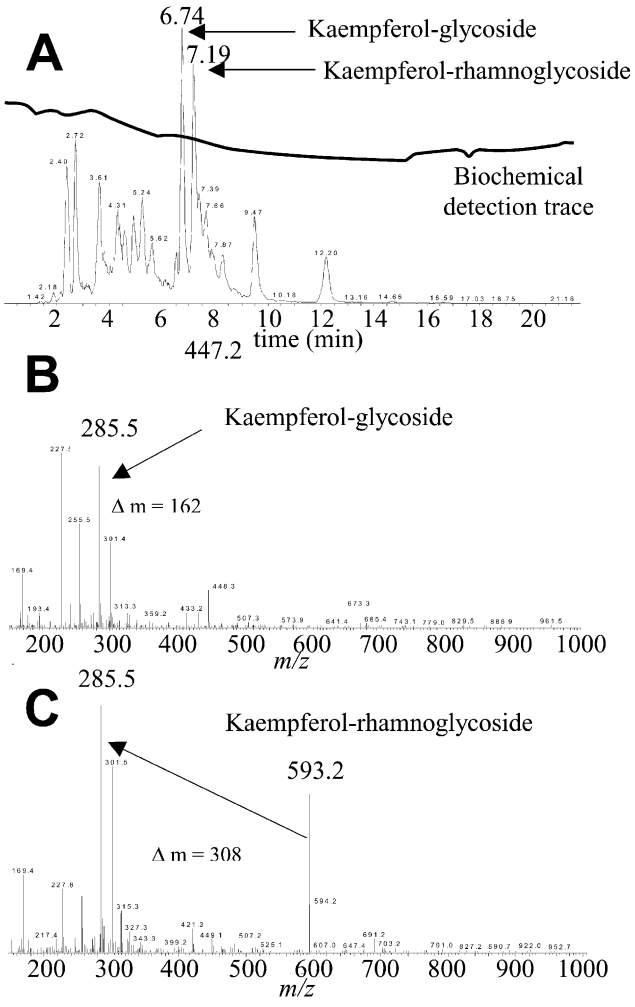

Fig. 7. Biological activity glycosylated phytoestrogenic compounds. A: MS RIC for m/z 285.5 and bioactivity profile of untreated pomegranate extract. B: MS spectrum at t_R 6.74 min. C: MS spectrum at t_R 7.19 min.

3. Conclusion

The LC-BCD–MS method described here demonstrates the advantage of providing both biological activity and relevant chemical data in parallel during a single chromatographic run. Not only is there a significant reduction in resources required, but a profound acceleration of the time needed for dereplication is achieved as well. Despite the loss in sensitivity, caused by bandbroadening and dilution of the HPLC effluent by the counter gradient, three weak affinity estrogenic compounds were detected in acid hydrolyzed pomegranate peel extract by applying LC-BCD–MS. The presence of luteolin, quercetin and kaempferol in pomegranate peel was not reported previously in the literature.

4. Experimental

4.1. Chemicals

Coumestrol was purchased from Eastman Kodak (Rochester, USA). Human recombinant β-estrogen receptor (β-ER) was obtained from Panvera Corporation (Madison, USA). Elisa blocking reagent was bought from Boehringer Mannheim (Almere, The Netherlands). Methanol p.a. and acetonitrile p.a. were obtained from Rathburn (Walkerburn, Scotland). Ethylacetate p.a. was bought from J.T. Baker (Deventer, The Netherlands). Ammonium acetate, quercetin dihydrate, luteolin, kaempferol, butylated hydroxyanisole (BHA) and genistein were purchased from Sigma Aldrich B.V. (Zwijndrecht, The Netherlands). PBS tablets were bought from ICN Biomedicals Inc. (Aurora, USA). 0.1 g C18 sep-pak cartridges were purchased from Isolute (Lakewood, CO, USA). C18 ADS restricted access material was kindly donated by Merck (Darmstadt, Germany).

4.2. Apparatus

4.2.1. On-line biochemical detection (LC-BCD)

LC-BCD–MS analysis of the pomegranate extract was performed on a Kiadis HRS 340B-screening instrument (Leiden, The Netherlands). In total, seven Knauer K-500 LC-pumps (Berlin, Germany) were used to deliver respectively the BioGradient (Schobel et al., 2001), HPLC column regeneration, β-ER and coumestrol solutions. The β-ER and coumestrol solutions were transferred in 10 ml Pharmacia BioTech superloop cartridges (Uppsala, Sweden) and were connected to the outlet of Knauer LC-pumps. The superloops were kept at 4 °C. The biochemical solutions, β-ER (100 nM) and coumestrol (500 nM), were added to the continuous-flow bioassay at a flow rate of 25 μl/min. Both solutions were prepared in phosphate buffered saline (PBS), which contained 0.4 g/l of blocking reagent. HPLC separations were performed on a 100×3.2 mm stainless steel Phenomenex® column packed with PRODIGY™ ODS(3) 5 μm, 100 Å particles. Pomegranate extracts were analyzed using a 7 min 20–35% acetonitrile gradient, followed by an 8 min post-gradient. The BioGradient setup consisted of four Knauer K-500 LC-pumps. Two LC-pumps delivered the solvent gradient, whereas the remaining pumps were positioned behind the analytical column and delivered makeup solutions. The effluent flow rate of the BioGradient was set at 1.2 ml/min and consisted of 10% acetonitrile concentration. The maximum flow rate over the HPLC column was set at 0.5 ml/min. The injection volume equalled 50 μl. After each analysis the analytical column was regenerated with 90% acetonitrile for 15 min and equilibrated at the chromatographic starting conditions for 5 min. The BioGradient effluent was split in a 1:24 ratio; 50 μl/min were directed towards the on-line biochemical assay, whereas the remaining 1150 μl/min were introduced into a ThermoFinnigan DECA MS detector (San Jose, CA, USA).

Fluorescence detection was performed with an Agilent HP1100 series (Waldbronn, Germany) fluorescence detector (excitation wavelength 340 nm and emission wavelength 410 nm). Liquid handling was performed with a Cavro MSP9500 autosampler (Sunnyvale, CA, USA) equipped with a Valco stainless steel six-port injection valve (Schenkon, Switzerland). Knitted polytetrafluorethylene (PTFE), 0.5 mm i.d., open tubular coils were used to allow the biochemical reagents and sample to interact for certain periods of time.

4.2.2. LC-BCD coupled to mass spectrometry (LC-BCD–MS)

After chromatographic separation, 1150 μl/min of the BioGradient effluent were introduced into a ThermoFinnigan DECA MS detector equipped with an APCI interface. The vaporizer and capillary temperature were set at 450 and 250 °C. The MS detector was operated in negative ion MS/MS data dependent scan mode. During full scan mode, ion intensities were measured over an 80–1000 m/z range. The m/z isolation window for the parent mass during MS/MS measurements was set at 1.5. The MS/MS activation amplitude equaled 60%. Intense background ions were specified up front and were ignored automatically during data dependent MS/MS analysis. The collection of MS and biochemical data was synchronized by contact closures, which were programmed in the control software.

4.3. On-line characterization screening

4.3.1. Description bioassay format

The bioassay format used was similar to those reported previously with a few modifications (Fig. 1) (Oosterkamp et al., 1996). In brief, 50 μl/min of the BioGradient effluent were first mixed with 25 μl/min of β-ER. The mixture was allowed to interact for a period of 40 s. Subsequently, the remaining binding sites on β-ER, were titrated by adding coumestrol, a natural autofluorescent ligand, to the reaction mixture at 25 μl/min. Similarly, the mixture was allowed to react for 40 s after which the extent of fluorescence enhancement, caused by the affinity interaction between coumestrol and β-ER, was monitored continuously at excitation and emission wavelengths of 340 and 410 nm, respectively. Depending on concentration and potency, estrogenic compounds reduce the amount of affinity complexes formed between coumestrol and β-ER. Consequently, as the fluorescence intensity is decreased temporarily, the presence of estrogenic activity in a sample is observed as a negative peak in the biochemical detection readout.

Although the homogeneous bioassay format described above does not require the separation of β-ER bound and unbound coumestrol, a 2×5 mm C18 ADS restricted access column was inserted in the biochemical assay. This way, background fluorescence, originating from autofluorescent compounds in the sample and unbound coumestrol, was reduced dramatically and enhanced bioassay sensitivity significantly.

4.3.2. Dereplication of bioactive compounds

For identification purposes a MS database was created, which contained negative ion APCI MS and MS/MS data of a large number of known, commercially available estrogenic compounds, e.g. steroids, mycoestrogens and phytoestrogens. As a first step towards identification, the MS and MS/MS data of the detected bioactive compounds was used as a database search query. Molecules, which showed matching molecular weight and MS/MS fingerprint, were analyzed by LC-BCD–MS. The retention times of these reference compounds were compared with those of the estrogenic compounds in the pomegranate extract. This way, based on matching retention times and MS and MS/MS data, known bioactive compounds were identified rapidly.

Alternatively however, automated fraction collection was enabled in order to support the isolation of bioactive molecules not known to the MS library. This way, additional chemical data like DAD, IR, MS^n and NMR spectra could be obtained to support further structure elucidation, if required.

4.4. Preparation of pomegranate sample

4.4.1. Extraction of aqueous pomegranate solution

Pomegranate (*Punica granatum*) pericarps (peels) were extracted by boiling in water for 45 min, decanting the liquor and concentrating over an open flame to 10% of its original volume (Justesen et al., 1998). This concentrated aqueous pomegranate pericarp extract was combined with two times its volume of ethylacetate, shaken vigorously and allowed to stand for 8 h. After extraction the ethylacetate phase was separated from the aqueous phase and evaporated under nitrogen at 40 °C. The polyphenolic compounds were resuspended in a small volume of methanol.

4.4.2. Acid hydrolysis of pomegranate extract

Acid hydrolysis of aqueous pomegranate pericarp extract was performed as described previously, with only a few modifications (Rehwald et al., 1994). In short, 8 ml of 62.5% aqueous methanol (containing 2 g/l BHA) were added to 3 ml of tenfold diluted pomegranate extract. Subsequently, 2 ml of 6 M HCl were added to the solution. The reaction mixture was refluxed for 2 h at 90 °C, after which the solution was allowed to cool in the refrigerator. The solution was diluted ten times with deionized water. Aliquots of 10 ml were passed over 0.1 g C18 Sep-pak cartridges and subsequently flushed with 5 ml of 2 mM ammonium acetate. Trapped compounds were eluted with 3 ml of methanol. The methanol fractions were pooled and evaporated under nitrogen. After evaporation, the remaining pellet was resuspended in a small volume of methanol.

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