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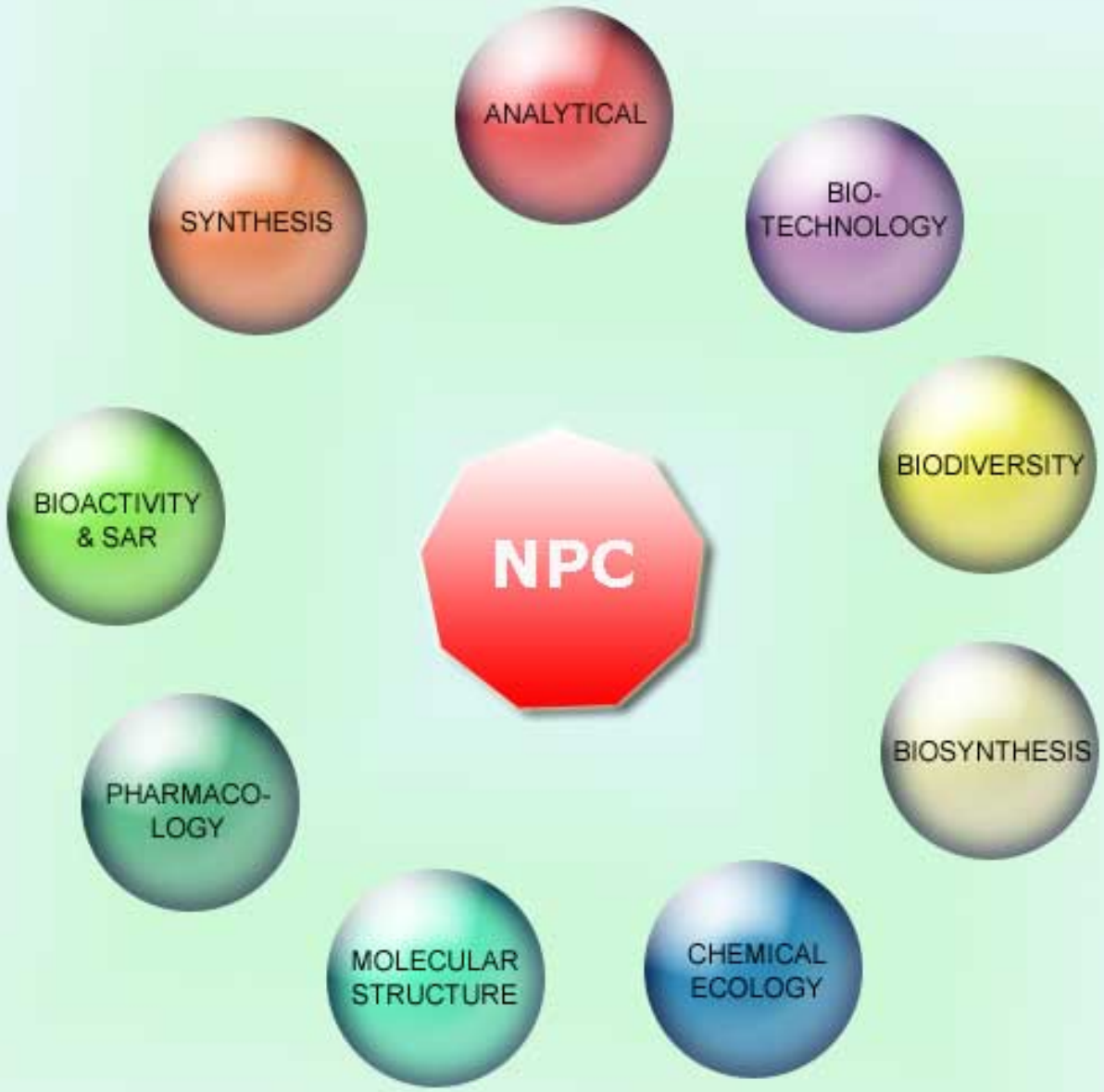

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A Semi-quantitative FIA-ESI-MS Method for the Rapid Screening of *Hypericum perforatum* Crude Extracts

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A method based on FIA-ESI-MS has been developed to profile the major constituents of *Hypericum perforatum* extracts. The objective was to obtain simultaneous semi-quantitative data on hypericin, chlorogenic acid, rutin, quercitrin, quercetin and hyperforin contents for a high-throughput screening of the raw plant material. The principal drawback of FIA-ESI analysis of complex mixtures involves ion suppression effects: the ionization of some components of the mixture can be severely suppressed by ionization of others. The results show that this problem can be alleviated using a new approach to generate calibration curves.

Keywords: extract screening, herbal medicine, antidepressive activity, FIA-ESI-MS, *Hypericum perforatum*.

Hypericum perforatum L. (Clusiaceae) is a herbaceous perennial plant, well known as a medicinal plant since antiquity [1a]. Many therapeutic properties have been attributed to it, such as antibacterial, antiviral, anti-inflammatory, analgesic, and anti-depressive. Nowadays, the use of *Hypericum* extracts is related mainly with their anti-depressive applications [1b]. *H. perforatum* contains components belonging to different natural product classes (Figure 1), including naphthodianthrones (hypericins), prenylated phloroglucinols (hyperforins), flavonol glycosides, biflavones, proanthocyanidins and phenylpropanes; the synergic effect of these constituents could explain the multiple medicinal uses.

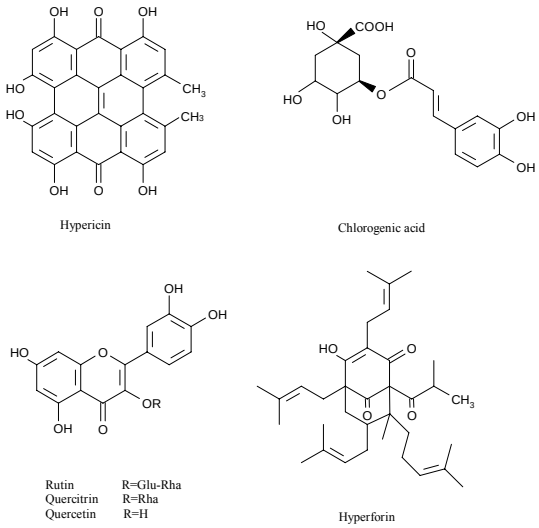

Figure 1: Major components of *H. perforatum*.

In a recent work [2] we have pointed out different chemical profiles among and within three subspecies of *H. perforatum* at different developmental stages. The chemical profile of Hyperici herba extract, as of other plant extracts, is, therefore, of pertinent interest in choosing the raw plant material to use in the preparation of commercial products. In the screening of metabolite profile, high analysis speeds and short method development times are crucial factors. A multitude of different HPLC-MS methods have been proposed for the complete characterization of *H. perforatum* extracts [3-9]. Most of these published methods have used mass spectrometry (MS) detection for identification and confirmation purpose only. Only three methods used MS for quantitative analysis. Mauri and Pietta [5b], using electrospray ionization (ESI) as the interface between HPLC and mass spectrometry, developed a rapid method for the analysis of naphthodianthrones and phloroglucinols in the negative mode, but only hypericin was quantified. Tolonen *et al*. [8a] reported quantification of hypericins and hyperforin. Chandrasekera *et al*. [9a] developed an HPLC-ESI-MS method for the simultaneous separation, identification and quantification of the major constituents of Hyperici herba extract, but they stressed specific problems correlated to HPLC analysis due to the physico-chemical properties of the naphthodianthrones.

In our laboratory it has been demonstrated that hypericin can be retained in different ratios by various RP-18 column packing and the registered peak areas showed coefficient of variation (CV) values of 50% [10a]. Mass spectrometry is considered to be a generic analytical technique with a relatively short development time. Furthermore, when applying flow injection analysis (FIA) for sample introduction, the requirements for high throughput are met. Therefore, our attention was focused on developing a single-step approach based on FIA-SIM-MS in order to provide semi-quantitative data for the rapid screening of *H. perforatum* raw materials.

In a our previous paper, a semi-quantitative method based on FIA-SIM-MS to estimate the production yields of vincristine and vinblastine in *Catharanthus roseus* extracts has been reported [10b]. The principal drawback of FIA-ESI analysis of a complex mixture involves ion suppression effects, so that the sensitivity factors for the single component calibration solution may not be applicable to mixtures due to one component suppressing the signal of another. In the case of *C. roseus*, it was demonstrated that the ion suppression effect does not alter the order of magnitude of vincristine and vinblastine signals.

The first aim of the present work was to test the presence of these suppression effects in the mixture of the major components of *H. perforatum* extracts. At first, pure diluted solutions (2.5 μg/mL) of hypericin, chlorogenic acid, rutin, quercitrin, quercetin and hyperforin, respectively were analysed by FIA-ESI-MS. Then, a solution containing the same concentration of each of the standards used in the previous experiment was prepared, and the signal intensity for each component in the mixture was measured. All the measurements were performed in selected ion monitoring (SIM) mode for the $[M-H]^-$ ions at *m/z* 503, 353, 609, 301, 535 and 447. Comparison of the respective signal intensities (peak areas) of the individual standard solutions with those of the mixture solution did not indicate an ion suppression effect on quercitrin, but the occurrence of ion suppression effects on hypericin, chlorogenic acid, rutin and quercetin due to hyperforin. In order to estimate the extent of these effects, the peak areas of the suppressed components in a solution containing the same concentration of these components plus quercitrin, with different amounts of hyperforin (0.05-1.5 μg/mL) were measured. It was observed that the suppression effects were always of the same order of magnitude, even when the ratio of hyperforin/suppressed components was 1:50. The suppression factors were ~2 for chlorogenic acid, rutin, quercetin, and ~4 for hypericin. In order to evaluate the precision of the peak intensity measurements (and, thus, the precision of our ion suppression estimates), coefficients of variation were calculated for a series of three measurements:

$$CV = (S/A) \times 100,$$

where S is the standard deviation of the peak area and A is the mean area of the peak determined by SIM. CVs were 8-12% for measurements performed with 1 μg/mL of hyperforin and 2.5 μg/mL of all other components.

On the basis of the obtained data, we decided to try a new approach to generate the calibration curves for FIA. Considering that the sensitivity factors for the single component in solution may not be applicable to mixtures containing a component suppressing the signal of another, standard mixture solutions containing all the components considered in relative different amounts were used. Therefore, the amount of each component in the standard mixture solutions was selected randomly in order to verify the correlation between the peak area and the concentration of each component, when present in mixture solutions in different ratios. A solution containing the same amount (2 μg/mL) of each of the considered compounds was used as external standard.

The calibration curves were obtained using SIM mode, plotting the relative peak areas for each $[M-H]^-$ ion with respect to the corresponding values for the external standard mixture solution *versus* the corresponding concentration ratio. The linearity of response was determined for five concentration levels for each component. For each point of the calibration curves, triplicate measurements were made, each loop injection being followed by a flow of 2 mL (8 min at 250 μL/min) of pure carrier solvent in order to prevent any memory effects. A linear range from 0.2 to 2.5 μg/mL for hypericin and from 1.5 to 10 μg/mL for all the other components was observed (loop injection 10 μL of solutions) (Table 1).

In *Hypericum* extracts the content of these characteristic constituents varies significantly in samples from different habitats and at different plant development stages. The extracts from plant material collected at three different ontogenetic stages were then analyzed. The extracts were subjected to ESI-MS/MS analysis by direct infusion; the ions at *m/z* 503, 353, 609, 447, 301 and 535 were identified as hypericin, chlorogenic acid, rutin, quercitrin, quercetin and hyperforin on the basis of fragmentation patterns. The semi-quantitative determinations were performed under the same conditions used to generate the calibration curves. The peak areas (three replicate measurement means) showed CV values of 10-13%. The amounts of hypericin, chlorogenic acid, rutin, quercitrin, quercetin and hyperforin are reported in Table 2.

Table 1: Linear calibration curves of major constituents of *Hypericum perforatum*.

	concentration (μg/mL)	y=ax+b, the linear model[a]		regression coefficient (r^2)	coefficient of variation (CV %)
		slope (a)	intercept (b)		
Hypericin	0.2 to 2.5	0.552	+0.507	0.9987	20
Chlorogenic acid	1.5 to 10	0.435	+0.484	0.9938	23
Rutin	1.5 to 10	0.952	-0.06	0.9976	10
Quercitrin	1.5 to 10	1.13	-0.118	0.9998	9
Quercetin	1.5 to 10	1.04	-0.056	0.9999	18
Hyperforin	1.5 to 10	0.566	+0.441	1	11

[a]y: concentration, x: peak area ratio.

Table 2: Amounts (% w/w) of major constituents of *H. perforatum* extracts determined by FIA-SIM-MS.

	hypericin	chlorogenic ac.	rutin	quercitrin	quercetin	hyperforin
Floral budding	0.005a	1.29a	n.d.	0.38b	0.13b	0.97a
Full flowering	0.033b	1.27a	n.d.	0.26a	0.10b	1.31b
Fresh fruits	0.007a	1.26a	n.d.	0.35b	0.088a	0.91a

Since the suppression effects could lead to underestimation of the content of some extract components, the content of quercetin (a suppressed component) in the same samples was determined also by HPLC-UV analysis (European Pharmacopoeia-approved modality) and compared with the results obtained by FIA-ESI-MS. The quantitative analysis was performed only on quercetin because, under the analytical conditions used, it showed the best resolution and peak shape. The calibration curve was obtained using standard solutions of quercetin; linearity was in the range 5-50 μg/mL. The content of quercetin determined by HPLC-UV analysis was similar to that obtained by FIA. Since suppression effects presumably always lead to underestimation of the content of suppressed components, these data indicate that, in this case, suppression effects are accounted for. The approach involving the use of mixture solutions of all components to generate calibration curves could be of crucial importance to FIA-ESI-MS semi-quantitative analysis of complex matrices, even in presence of ion suppression effects.

The obtained results indicate that FIA-ESI-MS is a simple, rapid and valid method to gain access to molecular diversity from different natural sources in the first screening of *Hypericum* crude extracts prior to full quantification using properly validated methods. In this phase, it is crucial not only to obtain a qualitative profile, but also to have quantitative information about the main constituents. This is particularly true when little is known about the relationship of these constituents to actions and efficacies, as in the case of *Hypericum*. Therefore, semi-quantitative data can be applied to the selection of the appropriate raw material, and also in determining ontogenetic stage variations.

Experimental

Chemicals: Reference standards: chlorogenic acid, rutin, quercetin, quercitrin and hypericin (Extrasynthèse-Genay, France), and hyperforin (Addipharma-GmbH Wandalenweg, Germany). The purity of reference standards was tested by HPLC [2].

Plant material: Aerial parts of native plants of *H. perforatum* L. at floral budding, full flowering and fresh fruiting were harvested in Camin village near Padova (Italy). A voucher specimen of the plants (5168 PAD) is deposited in the Botanical Garden of the University of Padova.

Extraction: Dried and powdered samples (0.1 g dry weight) were sonicated (1 h) at room temperature in 100 mL methanol. As far as possible, all extraction procedures were performed under protection from daylight. The extracts were stored in darkness at 0°C and brought to room temperature before use. Aliquots (3.0 mL) were filtered through a 0.20 μm cellulose filter (Advantec MFS, CA, USA) and directly analysed.

Analytical procedure: Calibration curves for FIA analysis were determined using a series of standard mixture solutions containing varying amounts of stock solutions of hypericin, chlorogenic acid, rutin, quercitrin, quercetin and hyperforin. A calibration curve for HPLC-UV analysis was determined using a series of solutions containing varying amounts of the stock solution of quercetin. The quantitative data are the means of three measurements.

Mass spectrometry: All mass spectrometric measurements were performed using a LCQ (Thermoquest, San José, CA, USA) ion trap mass spectrometer, operating under negative ion ESI conditions. The ESI source parameters were: spray

voltage 3 KV, capillary temperature 200°C, N_2 sheath gas flow 60 a.u., N_2 auxiliary gas flow 20 a.u. For quantitative determinations, flow-injection analysis (FIA) was performed by loop injection (10 µL of sample solution) in a flow of methanol supplied at 250 µL/min to the ESI source by a Spectra Series (Thermoseparation, San José, CA, USA) HPLC pump. For quantification, the mass spectrometer was operated in selected ion monitoring (SIM) mode, scanning with a window of 4 Th centered on ions at *m/z* 503 for hypericin, *m/z* 353 for chlorogenic acid, *m/z* 609 for rutin, *m/z* 447 for quercitrin, *m/z* 301 for quercetin and *m/z* 535 for hyperforin. The injection time was 200 ms.

HPLC-UV: HPLC analysis was performed on a ChromQuest (Thermoseparation, San Josè, CA, USA) pump P4000 equipped with a photodiode array detector UV6000. The chromatographic data were recorded and processed with a ChromQuest Chromathography Workstation. The quantification of quercetin was performed using a Nova-Pak RP-18 4µm (Waters Milford, CA, USA) column (150 x 3.9 mm) protected by a guard column (Alltech Italia, Milano, Italy) (20 x 2 mm I.D.). The mobile phase consisted of 0.01% (v/v) phosphoric acid (A), methanol (B) and acetonitrile (C). The gradient was as follows: from A:C (95:5, v/v) to A:C (85:15, v/v) in 10 min, then to A:B:C (70:10:20, v/v/v) in 20 min, then to A:B:C (10:15:75, v/v/v) in 10 min, then to A:B:C (5:15:80, v/v/v) in 15 min. The flow rate was kept at 1 mL/min and the injection volume was 20 µL. The chromatograms were acquired at 370 nm [2].

Statistical analysis: Data were subjected to analysis of variance (ANOVA) to assess significant differences; significance between values was tested by Tukey's test at the confidence level of $P \leq 0.05$.

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References

[1] (a) Barnes J, Anderson LA, Phillipson JD. (**2001**) St John's Wort (*Hypericum perforatum* L.): a review of its chemistry, pharmacology and clinical properties. *Journal of Pharmacy and Pharmacology*, ***53***, 583-600; (b) Linde K, Berner MM, Kriston L. (**2008**) St John's wort for major depression. *Cochrane Database of Systematic Reviews*, ***4***, CD 000448.

[2] Filippini R, Piovan A, Borsarini A, Caniato R. (**2010**) Study of dynamic accumulation of secondary metabolites in three subspecies of *Hypericum perforatum*. *Fitoterapia*, ***81***, 115-119.

[3] Piperopoulos G, Lotz R, Wixforth A, Schmierer T, Zeller KP. (**1997**) Determination of naphthodianthrones in plant extracts from *Hypericum perforatum* L. by liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography B*, ***695***, 309-316.

[4] Brolis M, Gabetta B, Fuzzati N, Pace R, Panzeri F, Peterlongo F. (**1998**) Identification by high performance liquid chromatography-diode array detection-mass spectrometry and quantification by high performance liquid chromatography-UV absorbance detection of active constituents of *Hypericum perforatum*. *Journal of Chromatography A*, ***825***, 9-16.

[5] (a) Hansen SH, Jensen AG, Cornett C, Bjornsdottir I, Taylor S, Wright B, Wilson ID. (**1999**) High-performance liquid chromatography on-line coupled to high-field NMR and mass spectrometry for structure elucidation of constituents of *Hypericum perforatum*. *Analytical Chemistry*, ***71***, 5235-5241; (b) Mauri P, Pietta P. (**2000**) High performance liquid chromatography/ electrospray mass spectrometry of *Hypericum perforatum* extracts. *Rapid Communications in Mass Spectrometry*, ***14***, 95-99.

[6] Liu FF, Ang CYW, Heinze TM, Rankin JD, Beger RD, Freeman JP, Lay JO. (**2000**) Evaluation of major active components in St. John's wort dietary supplements by high-performance liquid chromatography with photodiode array detection and electrospray mass spectrometric confirmation. *Journal of Chromatography A*, ***888***, 85-92.

[7] Ganzera M, Zhao J, Khan IA. (**2002**) *Hypericum perforatum* – Chemical profiling and quantitative results of St. John's wort products by an improved high-performance liquid chromatography method. *Journal of Pharmaceutical Sciences*, ***91***, 623-630.

[8] (a) Tolonen A, Uusitalo J, Hohtola A, Jalonen J. (**2002**) Determination of naphthodianthrones and phloroglucinols from *Hypericum perforatum* extracts by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, ***16***, 396-402; (b) Seger C, Römpp H, Sturm S, Haslinger E, Schmidt PC, Hadacek F. (**2004**) Characterization of supercritical fluid extracts of St. John's Wort (*Hypericum perforatum*) by HPLC-MS and GC-MS. *European Journal of Pharmaceutical Sciences*, ***21***, 453-463.

[9] (a) Chandrasekera DH, Welham KJ, Ashton D, Middleton R, Heinrich M. (**2005**) Quantitative analysis of the major constituents of St. John's wort with HPLC-ESI-MS. *Journal of Pharmacy and Pharmacology*, ***57***, 1645-1652; (b) Gadzovska S, Maury S, Ounnar S, Righezza M, Kascakova S, Refregiers M, Spasenoski M, Joseph C, Hagège D. (**2005**) Identification and quantification of hypericin and pseudohypericin in different *Hypericum perforatum* L. *in vitro* cultures. *Plant Physiology and Biochemistry*, ***43***, 591-601; (c) Isacchi B, Bergonzi MC, Carnevali F, van der Esch SA, Vincieri FF, Bilia AR. (**2007**) Analysis and stability of the constituents of St. John's wort oils prepared with different methods. *Journal of Pharmaceutical and Biomedical Analysis*. ***45***, 756-761.

[10] (a) Piovan A, Filippini R, Borsarini A, Caniato R. (**2004**) Flow injection analysis mass spectrometry, a tool to investigate the problems in the quantitative analysis of hypericin using reversed-phase high-performance liquid chromatography. *Rapid Communications in Mass Spectrometry*, ***18***, 131-132; (b) Favretto D, Piovan A, Filippini R, Caniato R. (**2001**) Monitoring the production yields of vincristine and vinblastine in *Catharanthus roseus* from somatic embryogenesis. Semiquantitative determination by flow-injection electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry*, ***15***, 364-369.

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