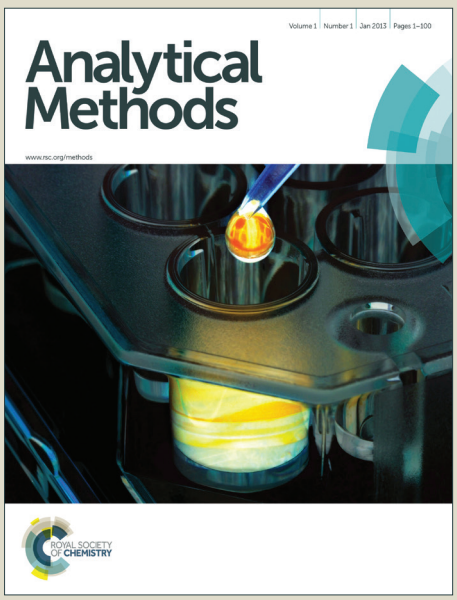
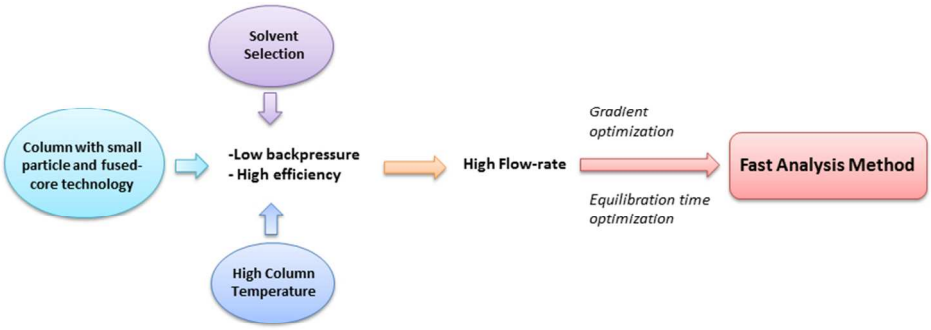
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods



254x190mm (96 x 96 DPI)

Fast analysis of β-ecdysone in Brazilian ginseng (*Pfaffia glomerata*) extracts by high-performance liquid chromatography using a fused-core column

Mauricio A. Rostagno[a]*, Isabel C. N. Debien[a], Renata Vardanega[a], Gislaine C. Nogueira[a], Gerardo F. Barbero[b], M. Angela A. Meireles[a]

[a]LASEFI/DEA/FEA (School of Food Engineering)/UNICAMP (University of Campinas), Rua Monteiro Lobato, 80, 13083-862 Campinas, São Paulo, Brazil

[b]Analytical Chemistry Department, Faculty of Sciences, University of Cadiz, Agrifood Campus of International Excellence (CeiA3), P.O. Box 40, 11510 Puerto Real, Cádiz, Spain

Correspondence: Dr. Mauricio Ariel Rostagno, LASEFI/DEA/FEA (School of Food Engineering)/UNICAMP (University of Campinas), Rua Monteiro Lobato, 80, 13083-862 Campinas, São Paulo, Brazil

E-mail: rostagno@fea.unicamp.br/

Tel: +55 19 35210100; Fax: +55 19 35214027

Abstract

The recent development of fused-core technology in HPLC columns is enabling faster and highly efficient separations. This technology was evaluated for the development of a fast analysis method for β-ecdysone in extracts of *Pfaffia glomerata*. A step-by-step strategy was used to optimize temperature (30-55 ºC), flow rate (1.0-2.0 mL min^{-1}), mobile phase composition (mixtures of water and methanol or acetonitrile) and equilibration time (1-5 min). A gradient method has been developed using two solvents: 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile. Optimized conditions provided a method for the separation of β-ecdysone in approximately 2 min with a total analysis time (sample-to-sample) of 9 min, including the return to initial conditions and the re-equilibration of the column. Evaluation of chromatographic performance revealed excellent intraday and interday reproducibility (> 99.5%), resolution (2.78), selectivity (1.13), peak symmetry (1.09) while presenting low limits of detection (0.20 $mg.L^{-1}$) and quantitation (0.67 $mg.L^{-1}$). The robustness of the method has also been calculated according to the concentration / dilution of the sample. Several sample solvent were evaluated and the best chromatographic results were obtained using methanol 80% in water. Finally, the developed method was validated with different extracts of *Pfaffia glomerata* samples.

Keywords: Phytoecdysones, β-ecdysone, Analysis, HPLC, Fused-core columns

1. Introduction

Ecdysteroids are steroid hormones that were first found in insects and later identified in over 120 plant families, in which their magnitude is higher [1]. The ecdysteroids produced by insects are known as zooecdysteroids (ZEs) and those produced from plants are known as phytoecdysteroids (PEs). Plants and insects rarely have the same ecdysteroids and thus, they play different functions in each one. It is known that ZEs are present at all stages of insect development, regulating many biochemical and physiological process, whereas in plants the function of PEs is still unknown. However, their presumed function is to contribute to the deterrence of invertebrate predators and as a source of polihydroxylated phytoesterols for cell growth and proliferation. In addition, PEs are apparently non-toxic to mammals and may have a number of beneficial pharmacological and medicinal applications [2-4]. Among the PEs, β-ecdysone (Figure 1), is actually recognized as the major biologically active ecdysteroid in most invertebrate systems [2].

In 1967, Takemoto et al. firstly found β-ecdysone in plants from the roots of *Achyranthes fauriei* [5]. More recently, β-ecdysone was isolated in roots of *Pfaffia glomerata* (Amaranthaceae), a traditional Brazilian medicinal plant [6]. This plant has been used to substitute the one known as "Asian" ginseng (*Panax ginseng* – Araliaceae). Due to the similar morphology of its roots to the Asian ginseng, they are popularly known as Brazilian ginseng [7]. However, the "Asian" and the "Brazilian" ginseng have different chemical composition and Ecdysteroids are present only in the later genus. Several pharmacological and medicinal studies suggested that *P. glomerata* extracts have potential analgesic and anti-inflammatory [8], gastroprotective [9], antinociceptive [10], anti-glycemic [11] and anti-microbial [12] properties and that it may also act as a melanogenisis inhibitor [13] and as a central nervous system depressant [14]. In fact, some pharmaceutical companies produce phytopharmaceuticals containing *P. glomerata* micronized roots and/or extracts reporting β-ecdysone as main active compound.

Without doubt, it is important to have reliable and robust analysis methods in order to allow the correct quantitation of the concentration of β-ecdysone in plants and derived products for the assessment of their biological role. Analysis of phytoecdysones can be achieved by high-performance liquid chromatography in normal phase using solvents like ethanol and dichloromethane on a diol stationary phase or in reversed-phase using mixture of water with

methanol or acetonitrile on a C_{18} column. Usually, column temperature is maintained slightly above room temperature and low flow-rates are used due to the high backpressure of the column. Conventional particle columns (250 x 4.6 mm; 5µm) have been used for the separation and analysis of PEs from a variety of samples, including Asparagus, Achyranthes root (*Radix achyranthis bidentatae*), Spanish catchfly (*Silene otites*) and Brazilian ginseng (*Pfaffia glomerata*), among others. Most methods require between 3 minutes to 18 minutes for the separation of β-ecdysone but also require the cleaning and conditioning of the column before the next injection [1, 7, 15-18].

The technology of HPLC stationary phases have seen a constant development through the last decades, leading to new chemistries and types of packing materials. The reduction of particle size can greatly improve chromatographic separations but the size of the particles reaches the limits of conventional HPLC systems around 3-5 µm. Therefore, to explore smaller particles in the stationary phase, systems capable of operating at higher pressures are needed. Recently, a new type of particle for HPLC separations was developed which allows using smaller particles while operating at normal HPLC pressures. They are termed fused-core particles, and are formed by a solid core. Due to their characteristics, they generate a lower pressure and therefore it is possible to explore smaller particles to improve the performance obtained and speed-up the analysis [19-21].

This technology has been successfully used for the development of fast analysis methods of other potentially bioactive substances. Compared to conventional HPLC methods, methods employing fused-core columns usually achieve reduction of analysis time 3-4 times [22-24].

Clearly, the performance of methods currently being used by researchers can be greatly improved by the use of this new column technology. Unfortunately, this technology has not yet been explored for the analysis of PEs. In this context, the objective of this work was to evaluate the feasibility of using a fused-core column to develop a fast method for the analysis of β-ecdysone in Brazilian ginseng extracts by HPLC.

2. Experimental

2.1. Chemicals and Reagents

Acetic acid (Merck, Darmstadt, Germany), acetonitrile (Scharlab, S. L. Barcelona, Spain) and methanol (Sigma Aldrich, São Paulo, Brazil), and) were HPLC grade. Ultra-pure water was supplied by a Milli-Q Advantage 8 water purifier system (Millipore, Bedford, MA, USA). The reference standard of β-ecdysone (20-hydroxyecdysone; ≥ 93%) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Stock solutions were prepared in 80% aqueous methanol and stored at -32 ºC.

2.2. Samples

P. glomerata roots were cultivated in the experimental field of CPQBA (Campinas, Brazil) and collected on November 17, 2008, being 7 years old. They were washed and dried in a forced air circulation dryer at 40 ºC for 5 days. The dried roots (10.8% moisture) were then comminuted in a pulse mill (Marconi, model MA 340, Piracicaba, Brazil) for few seconds. The remaining particles were milled again, this time using a knife mill (Tecnal, model TE 631, Piracicaba, Brazil) for 2 seconds at 18,000 rpm and finally, they were separated according to their size using sieves (Series Tyler, W.S. Tyler, Wheeling, IL). The milled roots were stored in freezer (Metalfrio, model DA 420, São Paulo, Brazil) at -20 ºC until being used as sample.

A commercial extract of *Pfaffia glomerata* was purchased from a local pharmacy. The extract was compressed as a pill inside a blister package. According to the manufacturer (Herbarium Botanic Laboratory LTDA, Colombo, PR, Brazil) each pill contained 300 mg of dry *P. glomerata* roots extracts containing 0.96% β-ecdysone, which corresponds to 2.88 mg of β-ecdysone in each pill.

2.2.1. Extract of Pfaffia glomerata obtained experimentally

The extract used for the development of the analysis method was obtained by pressurized liquid extraction (PLE) using the apparatus shown in Figure 2. Approximately 10 grams of *P. glomerata* roots with particles of 8 µm of diameter was placed in the 415 mL extraction cell. The empty space of the cell was filled with a Teflon column. The cell containing the sample was heated by a jacket connected to a thermostatic bath, which was set to the extraction temperature

(120°C). The extracting solvent, ethanol 80% (ethanol and water 80:20 v/v)) was pumped by a HPLC pump (Thermoseparation Products, Model ConstaMetric 3200 P/F, FL, USA) into the extraction cell until reach the extraction pressure (10 MPa). After pressurization, the *P. glomerata* roots with pressurized solvent were kept statically at the extraction conditions for 10 minutes. Thereafter, the block valve (Autoclave Engineers, Model 10V2071, Erie, PA, USA) was opened and the pressure was maintained constant by a heated micrometric valve (Autoclave engineers, Model 10VRMM). The flow rate of extracting solvent was fixed in 9.5 mL min^{-1}. The extracts were collected into a glass flask immersed in ice bath at ambient pressure until achieving a S/F (solvent mass/feed mass) of 16 (approximately 20 min). The extracts were filtered through 0.45 µm nylon syringe filter (Jet Biofil, Model FNY-402-030, Kyoto, Japan) and diluted 10 times with methanol 80% before the HPLC analysis

For the comparison of the sample solvent, the protocol used for the extraction of β-ecdysone consisted in the extraction of a single macerated pill with 25 mL of solvent for 30 min. The solvents used were mixtures of methanol, ethanol and acetonitrile and water in three levels; 100% of organic solvent (i.e. pure methanol, pure ethanol and pure acetonitrile); 90% of organic solvent [i.e. 90% methanol and 10% water (v/v); 90% ethanol and 10% water (v/v); 90% acetonitrile and 10% water (v/v)] and 80% of organic solvent [i.e. 80% methanol and 20% water (v/v); 80% ethanol and 20% water (v/v); 80% acetonitrile and 20% water (v/v)]. Extractions were carried out on an ultrasonic bath (frequency 40 kHz, power 135 W) (Unique, model Max Clean 1400, Indaiatuba, SP, Brazil). After the extraction, an aliquot was collected and filtered through 0.45 µm nylon syringe filter (Jet Biofil, Model FNY-402-030, Kyoto, Japan) before the HPLC analysis [25].

2.4. High-performance liquid chromatography

The analyses were carried out on a HPLC system (Waters Corp., Milford, Massachusetts), consisting of a separation module (2695) with integrated column heater and auto-sampler and a photodiode array detector (2998). Separation of compounds was carried out on a fused-core type column (Poroshell 120 EC-C_{18}, 100 × 4.6 mm, 2.7 µm, Agilent Technologies, Little Fall, DE, USA). UV absorbance was monitored from 200 to 400 nm and injection volume was 10 µL. The software for instrument control and data acquisition was Empower 3. Identification of β-ecdysone was achieved by the comparison of retention times and

UV spectra of separated compounds with the authentic standard. Quantification was carried out by integration of the peak areas at 246 nm using the external standardization method. The standard curve was prepared by plotting the concentration (0.1; 0.5; 1; 10; 50; 100 and 200 mg.L-1) against area of the peak. Regression equations and correlation coefficient (r^2) were calculated using Microsoft Excel 2010 software. Detection and quantitation limits (LOD and LOQ, respectively) were determined by considering a value 3 times the deviation of background noise obtained from blank samples (n = 10) dividing by the slope of the calibration curve line and a value 10 times the deviation of background noise obtained for blank samples (n = 10) dividing by the slope of the calibration curve line, respectively [26].

3. Results and discussion

3.1. Selection of conditions

For the initial separation, a series of runs using a linear gradient of solvent B from 0% to 100% in 10 minutes was used. The maximum analysis time was fixed in 10 minutes. For the optimization of the method, different mobile phase composition (mixtures of methanol or acetonitrile and water), temperature (30-55 ºC), flow rate (1.0-2.0 mL min-1) and equilibration time (1-5 min) were tested. Column efficiency was evaluated on basis of retention time, peak width, k prime, selectivity, symmetry factors and width @ baseline and resolution of the critical pair of peaks: β-ecdysone and the unidentified peak eluting near β-ecdysone. The mobile phase selection was based on a previous series of experiments using water (solvent A) and methanol or acetonitrile (solvent B) with different amounts of acetic acid (0-2%) in both solvents (A and B). Using pure acetonitrile, the system pressure was 35.6% lower than when using pure methanol (1.2 mL min^{-1} and 25 ºC). A similar difference in pressure (33.2%) was also obtained by mixing these solvents and water during the gradient, although at different proportions. The highest pressure using mixtures of methanol or acetonitrile and water was obtained with 40% of methanol (4342 psi) and with 20% of acetonitrile (2714 psi). Due to the lower viscosity and the lower backpressure generated, acetonitrile was selected as solvent of mobile phase B. The lower pressure generated will ultimately allow using higher flow rates to reduce analysis time. On the other hand, the amount of acetic acid in the mobile phase also influenced separation and the mobile phase composition. A significant gain in resolution was observed using acidified water

(0.1% acetic acid) (solvent A) and acidified acetonitrile (0.1% acetic acid) (solvent B). Higher amounts of acetic acid in the solvents generated a loss of resolution of chromatographic peaks.

It was observed that increasing temperature of the column the pressure generated was greatly reduced allowing the increment of flow-rate. The gradient duration was adjusted proportionally to the increase in flow-rate. The use of higher temperatures is a useful tool to reduce analysis time since mobile phase viscosity is significantly reduced which in turn decreases the pressure drop across the column allowing higher linear velocities of the mobile phase. In addition, as known by the Strokes-Einstein relationship, the diffusion coefficient is directly proportional to the absolute temperature and inversely proportional to the viscosity. The lower viscosity and higher diffusivity of a mobile phase at high temperatures produce much lower mass transfer resistance, thereby by decreasing the peak width and leading to flatter van Deemter curves. A flatter van Deemter curve allows the use of higher linear velocities without affecting column efficiency [27]. Therefore, by increasing column temperature there is an improvement of analyte resolution through an increased diffusion coefficient of the mobile phase and a lower mass transfer resistance. However, it is also important not to exceed the column maximum operating temperature (60 ºC) since it may significantly reduce expected column life. Therefore 55 ºC was selected as the maximum working temperature. Based on these principles and limitations, column temperature was gradually increased from 30 to 55 ºC, in 5 ºC intervals. Increasing column temperature to 35, 40, 45, 50 and 55 ºC resulted in a mean reduction of retention time of analytes. It was also observed that by increasing the temperature of the column produced a narrowing of the peak width, increased peak height and better resolution in the separation of β-ecdysone.

Once optimum temperature was selected, the reduced column backpressure allowed exploring flow rate in order to shorten analysis time. Consequently, flow rate was step-by-step increased from 1.0 to 2.0 mL min^{-1}. Maximum flow rate was determined by the system pressure's limitation, which was 5000 psi. As flow rate was increased, a proportional reduction of the gradient was applied in order to maintain separation of two peaks. For example, if flow rate was doubled, the gradient time was reduced to half while maintaining the same percentage of solvents of the mobile phase. It was observed that by increasing the flow rate, the analysis time is shortened and the peak width is reduced maintaining an optimum separation of the two

chromatographic peaks. Therefore, shorter analysis times were achieved by using higher temperatures (55 ºC) and flow-rates (2.0 mL min^{-1}).

Total analysis time is the amount of time from injection to injection and includes the run time, column clean-up and re-equilibration time. Re-equilibration time is necessary in gradient HPLC in order to ensure that the column environment has returned to initial stable conditions. This condition is particularly important when using gradient elution since the difference between initial and final organic composition of the mobile phase is significant. The importance of equilibration time is even greater since the failure to optimize re-equilibration time can lead to unnecessary overextension of analysis time, with the increased cost and reduced sample throughput associated. Usually, equilibration time is recommended by manufacturers on basis of the column volume and flow rate. Standard recommendations are approximately 10 times the column volume, although it depends on the applications and more importantly, the mobile phases and gradient used. In this study, all previous sets of experiments were carried out using 5 min between runs, which is equivalent to approximately 42% of the total method duration (including elution, clean-up and re-equilibration times) and equivalent to 23.2 volumes of the column. Therefore, in order to keep this equilibration time as low as possible, and consequently reduce the total method duration, shorter re-equilibration times (1-4 min) were evaluated. Equilibration time was implemented as a delay after the mobile phase composition returned to initial conditions (7.0 min), after which a new sample (methanolic extract) was injected in the column. By using 5 min to re-equilibrate the column between runs provided a mean (n=18; interday) area and retention time variability lower than 0.24 and 0.20% respectively. By reducing the equilibration time to 4, 3, 2 and 1 min resulted on a mean area variability lower than 0.20, 0.29, 0.43 and 0.81%, and mean retention time variability lower than 0.23, 0.26, 0.37 and 0.53%, respectively. Although the use of very short re-equilibration times variability was within the normal range, a slight higher reproducibility for the analysis of β-ecdysone was observed by using equilibration times higher than 2 min, and therefore 2 minutes can be considered as the most appropriate re-equilibration time in order to achieve the highest possible reproducibility while not over extending total run time. This equilibration time is equivalent to 9.3 times the column´s volume and slightly lower than recommended.

To sum up, the gradient was optimized for the separation at 55 ºC, a flow rate of 2.0 mL min^{-1} and 2 minutes of re-equilibration time, achieving the best balance between analysis time and separation of the critical pair of peaks (β-ecdysone and the unidentified peak eluting near β-ecdysone). According to these results and after developing several trial-and-error experiments to optimize the gradient, best separation of these two peaks was achieved in approximately 2 min. Best separation gradient profile was 0 min, 5% B; 0.5 min, 10% B; 2.0 min, 12.5% B; 3.0 min, 15% B; 4.0 min, 80% B; 5.0 min; 100% B; 6.0 min, 100% B; 7.0 min, 5% B. The gradient includes 1 min at 100% of mobile phase B for column clean-up (5.0-6.0 min) and 1 min to return to initial conditions (6.0-7.0 min).

3.2. Characteristics of the method

A representative chromatogram of the methanolic extract is presented in Figure 3 and the chromatographic properties of the developed method are reported in Table 1. By applying the developed method, retention time of β-ecdysone and the unidentified peak eluting near β-ecdysone were 2.01 and 2.11 min respectively. The method is completed in 5 minutes and total analysis time (sample-to-sample) is 9 minutes, including the return to initial conditions and the re-equilibration of the column. Resolution of β-ecdysone and the unidentified peak was calculated as 2.48 and 1.45, respectively. The developed method achieved a good separation of this pair of peaks. It is considered a base-line separation when resolution reaches a value of 1.5. In the case of the critical pair of peaks a near base-line separation was achieved, also a good separation between β-ecdysone and the preceding peak. The width of peaks, k prime, selectivity and symmetry factors were also calculated and are shown in Table 1. Symmetry Factor of the peak of β-ecdysone is 1.09 indicating a slight tailing caused by the partial overlapping of β-ecdysone with the unidentified peak. UV-Vis spectrum of the standard of β-ecdysone and the spectrum of the real sample of β-ecdysone (methanolic extract) are shown in Figure 3. It is observed that they are similar and both have its absorption maximum at 246 nm. LOD and LOQ of β-ecdysone were also calculated, giving values of 0.20 and 0.67 mg L^{-1} respectively.

The repeatability and reproducibility of the developed method was studied in relation to the peak area and the retention time of β-ecdysone. A total of 30 HPLC analyses of the same sample, a methanolic extract of pills of *Pfaffia glomerata*, were performed on three successive

days (10 analyses per day). The intraday and interday relative standard deviations of retention time were lower than 0.14% and 0.47%, respectively. The relative intraday and interday standard deviations of peak area were lower than 0.15% and 0.25%, respectively.

3.3. Robustness of the method: sample concentration/dilution of the sample

Most samples that were analyzed were expected to contain some amount of organic solvent, as they are obtained by extraction methods, which employ organic solvents, mainly methanol or ethanol. Furthermore, in several methods, a final concentration step is included to increase the analytical signal in the detection systems, thus changing the initial solvent concentration. As a result, the robustness of the chromatographic method related to the sample dilution should be checked. Different dilutions (2, 3, 4 and 5) with methanol of the initial sample were studied.

The robustness for chromatographic resolution, concentration, width of the peak, K prime, selectivity, symmetry factor and also peak retention time for β-ecdysone and the unidentified peaks eluting before (unidentified peak #1) and after β -ecdysone (unidentified peak # 2) was established (Table 2). These parameters were calculated by Empower 3 software. The volume of extract injected was 10 µL. Each analysis was performed in triplicate.

Regarding peak resolution, the developed method showed that a lower concentration of the sample improved the separation between β-ecdysone and the unidentified peak. It was further verified that a lower concentration of the sample did not affect the reproducibility of the method. It was also confirmed that the sample dilution did not significantly affect the retention time, width of the peaks, K prime, selectivity and symmetry factor of the tree peaks studied. Finally, it should say that ideally the sample should be with a concentration below 100 mg L^{-1}. Furthermore, in terms of chromatographic performance there are no significant differences between 25 and 50 mg L^{-1}.

3.4. Sample solvent

Several extractions of a commercial extract of *Pfaffia glomerata* sold in pills were been performed with different solvents, as detailed in Section 2.2.1.

The solvents used in the extractions were mixtures of methanol, ethanol and acetonitrile and water [80-100% of organic solvent and 0-20% of water (v/v)]The extracts obtained with each solvent were analyzed using the chromatographic method developed to test whether the extraction solvent affects the chromatographic separation of the peaks studied. The studied parameters were the retention time, the amount of β-ecdysone extracted, the RSD of the amount of β-ecdysone extracted, width of the peaks, K prime, selectivity, resolution and symmetry factor for the chromatographic peaks studied. The obtained results for each solvent are shown in Table 3. Table 3 shows huge differences between different solvents. However, there was no significant difference ($p < 0.05$) between some of the factors while others were heavily influenced depending of the solvent. It is noteworthy that the most important aspect is the separation (i.e. resolution) of b-ecdysone from the unidentified peak #2) and clearly methanol is the best solvent. There were small differences between pure methanol and mixtures with water (10-20%) but these small when compared to the differences to the other solvents and therefore it is safe to assume that any of these solvents are adequate to be used in the analysis of b-ecdysone. This observation was inserted in the text. On the other hand, as can be observed in Table 3, methanol (100, 90 and 80%) was also the best solvent for the extraction of β-ecdysone and to dissolve the sample. High concentrations of ethanol (100, 90 and 80%) significantly affected the peak shape and therefore, such a high concentrations of ethanol should be avoided. In that case, higher amounts of water reduced this effect, but the chromatographic peaks had a lower resolution than methanol (100, 90 and 80%). Acetonitrile (100, 90 and 80%) is not a good solvent choice for the extraction. When acetonitrile is used as the extraction solvent, the sample should be preferably evaporated and re-dissolved in 80% methanol. Table 3 shows that the extractions with methanol (90 and 80%) produced higher extraction yields, lower RSD of the amount of β-ecdysone extracted, smaller peak widths and best resolution for the chromatographic peaks. This effect was more pronounced when methanol 80% was used as the extraction solvent.

However, it is important to highlight that the extraction "sample" was a commercial extract and therefore these are not extraction yields *per si* and the results reflect the ability of the solvent to dissolve β-ecdysone and how the sample solvent affects the chromatographic performance of the method.

3.5. Comparison with other methods

There are numerous works in the literature were β-ecdysone was analyzed by HPLC using UV-Vis detection. Most of them use C18 columns. These conventional methods of analysis have a higher time of analysis and less peak resolution of the β-ecdysone. Hwan Boo et al [28] analyzed β-ecdysone on *Achyranthes japonica* Nakai plants. They obtained a correct separation of β-ecdysone in 18 minutes. Serra et al [29] quantified β-ecdysone in different parts of *Pfaffia glomerata* by HPLC. They managed shorten the analysis time of β-ecdysone to 9.5 minutes maintaining a correct separation of the peak. Flores et al [30] analyzed β-ecdysone in a time of 2.7 minutes, but with a very poor chromatographic resolution of the peak, which had a large tail. The reported times are relative to retention time of of β-ecdysone. The developed method for the analysis of β-ecdysone by HPLC with fused-core columns resulted in shorter analysis time and a higher sample processing capability when compared to these previous methods and represent a step forward in the analytical methodology available. The employed strategy consisting of using high column temperature, solvents with low viscosity, in combination with a fused-core column allows the usage of shorter columns with smaller particles and higher flows-rates, resulting in a shortening of the time of analysis with a high resolution of the chromatographic peak of β-ecdysone.

3.6. Application to real samples

The developed HPLC method was applied to the analysis of 10 different Brazilian ginseng (*Pfaffia glomerata*) extracts samples in order to determine the β-ecdysone (Table 4) content. The extracts were obtained by pressurized liquid extraction (PLE) as discussed in section 2.2.1. The concentration of β-ecdysone in these extracts ranged from 29.1 to 37.7 mg L^{-1} while the content β-ecdysone in the raw material ranged from 4.7 to 6.0 $mg.g^{-1}$. These results indicate that the developed method is efficient and reliable for the analysis of β-ecdysone in Brazilian ginseng extracts.

4. Conclusions

In the present study, a step-by-step optimization strategy of chromatographic parameters (mobile phase composition, temperature of the column, flow rate, gradient and re-equilibration time) was used to develop a fast and reproducible analysis method for the determination of β-ecdysone in Brazilian ginseng extracts. Separation of these compounds was achieved in approximately 2 min and total analysis time, including column clean-up, and re-equilibration time, was 9 min. The optimized method showed and excellent chromatographic performance in terms of resolution, selectivity, peak symmetry, reproducibility, quantification and detection levels and was successfully used for the analysis of different real samples with similar performance. The developed method has presented an excellent robustness according to the concentration / dilution of the sample and the injection volume. It has also been obtained that the best solvent for the extraction process and the dilution of the samples is methanol 80% in water. The combination of state-of-the art column technology and optimized conditions significantly increased sample throughout in standard chromatographic systems when compared to conventional methods. Based on the results gathered during the method development, it is clear that fused-core column technology has a great potential to deliver faster and more sensitive methods for the analysis of β-ecdysone and other natural products.

Acknowledgments

The authors acknowledge funding from FAPESP (Projects 2012/10685-8 and 2013/04304-4) and from CNPq (Project 470916/2012-5). Authors are also thankful for scholarships provided by FAPESP (2013/15049-5) and CNPq (560914/2010-5, 140282/2013-0 and 151165/2010-6).

References

1. M. Báthori, *TrAC Trends in Analytical Chemistry*, 1998, **17**, 372-383.
2. L. Dinan, *Phytochemistry*, 2001, **57**, 325-339.
3. R. A. Festucci-Buselli, L. A. S. Contim, L. C. A. Barbosa, J. J. Stuart, R. F. Vieira and W. C. Otoni, *Brazilian Journal of plant physiology*, 2008, **20**, 305-311.
4. L. Dinan, T. Savchenko and P. Whiting, *Cellular and Molecular Life Sciences*, 2001, **58**, 1121-1132.
5. T. Takemoto, S. Ogawa and N. Nishimoto, *I. Yakigaku Zasshi*, 1967, **87**, 1463-1468.
6. Y. Shiobara, S.-S. Inoue, K. Kato, Y. Nishiguchi, Y. Oishi, N. Nishimoto, F. de Oliveira, G. Akisue, M. K. Akisue and G. Hashimoto, *Phytochemistry*, 1993, **32**, 1527-1530.
7. A. R. Zimmer, F. Bruxel, V. L. Bassani and G. Gosmann, *Journal of Pharmaceutical and Biomedical Analysis*, 2006, **40**, 450-453.
8. A. G. Neto, J. M. L. C. Costa, C. C. Belati, A. H. C. Vinhólis, L. S. Possebom, A. A. Da Silva Filho, W. R. Cunha, J. C. T. Carvalho, J. K. Bastos and M. L. A. e Silva, *Journal of Ethnopharmacology*, 2005, **96**, 87-91.
9. C. S. Freitas, C. H. Baggio, J. E. Da Silva-Santos, L. Rieck, C. A. de Moraes Santos, C. C. Júnior, L. C. Ming, D. A. Garcia Cortez and M. C. A. Marques, *Life Sciences*, 2004, **74**, 1167-1179.
10. C. S. Freitas, C. H. Baggio, A. Twardowschy, A. C. d. Santos, B. Mayer, A. P. Luiz, C. A. M. d. Santos, M. C. A. Marques and A. R. S. d. Santos, *Journal of Ethnopharmacology*, 2009, **122**, 468-472.
11. N. R. Sanches, R. Galletto, C. E. Oliveira, R. B. Bazotte and D. A. G. Cortez, *Acta Scientiarum*, 2001, **23**, 613-617.
12. A. G. Neto, A. A. da Silva Filho, J. M. L. C. Costa, A. H. C. Vinholis, G. H. B. Souza, W. R. Cunha, M. L. A. E. Silva, S. Albuquerque and J. K. Bastos, *Phytomedicine*, 2004, **11**, 662-665.
13. S. Nakamura, G. Chen, S. Nakashima, H. Matsuda, Y. Pei and M. Yoshikawa, *Chem. Pharm. Bull.*, 2010, **58**, 690-695.
14. F. de-Paris, G. Neves, J. B. Salgueiro, J. Quevedo, I. Izquierdo and S. M. K. Rates, *Journal of Ethnopharmacology*, 2000, **73**, 261-269.
15. I. D. Wilson, E. D. Morgan, R. Lafont, J. P. Shockcor, J. C. Lindon, J. K. Nicholson and B. Wright, *Chromatographia*, 1999, **49**, 374-378.
16. B. T. Zhao, S. Y. Jeong, D. C. Moon, K. H. Son, J. K. Son and M. H. Woo, *Archives of Pharmacal Research*, 2012, **38**, 1449-1455.
17. J. Li, H.-J. Li, P. Li and H. Qi, *Biomedical Chromatography*, 2007, **21**, 823-828.
18. A. Vanyolos and M. Báthori, *Current Pharmaceutical Analysis*, 2008, **4**, 162-175.
19. N. Manchón, M. D'Arrigo, A. García-Lafuente, E. Guillamón, A. Villares, J. A. Martínez, A. Ramos and M. A. Rostagno, *Analytical and Bioanalytical Chemistry*, 2011, **400**, 1251-1261.
20. A. Vaast, K. Broeckhoven, S. Dolman, G. Desmet and S. Eeltink, *Journal of Chromatography A*, 2012, **1228**, 270-275.
21. J. J. Kirkland, S. A. Schuster, W. L. Johnson and B. E. Boyes, *Journal of Pharmaceutical Analysis*.
22. M. A. Rostagno, N. Manchón, M. D'Arrigo, E. Guillamón, A. Villares, A. García-Lafuente, A. Ramos and J. A. Martínez, *Analytica Chimica Acta*, 2011, **685**, 204-211.

23. N. Manchón, M. D'Arrigo, A. García-Lafuente, E. Guillamón, A. Villares, A. Ramos, J. A. Martínez and M. A. Rostagno, *Talanta*, 2010, **82**, 1986-1994.
24. A. M. Farías-Campomanes, M. A. Rostagno and M. A. A. Meireles, *The Journal of Supercritical Fluids*, 2013, **77**, 70-78.
25. M. A. Rostagno, M. Palma and C. G. Barroso, *Journal of Chromatography A*, 2003, **1012**, 119-128.
26. G. L. Long and J. D. Winefordner, *Analytical Chemistry*, 1983, **55**, 712A-724A.
27. L. Nováková and H. Vlčková, *Analytica Chimica Acta*, 2009, **656**, 8-35.
28. K. H. Boo, D. Lee, G. L. Jeon, S. H. Ko, S. K. Cho, J. H. Kim, S. P. Park, Q. Hong, S.-H. Lee, D.-S. Lee and K. Z. Riu, *Bioscience, Biotechnology and Biochemistry*, 2010, **74**, 2226-2231.
29. L. Z. Serra, D. F. Felipe and D. A. G. Cortez, *Brazilian Journal of Pharmarcognosy*, 2012, **22**, 1349-1354.
30. R. Flores, D. Brondrani Jr., V. Cezarotto Jr., S. R. Giacomelli and F. T. Nicoloso, *In vitro Cellular & Developmental Biology- Plant*, 2010, **46**, 210-217.

Figures

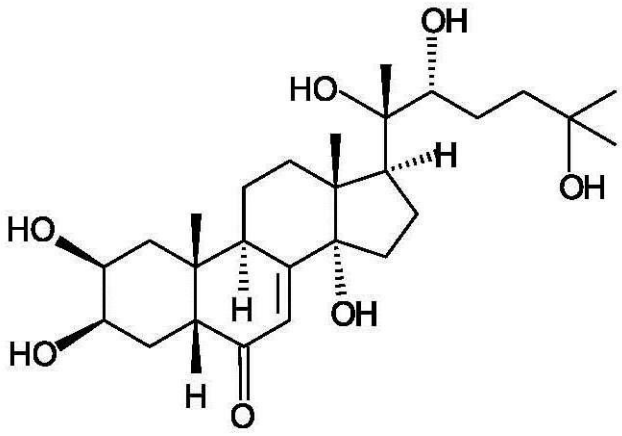


Figure 1. Chemical structure of β-ecdysone

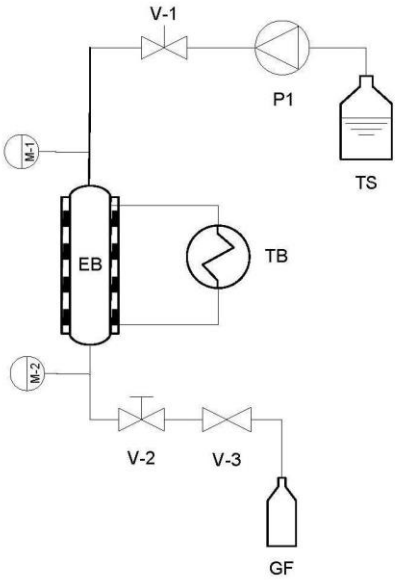


Figure 2. Schematic diagram of the pressurized liquid extraction unit. TS: Tank of solvent; P1: High pressure pump; V-1 and V-2: block valve; V-3: micrometric valve; M-1 and M-2: manometers; EB: extraction bed; TB: thermostatic bath; GF: glass flask.

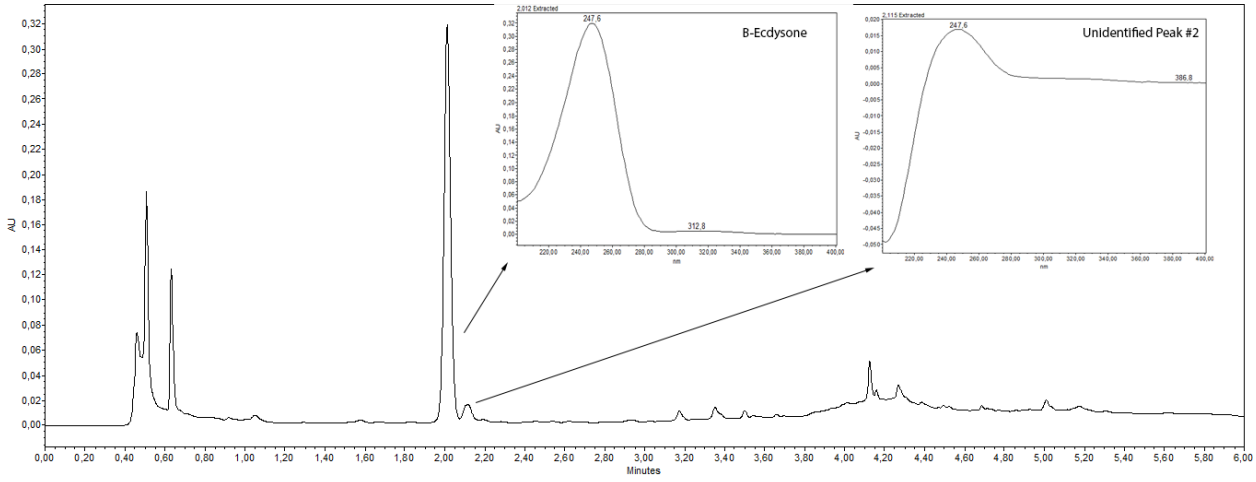


Figure 3. Representative chromatogram of a real sample obtained using the optimized method.

Table 1- Chromatographic characteristics of the developed method

Name	RT	Width	K Prime	Selectivity	Resolution	Symmetry Factor
Unidentified peak 1	1.76	5.43	3.10			
β-ecdysone	2.01	9.93	3.49	1.13	2.78	1.09
Unidentified peak 2	2.11	5.55	3.72	1.06	1.45	

Table 2 – Effect of sample concentration on the chromatographic performance of the developed method

Dilution	Compound	RT (min)	Concentration (mg/L)	RSD (%)	Width (sec.)	K Prime	Selectivity	Resolution	Symmetry Factor
1X	Unidentified peak 1	1.58	106.27	0.20	7.18	2.95			0.99
	β -Ecdysone	2.01			9.39	4.03	1.37	6.67	1.08
	Unidentified peak 2	2.11			5.36	4.28	1.06	1.47	
2X	Unidentified peak 1	1.58	54.46	0.19	9.18	2.95			0.78
	β -Ecdysone	2.02			9.33	4.06	1.38	6.06	1.09
	Unidentified peak 2	2.12			5.13	4.31	1.06	1.53	
3X	Unidentified peak 1	1.58	35.95	0.22	8.90	2.95			0.79
	β -Ecdysone	2.03			10.85	4.06	1.38	6.06	1.09
	Unidentified peak 2	2.13			5.25	4.31	1.06	1.53	
4X	Unidentified peak 1	1.58	26.61	0.21	9.20	2.95			0.78
	β -Ecdysone	2.03			10.55	4.07	1.38	6.06	1.10
	Unidentified peak 2	2.13			5.25	4.32	1.06	1.52	
5X	Unidentified peak 1	1.58	21.85	0.20	7.50	2.95			0.92
	β -Ecdysone	2.03			9.85	4.07	1.38	6.26	1.10
	Unidentified peak 2	2.13			5.18	4.32	1.06	1.52	

Table 3- Effect of sample solvent on the chromatographic performance of the developed method

Solvent	Compound	RT (min)	Concentration (mg/L)	Mass (mg)	RSD (%)	Width (sec.)	K Prime	Selectivity	Resolution	Symmetry Factor
80% MeOH	Peak # 1	1.58	106.27	2.65	1.07	7.18	2.95	1.37	6.67	0.99
	β -Ecdysone	2.01				9.39	4.03			1.08
	Peak # 2	2.11				5.36	4.28	1.06	1.47	
90% MeOH	Peak # 1	1.57	104.14	2.60	1.31	6.80	2.93	1.37	6.38	0.92
	β -Ecdysone	2.01				9.50	4.02			1.08
	Peak # 2	2.11				5.35	4.27	1.06	1.47	
MeOH	Peak # 1	1.57	88.94	2.22	1.55	9.90	2.93	1.37	5.87	0.77
	β -Ecdysone	2.01				9.45	4.02			1.08
	Peak # 2	2.11				5.45	4.27	1.06	1.38	
80% EtOH	Peak # 1	1.57	100.28	2.51	1.34	8.70	2.92	1.38	3.47	0.88
	β -Ecdysone	2.01				11.80	4.03			0.95
	Peak # 2	2.11				5.05	4.28	1.06	-	
90% EtOH	Peak # 1	1.68	80.04	2.00	1.67	8.65	3.20	1.26	2.63	1.30
	β -Ecdysone	2.01				13.55	4.03			0.86
	Peak # 2	2.11				5.10	4.28	1.06	-	
EtOH	Peak # 1	1.68	19.02	0.48	1.74	18.05	3.20	1.26	2.33	0.79
	β -Ecdysone	2.01				16.75	4.03			0.76
	Peak # 2	2.11				5.30	4.27	1.06	-	
80% ACN	Peak # 1	1.91	26.83	0.67	1.65	48.55	3.77	1.07	-	-
	β -Ecdysone	2.01				10.25	4.02			-
	Peak # 2	2.08				4.35	4.20	1.04	-	
90% ACN	Peak # 1	1.91	37.25	0.93	1.71	71.25	3.78	1.07	-	-
	β -Ecdysone	2.02				10.60	4.04			-
	Peak # 2	2.11				4.70	4.28	1.06	-	
ACN		-	-	-	-	-	-	-	-	-

Table 4. Concentration of β-ecdysone ($mg.L^{-1}$ ± RSD) in different Brazilian ginseng root samples

Sample	Extract ($mg.L^{-1}$ ± RSD)
1	37.7 ± 0.7
2	36.6 ± 0.7
3	35.1 ± 0.7
4	33.0 ± 0.5
5	33.4 ± 0.1
6	32.0 ± 0.7
7	31.2 ± 0.4
8	30.2 ± 0.7
9	29.1 ± 0.2
10	31.1 ± 0.1