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Research Article

# Analysis of isoflavones in soybeans employing direct analysis in real-time ionization–high-resolution mass spectrometry

A direct analysis in real-time (DART) ion source coupled to a high-resolution orbitrap mass spectrometer was used for the quantitative analysis of isoflavones isolated from soybeans. For the isolation of genistein, daidzein, glycitein, and their respective acetyl, malonyl, and glucoside forms, an extraction employing 80% aqueous MeOH enhanced by sonication was used. As far as the total isoflavones (expressed as aglycones) were to be determined, an acid hydrolysis with 80% aqueous EtOH and refluxing had to be employed, while in the latter case a good agreement of the results with the data generated by the UHPLC-orbitrap MS method was achieved, in the case of the analysis of non-hydrolyzed extracts, some overestimation of the results as compared with those generated by UHPLC-orbitrap MS was observed. A careful investigation of this phenomenon showed that the free aglycones originated from the conjugated forms of isoflavones in the DART ion source, thus contributing significantly to the ''free'' genistein/daidzein/glycitein signals during the DART analysis. Good recoveries (95–102%) and repeatabilities (RSD: 7–15%) were obtained at the spiking levels of 0.5, 1, and 0.05 g/kg, for daidzein, genistein, and glycitein, respectively. The limits of detection estimated for the respective analytes were 5 mg/kg.

**Keywords:** Ambient mass spectrometry / Direct analysis in real time / Isoflavones / Phytoestrogens / Soybean
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## 1 Introduction

Isoflavones are phytochemicals occurring in a variety of plants including soybeans [1]. On the basis of the structural similarity to the endogenous estrogen 17β-estrodiol, isoflavones have been implicated in potential health benefits related to cancer risk-lowering effect [2, 3], heart disease prevention [4], bone mass density increase to prevent osteoporosis [5], and the reduction of postmenopausal syndromes in women [6]. Owing to the purported beneficial effects of soy isoflavones, the use of soy products has become popular during the recent years [1].

Soybean may contain three free isoflavones: daidzein, genistein, and glycitein, which are also present as aglycones in three conjugated forms: (i) β-glucosides – daidzin, genistin, and glycitin; (ii) acetyl-β-glucosides – 6″-*O*-acetyl-β-daidzin, 6″-*O*-acetyl-β-genistin, and 6″-*O*-acetyl-β-glycitin; and (iii) 6″-*O*-malonyl-β-glucosides – 6″-*O*-malonyl-β-daidzin, 6″-*O*-malonyl-β-genistin, and 6″-*O*-malonyl-β-glycitin [7].

Several analytical approaches can be used for the analysis of isoflavones present in soybeans and soy-based foods. In particular, instrumental techniques such as gas chromatography (GC), liquid chromatography (LC), capillary electrophoresis (EC), and bioanalytical ones represented by immunoassay have been employed. Considering the frequency of their use, the methods employing liquid chromatography (HPLC) are dominating, obviously because good performance characteristics can be obtained for measurements of all isoflavone chemical forms [1, 8]. In older studies, the detectors coupled to HPLC were typically UV or UV-diode array (DAD), in the most recent years, mainly mass spectrometric (MS) detection employing for target analytes either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) has become common [8–12]. It should be noted that while the use of HPLC with conventional detection requires typically a purification step (e.g. solid-phase extraction, SPE) of crude extracts and/or careful optimization of HPLC separation to avoid co-elutions of target analytes with matrix co-extracts, some degree of simplification of sample preparation procedure is enabled using MS detection [8].

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**Abbreviations: DART**, direct analysis in real-time; **TPP**, triphenyl phosphate

 

In the recent years, a large number of novel ambient desorption ionization techniques, such as desorption electrospray ionization (DESI), atmospheric-pressure solids analysis probe (ASAP), direct analysis in real-time (DART), and many others have become available [13–15]. Their main advantages, compared with up-to-date conventional techniques (GC-MS, LC-MS), involve the possibility of direct sample examination in the open atmosphere, minimal or no sample preparation requirements, and remarkably high sample throughput. DART, which was investigated in this study, represents one of the APCI-related techniques employing a glow discharge for the ionization. Metastable helium atoms, originating in the plasma, react with ambient water, oxygen, or other atmospheric components to produce the reactive ionizing species [14]. The DART ion source was shown to be efficient for soft ionization of a wide range of both polar and non-polar compounds. Until now, several papers have been published describing various DART applications including rapid analysis of various substances occurring in foodstuffs and food crops [16–21].

In this study, the challenge to develop a rapid method for the specific determination of individual isoflavones in soybeans based on the use of ambient mass spectrometry has been addressed. A DART ion source coupled to a high-resolution orbitrap mass spectrometer has been investigated for their analysis.

## 2 Materials and methods

### 2.1 Chemicals and reagents

Standards (purity >98%) of target isoflavones daidzein, genistein, glycitein, daidzin, genistin, and glycitin together with triphenyl phosphate (TPP) used as an internal standard, were obtained from Dr. Ehrenstorfer (Augsburg, Germany) and Sigma-Aldrich (Taufkirchen, Germany). Working standard solutions were prepared in methanol (0.1 mg/mL).

Methanol (HPLC-grade) was supplied by Merck (Darmstadt, Germany). Ethanol (96%, v/v, p.a.), HCl (36%, p.a.), and NaOH (p.a.) were purchased from Lach-ner (Neratovice, Czech Republic). Water used as the components of mobile phase was purified with the use of a Milli-Q system (Millipore, Bedford, MA, USA).

Soybeans were purchased from retail market.

### 2.2 Sample preparation

#### 2.2.1 Analysis of free forms of isoflavones in soybeans

One gram of homogenized sample was placed into a 15 mL tube. Ten milliliters of 80% MeOH were added. The sample was sonicated for 30 min and centrifuged (10 000 rpm, 3 min). The supernatant was decanted into a 25-mL volumetric flask and the sample was extracted twice more with 10 and 5 mL of 80% MeOH. The volume was made up with 80% MeOH. A volume of 500 μL of the extract was mixed with 20 μL of the internal standard solution (5 μg/mL) and 480 μL of MeOH. The resulting content of the matrix was 20 mg/mL.

#### 2.2.2 Analysis of total isoflavones (as aglycone forms) in soybeans after acid hydrolysis

Homogenized sample (1 g) was mixed with 10 mL of hydrochloric acid (6 mol/L) and 40 mL of 96% v/v ethanol. After being heated for 4 h under reflux, the reaction mixture was transferred into a 50-mL volumetric flask. Two milliliters of the (acidic) extract were neutralized using 6 M NaOH up to pH ≈ 6. The volume (5 mL) was made up with 80% EtOH. Prior to the instrumental analysis, 40 μL of this extract were mixed with 20 μL of the internal standard solution (TPP, 5 μg/mL) and 940 μL of MeOH. Under this setup, the equivalent of matrix was 0.32 mg/mL.

### 2.3 DART-orbitrap MS analysis

The DART-orbitrap MS system consisted of a DART ion source (model DART-SVP) with a 12 Dip-It tip scanner autosampler (IonSense, Saugus, MA, USA) coupled to an Exactive high-resolution orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). A Vapur interface (IonSense, Saugus, MA, USA) was employed to hyphenate the ion source and the mass spectrometer. Low vacuum in the interface chamber was maintained by a diaphragm pump MZ 2 NT (Vacuubrand, Wertheim, Germany).

The DART ion source was operated in a positive-ion mode with helium as the ionizing medium at a pressure of 5.5 bar. The gas beam was heated to 550°C and the grid electrode voltage was set to +350 V. The parameters of the mass spectrometer were as follows: capillary voltage: +60 V; tube lens voltage: +120 V; skimmer voltage: +21 V; capillary temperature: 250°C. The acquisition rate was set to 2 spectra/s with corresponding mass resolving power of 50 000 FWHM ($m/z$ 200). A constant speed of 0.5 mm/s was used for the Dip-It tip scanner autosampler.

XCALIBUR software (v. 2.1) was used for instrument control, data acquisition, and data processing. Mass spectral data were obtained by averaging the mass spectra recorded during the exposure of the sample to the DART gas beam followed by the subtraction of background ions.

### 2.4 UHPLC-orbitrap MS analysis

The sample components were separated using an Accela UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) and a Hypersil Gold analytical column (100 mm × 2.1 mm id, 1.9 μm; Thermo, USA) held at 40°C. As the

 

mobile phase, 0.1% acetic acid in water (A) and 0.1% acetic acid in MeOH (B) were used. The gradient was as follows: start with 10% B, linear increase to 100% B in 7 min, keep up to 10 min, switching to 10% B in 10.1 min, and column equilibration for 3 min before the next injection start. The flow rate was 300 μL/min for electrospray ionization (ESI). The injection volume was 1 μL; the partial loop was used as an injection technique.

Optimal conditions for the heated electrospray interface (HESI-II, Thermo Fisher Scientific) were as follows: sheath gas/aux gas: 35/10 arbitrary units; capillary temperature: 250°C; heater temperature: 250°C; capillary voltage: +60 V/−60 V; tube lens voltage: +120 V/−120 V; skimmer voltage: +21 V/−21 V; and spray voltage +4 kV/−4 kV.

The system was operated in the full spectral acquisition mode in the mass range of $m/z$ 50–1000 at resolving power settings of 50 000 FWHM at an acquisition rate 2 spectra/s in the positive/negative ionization mode (switching). The external mass axis calibration without the use of the specific lock mass was employed.

## 3 Results and discussion

### 3.1 Optimization of DART-orbitrap MS parameters

In the first part of our experiments, the relationship between the setting of various DART operating parameters and the features of mass spectra generated under particular conditions was investigated. In general, helium beam temperature, flow rate, and desorption time are typically the major parameters affecting DART ion formation and effectiveness of their transmission into MS [16].

Protonated molecules $[M+H]^+$ were obtained under conditions of positive DART ionization when analyzing standards of target analytes (i.e. daidzein, genistein, and glycitein) dissolved in methanol. On the other hand, deprotonated molecules $[M-H]^-$ were recorded under conditions of negative DART ionization. Although the intensities of the ions were slightly higher in the negative-ion mode, in the final method, the positive ion mode was selected since the internal standard (TPP) used for quantification purpose was not ionizable under DART(−) conditions.

The impact of helium gas beam temperature was monitored for temperatures 250, 350, 450, and 550°C, the last of which provided the highest responses for all analytes tested. As regard helium flow rates, a constant pressure of 5.5 bar is recommended for the DART-SVP model, thus this value was kept during all experiments.

Another important factor optimized was the (thermal) desorption time. In this study, a 12 Dip-It tip scanner autosampler was used for sample introduction. This autosampler scans the glass-rod surfaces with samples through the DART gas stream at a constant speed. Within our experiments, a constant speed of 0.5 mm/s was found optimal providing the MS peak width of 5 s.

### 3.2 Analysis of soybean extracts

Once the DART parameters were optimized, the detection of isoflavones in soybean extracts prepared in two different ways was investigated. In the first extraction procedure, enhanced by sonication, 80% aqueous MeOH was employed. This approach allows the isolation of genistein, daidzein, glycitein, and their respective acetyl, malonyl, and glucoside (i.e. conjugate) forms [8, 22]. In the second case, the sample was refluxed with 80% aqueous EtOH into which hydrochloric acid was added. Under these conditions, acetyl, malonyl, and glucoside forms were hydrolyzed yielding their respective aglycones; thus, this extraction method allowed the determination of the total isoflavones expressed as aglycones [8, 22].

It should be noted that for the quantitative analysis using a DART ion source, it is recommended to use an internal standard to compensate variation of the ion intensities of analytes [16]. TPP, yielding $[M+H]^+$ at $m/z$ 327.0781, was used for this purpose. With regards to impossibility to get an isoflavones-blank soybean sample for preparation of the matrix-matched standard, we decided to use the standard addition method for quantification. In addition to this approach, a calibration using the standards in pure solvent was also evaluated. Finally, the use of isotopically labeled internal standards (currently available as $d_4$-genistein, $d_4$-daidzein) can also be an effective way to quantify, as well as to compensate for the losses of analytes during the extraction step. The application of these labeled

**Table 1.** Comparison of the results (mean ± standard deviation) generated by DART(+)-orbitrap MS and UHPLC-ESI(+)-orbitrap MS

| Analyte | Content (mg/kg) | | | |
|---|---|---|---|---|
| | Extraction A ($n=6$)[a] | | Extraction B ($n=6$)[b] | |
| | DART(+)-orbitrap MS | UHPLC-ESI(+)-orbitrap MS | DART(+)-orbitrap MS | UHPLC-ESI(+)-orbitrap MS |
| Genistein | 230 ± 46 | 12.6 ± 0.7 | 362 ± 80 | 320 ± 21 |
| Daidzein | 743 ± 133 | 21.9 ± 1.2 | 1123 ± 230 | 1030 ± 82 |
| Glycitein | 21.7 ± 5.2 | 2.9 ± 0.2 | 44 ± 11 | 37 ± 4 |

a) Extraction with 80% aqueous MeOH enhanced by sonication.
b) Extraction with 80% aqueous EtOH containing HCl (acid hydrolysis) and refluxing.

 

standards does not represent an ideal solution since in addition to a very high cost (20 € versus 3€, respectively, per 1 mg), an isotopic exchange (D–H) may occur, thus biasing the quantitative data.

The quantification results generated by DART(+)-orbitrap MS were also compared with a ''conventional'' technique represented by UHPLC-ESI(+)-orbitrap MS (Table 1). No statistical differences ($p = 0.05$) were obtained for these two techniques when analyzing the hydrolyzed extracts obtained by the second extraction procedure (80% aqueous EtOH containing HCl and refluxing). However, rather surprisingly, the data obtained by DART(+)-orbitrap MS when analyzing the first type of extracts (80% aqueous MeOH enhanced by sonication) were largely overestimated as compared with those generated by UHPLC-ESI(+)-orbitrap MS (Table 1). Since orbitrap MS represents a full mass spectra acquisition detector, retrospective data mining is possible with this instrumentation. Based on the exact mass measurement, we attempted at the identification of (non-targeted) acetyl, malonyl, and glucoside forms of the particular isoflavones.

**Table 2.** Exact masses (*m/z*) for particular aglycones and the acetyl, malonyl, and glucoside forms

| Analyte | Elemental composition | $[M+H]^+$ |
|---|---|---|
| Daidzein | $C_{15}H_{10}O_4$ | 255.0652 |
| Daidzin | $C_{21}H_{20}O_9$ | 417.1180 |
| Acetyl-daidzin | $C_{23}H_{22}O_{10}$ | 459.1285 |
| Malonyl-daidzin | $C_{24}H_{22}O_{12}$ | 503.1184 |
| Genistein | $C_{15}H_{10}O_5$ | 271.0601 |
| Genistin | $C_{21}H_{20}O_{10}$ | 433.1129 |
| Acetyl-genistin | $C_{23}H_{22}O_{11}$ | 475.1235 |
| Malonyl-genistin | $C_{24}H_{22}O_{13}$ | 519.1133 |
| Glycitein | $C_{16}H_{12}O_5$ | 285.0758 |
| Glycitin | $C_{22}H_{22}O_{10}$ | 447.1286 |
| Acetyl-glycitin | $C_{24}H_{24}O_{11}$ | 489.1391 |
| Malonyl-glycitin | $C_{25}H_{24}O_{13}$ | 533.1290 |

Figure 1 in the upper part shows the UHPLC-ESI(+)-orbitrap MS chromatograms of the exact masses corresponding to daidzein (*m/z* 255.0652), genistein (*m/z* 271.0601), and glycitein (*m/z* 285.0758). These chromatograms contained not only signals corresponding to the protonated molecules of particular aglycones ($t_R$ 5.49, 5.94, and 5.58 min, respectively), but also other signals at lower retention times as compared with those of aglycones. When extracting the exact masses for particular acetyl, malonyl, and glucoside forms (see Table 2), we observed the signals of their protonated molecules at the same retention times as those of the earlier eluting compounds. Based on these observations, we hypothesized that the overestimation of DART data was due to the fragmentation of the conjugate forms in the ion source. Since DART does not use a separation step prior the detection, the ion *m/z* 255.0652 might represent, in fact, a sum of daidzein (aglycone) as well as fragments of daidzein-conjugate forms. Similarly, *m/z* 271.0601 might constitute genistein (aglycone) and fragments of genistein-conjugate forms. To confirm our hypothesis, we analyzed using DART-(+)-orbitrap MS pure standards of particular glucosides (daidzin, genistin, and glycitin).

As Fig. 2 shows, the extent of fragmentation of glucosides was quite high. The ratios of $[M+H-glucosyl]^+$ and $[M+H]^+$ ions were 1:0.33, 1:0.35, and 1:0.07 for daidzin,

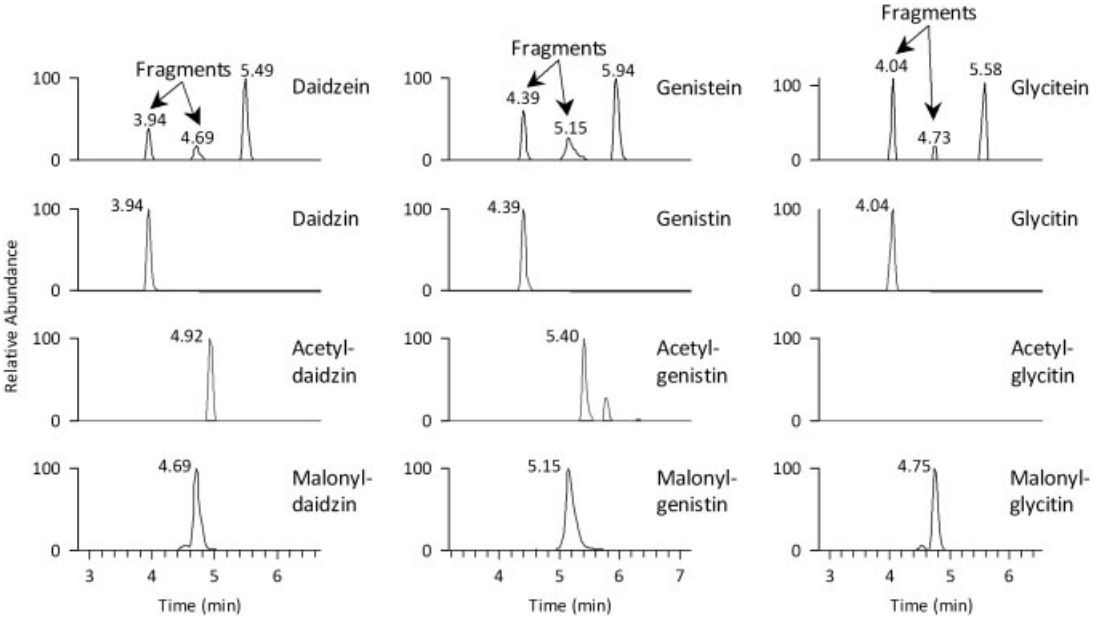


**Figure 1.** UHPLC-ESI(+)-orbitrap MS chromatogram documenting the presence of conjugate forms of isoflavones in soybean extract (without acid hydrolysis) and their fragmentation yielding ions corresponding to aglycones. The exact masses (*m/z*) for particular acetyl, malonyl, and glucoside forms and aglycones are summarized in Table 2.

 

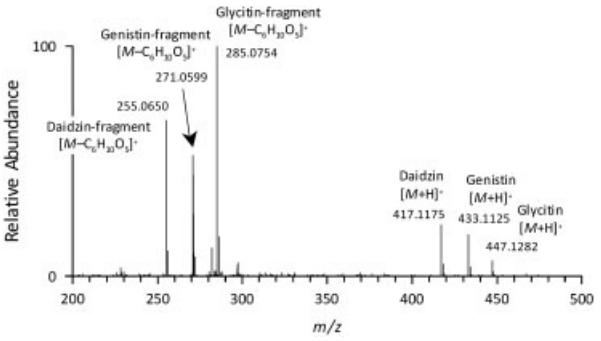


**Figure 2.** Fragmentation of glucosides (daidzin, genistin, glycitin) during DART ionization. The protonated molecules $[M+H]^+$ of particular glucosides fragmented to $[M+H-glucosyl]^+$ ions.

genistin, and glycitin, respectively. Thus the generated fragments $[M+H-glucosyl]^+$ contributed significantly to the signal of ''free'' aglycones. The fragmentation of conjugate forms (in a similar extent) was also observed by Wu et al. [12], who used LC-ESI(+)-quadrupole MS, on the other hand, almost no fragmentation was observed in a study conducted by Griffith and Collison with LC-ESI(+)-ion trap MS [9]. Employing LC-ESI(+)-orbitrap MS in this study, we observed quite low extension of fragmentation (5–9%) of glucoside forms as compared with DART(+)-orbitrap MS. The fragments formed during the DART ionization can be, therefore, explained as a result of high temperature during the desorption process. However, a high temperature was needed to obtain acceptable sensitivity during the analysis. In addition, even at the lowest desorption temperature tested (250°C) during the DART ionization, the extent of fragmentation was still high (ratios of $[M+H-glucosyl]^+$ and $[M+H]^+$ ions of all three glucosides tested were in the range of 1:(0.8–0.4)).

In any case, one should be aware that conjugate forms represent the major source of isoflavones in soybeans [1], thus their potential fragments (during DART-MS analysis) can significantly influence the generated data. Fortunately, rather than the content of ''free'' aglycones, the amount of ''total'' aglycones (determined either as a sum of ''free'' aglycones and conjugate forms multiplied by particular aglycone molecular equivalents, or as a sum of all aglycones obtained during acid hydrolysis) provides more informative data, mainly for calculation of estimated daily intake.

For the extraction accompanied by hydrolysis (80% aqueous EtOH with added HCl), the recoveries 95, 97, and 102%, and RSDs of 10, 7, and 15%, were achieved for genistein, daidzein, and glycitein, respectively, spiked at level 0.5, 1, and 0.05 g/kg, respectively. (*Note:* The natural content of genistein, daidzein, and glycitein (''total'' isoflavones) in the analyzed soybean sample was 0.36, 1.1, and 0.044 g/kg, respectively.)

Since the content of matrix in the examined sample extracts was quite low (0.32 mg/mL), we did not observe matrix effects (signal suppression/enhancement) during the

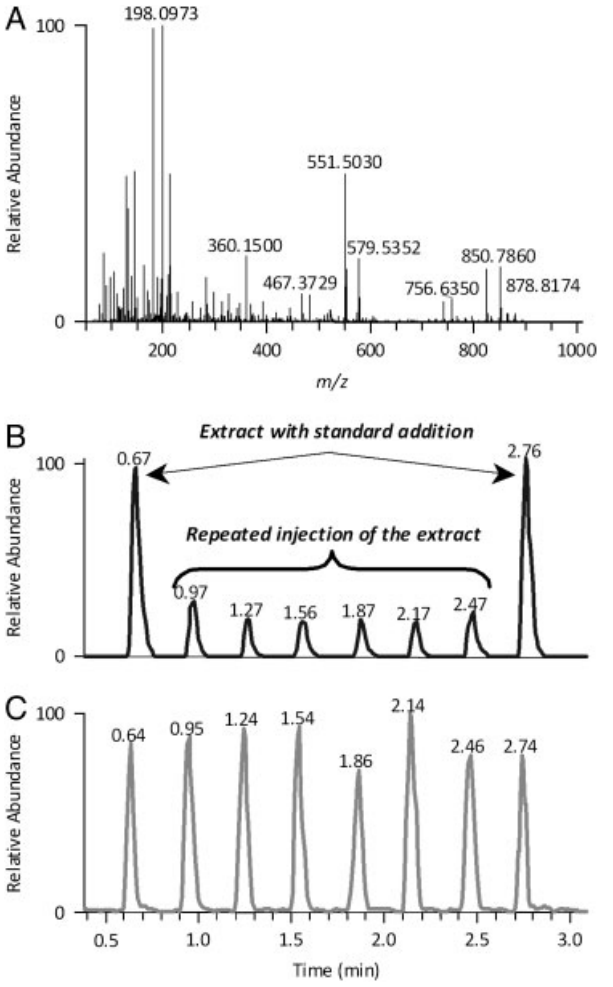


**Figure 3.** (A) Mass spectrum (*m/z* 50–1000) of a soybean extract (after acid hydrolysis) analyzed by DART(+)-orbitrap MS; (B) daidzein (*m/z* 255.0652) in soybean extracts at a level of 1.13 g/kg and the same extract with standard addition (+1.38 g/kg); (C) TPP (IS) (*m/z* 327.0781) at a concentration of 100 ng/mL.

DART(+)-orbitrap MS analysis; thus, the calibration using standards in pure solvent can be used as a more effective way for quantification of target analytes. The limits of detection were 5 mg/kg for all aglycones. These values were determined by analyzing the standards in pure solvent; in general, the MS peaks appeared if the detector signal was $>2\times10^3$, which represents a value just above the noise. Also, at least three data points per MS peak were required. Figure 3 illustrates on the example of daidzein (*m/z* 255.0652) the repeated injection of soybean extracts and extract with standard addition. To document the fluctuation of signal intensity, the internal standard TPP (*m/z* 327.0781) is also shown. The standard addition method provided linearity for target analytes up to 3 g/kg (corresponding to 960 ng/mL), which is sufficient for quantification purposes, since the content of ''total'' isoflavones expressed as aglycones in soybeans is typically below this level [1].

 

## 4 Concluding remarks

Ambient mass spectrometry employing DART(+)-orbitrap MS was used for quantitative analysis of isoflavones isolated from soybeans. A good agreement of DART(+)-orbitrap MS results with the data generated by UHPLC-ESI(+)-orbitrap MS method was achieved for the analysis of the extracts prepared by hydrolysis with 80% aqueous EtOH containing HCl and refluxing. On the other hand, the analysis of non-hydrolyzed extracts (extraction employing 80% aqueous MeOH enhanced by sonication) led to the overestimation of the DART(+)-orbitrap MS results for ''free'' isoflavones. This phenomenon could be explained as a result of the fragmentation of the conjugate forms into the aglycones during ionization, which thus contribute significantly to the signal of ''free'' aglycones during the DART analysis. In any case, the developed DART(+)-orbitrap MS method can be used for the rapid analysis (no chromatographic separation) of the amount of ''total'' aglycones after acid hydrolysis of the sample.

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*Mention of brand or firm names in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the Institute of Chemical Technology, Prague.*

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