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Comparison of liquid chromatography and supercritical fluid chromatography coupled to compact single quadrupole mass spectrometer for targeted *in vitro* metabolism assay

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ABSTRACT

The goal of this study was to evaluate the combination of powerful chromatographic methods and compact single quadrupole MS device for simple *in vitro* cytochrome P450 (CYP) inhibition assay, instead of more expensive triple quadrupole MS/MS detectors. For this purpose, two modern chromatographic approaches (ultra-high pressure liquid chromatography (UHPLC) and ultra-high performance supercritical fluid chromatography (UHPSFC)) were tested in combination with simple MS detector. To show the applicability for an *in vitro* CYP-mediated metabolism assay using the cocktail approach, a method was first developed in UHPLC–MS to separate a mixture of 8 probe substrates and 8 CYP-specific metabolites. A screening procedure was initially applied to determine the best combination of a column, an organic modifier and a mobile-phase pH, followed by fine tuning of the conditions (*i.e.*, gradient profile, temperature and pH) using HPLC modelling software. A similar sequential method development procedure was also evaluated for UHPSFC–MS. For method development, where peak tracking is necessary, the use of single quadrupole MS was found to be extremely valuable for following the investigated analytes. Ultimately, a baseline separation of the 16 compounds was achieved in both UHPLC–MS and UHPSFC–MS with an analysis time of less than 7 min. In a second series of experiments, sensitivity was evaluated, and LOQ values were between 2 and 100 ng/mL in UHPLC–MS, while they ranged from 2 to 200 ng/mL in UHPSFC–MS. Based on the concentrations employed for the current *in vitro* phase I metabolism assay, these LOQ values were appropriate for this type of application. Finally, the two analytical methods were applied to *in vitro* CYP-dependent metabolism testing. Two well-known phytochemical inhibitors, yohimbine and resveratrol, were investigated, and reliable conclusions were drawn from these experiments with both UHPLC–MS and UHPSFC–MS. At the end, the proposed strategy of optimized chromatography combined with simple MS device has been shown to potentially compete with the widely used combination of generic chromatography and highly selective MS/MS device for simple *in vitro* CYP inhibition assays. In addition, our analytical method may be easier to use in a routine environment; the instrument cost is significantly reduced and the two developed methods fit for purpose.

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1. Introduction

To efficiently eliminate xenobiotic compounds from the body, hepatic metabolism performs biochemical reactions to convert parent compounds into typically more hydrophilic metabolites. Many of these biotransformations are part of oxidative metabolism (phase I) which involves the cytochrome P450 (CYP) subfamily [1]. Environmental factors such as diet, drug therapy and toxic substances have a significant impact on CYP activities, causing a high inter-individual variability. In view of the key role of hepatic metabolism in most marketed drugs, the inhibition or induction of CYP activities may consequentially affect the therapeutic outcomes of the administered drug because of a severe impact on its pharmacokinetics. For example, when the activity of the major CYP responsible for drug metabolism is reduced, the excretion of the drug could become problematic and exceed the plasmatic drug concentrations to unexpectedly toxic levels [2]. Therefore, the evaluation of the CYP interaction potential of drugs, new chemical entities, toxic substances and phytochemicals must be performed to account for the existing risks during co-exposure.

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In the last decade, reliable high-throughput *in vitro* metabolism assays were successfully developed combining ultra-high pressure liquid chromatography (UHPLC) and mass spectrometry (MS). This method has currently become the prevailing analytical strategy for *in vitro* metabolism studies [3]. By using columns packed with sub-2 μm particles, it was possible to strongly reduce analysis times while maintaining similar chromatographic performance compared with conventional LC, which is an invaluable condition for developing high-throughput metabolic screening methodologies. Moreover, combination with MS detection has afforded additional sensitivity, selectivity, resolution and peak tracking capabilities, which are essential requirements for metabolism studies. Recently, such benefits have allowed designing high-throughput assays to assess CYP *in vitro* activities in human liver microsomes (HLMs) by using a cocktail approach. Compared with the classical methodology, which performs individual incubation of the CYP-specific substrates with HLMs, this strategy directly incubates the mixture of substrates for monitoring several CYP isoform activities within a single experiment, saving time and assay costs [4].

Even if liquid chromatography remains the gold standard as a separation technique, supercritical fluid chromatography (SFC) has made a remarkable comeback in the last few years. Indeed, modern SFC appears to be a powerful and green technique for the analysis of molecules exhibiting a broad range of polarity when the supercritical CO_2 mobile phase is modified with polar organic solvents (*e.g.*, methanol, isopropanol, *etc.*) and additives (ammonium hydroxide, ammonium formate, *etc.*) [5]. Practically, SFC can be used in both the reversed phase and the normal phase modes with the same mobile phase. Recently, ultra-high performance supercritical fluid chromatography (UHPSFC) has become commercially available through technological advances in pumping systems, backpressure regulation, and reduction of system void volumes (*i.e.*, extra-column and dwell volume) [6]. These technical improvements make UHPSFC compatible with the most recent stationary-phase technologies including columns packed with fully porous sub-2 μm particles, generating kinetic performance comparable to what is achieved in UHPLC. Thus, both fast analysis and enhanced chromatographic resolution can be easily achieved in UHPSFC. To date, the coupling of UHPSFC with MS has not been thoroughly investigated but appears promising, particularly in terms of increased sensitivity compared with UHPLC–MS [7]. It is also important to notice that UHPSFC–MS has not been employed until now for metabolism studies.

In vitro metabolism assays could be divided into two distinct categories, untargeted and targeted. Among the untargeted studies, metabolite profiling and metabolite identification (metabolite ID) have to be carried out with high-end MS devices (*i.e.*, orbitrap or QqTOF/MS) for investigating the numerous unknown metabolites derived from metabolism [8,9]. Over the past few years, significant efforts have been made to improve the acquisition rate, sensitivity and resolving power of high-resolution MS instruments, which are generally employed for these more sophisticated *in vitro* metabolism studies [10–12]. The most recent developments of quadrupole-based instruments have been focused on making them more sensitive and user-friendly, and the most widely used strategy for simple *in vitro* metabolism assay consists in performing generic LC conditions combined with state-of the-art MS/MS detector, to achieve a sufficient level of sensitivity and selectivity.

However, *in vitro* protocol conditions (*e.g.*, substrates concentration) are easily adaptable (sensitivity tuning) and the sample matrix is rather "simple" compared to *in vivo* experiments, as it contains only a very limited amount of proteins/enzymes that are mostly eliminated through centrifugation step (selectivity tuning).

Based on these observations, the goal of the present study was to evaluate whether fully optimized chromatographic methods combined with relatively simple, cheap and easy-to-use single quadrupole MS device could represent a valuable alternative to the current gold standard platform in the case of *in vitro* CYP inhibition study. Because the chromatographic step was extremely important in our methodology, both UHPLC and UHPSFC technologies were evaluated.

Suitable methods were developed for the separation of a mixture of 8 substrates and their CYP-specific metabolites. Then, the optimal separations achieved with these two analytical approaches were compared in terms of selectivity and sensitivity and finally applied for *in vitro* CYP-mediated metabolism experiments using HLMs and the cocktail approach.

2. Experimental

2.1. Chemical, reagents and columns

Methanol (MeOH) and acetonitrile (ACN) of ULC–MS grade were purchased from Biosolve (Valkenswaard, Netherlands), whereas isopropanol and heptane were purchased from VWR (Radnor, PA, USA). Pressurized liquid CO_2, 3.0 grade (99.9%), was purchased from PanGas (Dagmerstellen, Switzerland). Formic acid (98–100%) was obtained from Merck (Darmstadt, Germany) and glacial acetic acid from Biosolve. Ultrapure water was supplied by a Milli-Q Advantage A10 purification unit from Millipore (Bedford, MA, USA). Chlorzoxazone (98%), 6-hydroxychlorzoxazone (97%), 4′-hydroxyflurbiprofen (98%), hydroxybupropion (95%), 5-hydroxyomeprazole sodium salt (98%) and omeprazole (98%) were purchased from Toronto Research Chemicals (Ontario, Canada). Ammonium hydroxide, potassium hydroxide, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt (HEPES) (99.5%), dextromethorphan hydrobromide (99%), dextrorphan tartrate (98%), bupropion hydrochloride (98%), phenacetin (97%), acetaminophen (99%), flurbiprofen (99%), coumarin (99%), 7-hydroxycoumarin (99%), resveratrol (99%) were obtained from Sigma–Aldrich (Buchs, Switzerland), whereas methanolic stock solutions of midazolam and 1-hydroxymidazolam were purchased from Lipomed (Arlesheim, Switzerland). (S)-mephenytoin (99%) was obtained from Enzo Life Sciences (Lausen, Switzerland) and β-nicotinamide adenine dinucleotide 2′-phosphate reduced tetrasodium salt (NADPH) (96%) was obtained from Applichem (Darmstadt, Germany). Yohimbine hydrochloride (98%) was purchased from Carl Roth (Lauterbourg, France).

Pooled HLMs from 30 donors was purchased from BD Biosciences (Allschwil, Switzerland). Aliquots of HLMs were stored at −80 °C. A 50 mM NADPH stock solution was prepared in water and stored at −20 °C. Immediately before the CYP450-dependent metabolism assays, intermediate concentrations of the HLMs were prepared by diluting aliquots with the 50 mM HEPES (pH 7.4) buffer solution, which was prepared by dissolving the required amount of HEPES in water. The pH was adjusted to 7.4 with potassium hydroxide using a Seven Multi S40 Mettler Toledo pH meter (Greifensee, Switzerland). The substrate, metabolite and phytochemicals stock solutions at 1 mg/mL were prepared in MeOH and stored at −20 °C for maximum 6 months, because of the stability. Intermediate solutions were appropriately reconstituted daily in the 50 mM HEPES (pH 7.4) buffer solution.

The following UHPLC columns possessing identical dimensions (50 mm × 2.1 mm, 1.7 μm), were purchased from Waters (Milford, MA, USA): Acquity UPLC BEH C18, Acquity UPLC BEH Shield RP18, Acquity UPLC BEH Phenyl and Acquity UPLC CSH C18. The UHPSFC columns employed in this study were also purchased from Waters: Acquity UPC^2 BEH, Acquity UPC^2 BEH 2-EP, Acquity UPC^2 CSH Fluoro-Phenyl and Acquity UPC^2 HSS C18 SB. All the UHPSFC columns have dimensions of 100 mm × 3.0 mm, 1.7 μm, except the last one which possesses particle sizes of 1.8 μm.

2.2. Instrumentation

2.2.1. UHPLC system

Experiments were performed on an Acquity UPLC H-Class system from Waters (Milford, MA, USA). This low pressure mixing system was equipped with a pumping device, autosampler and column oven, which includes an active preheater. The quaternary solvent delivery pump was able to work up to pressure of 1000 bar and allows the selection of up to 4 different buffers or organic modifiers in any combination. The autosampler was a flow through needle (FTN) injection system with 15 μL needle. The injection volume was 2 μL and the measured dwell volume of the system was 375 μL. The mobile phase flow rate was set at 500 μL/min. Mobile phases were prepared as follows: ammonium formate 10 mM (pH 3.0) by adding an appropriate volume of formic acid and adjusting pH to 3.0 with ammonium hydroxide; ammonium acetate 10 mM (pH 6.0) by adding an appropriate volume of acetic acid and adjusting pH to 6.0 with ammonium hydroxide; and ammonium formate 10 mM (pH 9.0) by adding an appropriate volume of ammonium hydroxide and adjusting pH to 9.0 with formic acid. For modelling retention times, Drylab®2010 Plus modelling software (Molnar-Institute, Berlin, Germany) was employed.

2.2.2. UHPSFC system and interface configuration

The Waters Acquity UPC^2 system was equipped with a binary solvent delivery pump, an autosampler that included a 10 μL loop for partial loop injection, a column oven and a two-step (passive + active) backpressure regulator (BPR). The passive component maintains pressure higher than 104 bar while the active component allows further back pressure increase and fine backpressure adjustments. The injection volume was 2 μL and the measured dwell volume was 440 μL. The hyphenation interface and splitter for UHPSFC–MS are detailed in [7]. Briefly, the UHPSFC system was hyphenated with the MS detector using the following interface configuration: pre-BPR-split + make-up pump. This interface kit purchased from Waters was composed of two serial zero-dead-volume T-unions connected to column outlet. CO_2 miscible make-up liquid delivered by a Waters Isocratic Solvent Manager (ISM) make-up pump was added and mixed to the chromatographic effluent in the upstream T-union, while the downstream T-union acted as a flow splitter. A fraction of the total flow was directed from the downstream T-union to the ESI source, while the remaining mobile phase was directed to the BPR.

The different organic modifiers were prepared as follows: ammonium formate 10 mM was added to methanol, methanol with 2% water, isopropanol and isopropanol with 2% water.

2.2.3. QDa detector

Both UHPLC and UHPSFC were combined with a single quadrupole Acquity QDa detector (Waters, Milford, MA, USA) fitted with a Z-spray electrospray (ESI) ionization source. Being a pre-optimized detector, various ESI parameters, such as desolvation and cone gas (nitrogen) flows were not adjustable and were fixed at 1200 L/h and 150 L/h, respectively. A few ESI parameters (depending on compound and mobile phase flow rate) were manually adjusted, *i.e.*, probe temperature (up to 600 °C), capillary (0.3–1.5 kV for positive ionization mode and 0.3–0.8 kV for negative ionization mode) and cone voltage (0–100 V). In this study, the probe temperature was set at 600 °C, whereas the capillary voltage was set at 1.2 kV (1.0 kV for UHPSFC–MS) and 0.8 kV in ESI positive and negative ionization modes, respectively. In the first step, the MS device was operated in scan mode (mass range m/z of 50–500 Da) for selecting the most appropriate m/z of each analyte for further single ion recording (SIR) detection. Then, the cone voltages were optimized for each compound in the corresponding ESI polarity mode for both UHPLC–MS and UHPSFC–MS experiments and values were reported in Table 1. During MS detection in SIR mode, data acquisition rate was automatically adjusted by the software to generate sufficient data acquisition points for each chromatographic peak, taking into account the number of SIR to monitor and the ESI polarity switching time (25 ms). The gain parameter was fixed at 1. Data acquisition, data handling and instrument control were performed by the Empower v. 4.1 software (Waters, Milford, MA, USA).

2.3. Substrate cocktail incubation procedure

The cocktail approach for elucidating the impact of phytochemicals towards CYP probe reactions was performed as described in [13] with some minor modifications. Briefly, each incubation mixture (100 μL) contained 0.5 mg/mL proteins of pooled HLMs, 50 mM HEPES (pH 7.4), 2 mM NADPH and 8 probe substrates for major hepatic CYPs. The CYP probe substrates and their final concentrations in the incubation were: phenacetin (CYP1A2, 50 μM or 9710 ng/mL), coumarin (CYP2A6, 5 μM or 730 ng/mL), bupropion (CYP2B6, 5 μM or 1200 ng/mL), flurbiprofen (CYP2C9, 5 μM or 1220 ng/mL), omeprazole (CYP2C19, 40 μM or 13,820 ng/mL), dextromethorphan (CYP2D6, 5 μM or 1360 ng/mL), chlorzoxazone (CYP2E1, 40 μM or 6780 ng/mL) and midazolam (CYP3A, 2.5 μM or 810 ng/mL). The chemical structures of the substrates and their CYP-specific métabolites are reported in Fig. 1. Compared to the original cocktail approach [13], omeprazole was preferred over (S)-mephenytoin due to its higher microsomal metabolic rate at lower concentration and the better analytical response of the CYP-specific metabolite. During preliminary experiments, sensitivity obtained with both analytical strategies was not sufficient for the detection of 4′-hydroxymephenytoin produced under incubation conditions and using (S)-mephenytoin at 100 μM (or 21,830 ng/mL). Indeed, this metabolite possessed LOD and LOQ values of approximatively 400 ng/mL and 900 ng/mL, respectively. Omeprazole was incubated at 40 μM to reduce the risk of interaction with other substrates of the cocktail [4]. Two phytochemicals, namely yohimbine and resveratrol, were individually added to the incubation mixture to obtain final concentrations of 10 μM. The control incubation did not contain phytochemicals. After a pre-incubation for 3 min at 37 °C, the CYP450-dependent phase I reactions were initiated by adding an excess of NADPH. The incubation takes place during 20 min at 37 °C under agitation (400 rpm). The enzymatic reaction was stopped by adding 100 μL of ice-cold ACN to the reaction medium. The precipitated proteins were removed by centrifugation (5 min at 10,000 rpm), and an aliquot (150 μL) of the resulting supernatant was transferred to a vial for analysis. Triplicate incubations were carried out. Finally, the impact of phytochemicals on CYPs activities were estimated and discussed.

3. Results and discussion

When studying *in vitro* metabolism using the cocktail approach, there is a need to develop fast and sensitive methods able to discriminate a number of substrates and metabolites. There are two strategies to develop such an assay. The first possibility is to use generic, non-optimized chromatographic conditions, together with the latest generation of MS/MS device offering very high sensitivity and selectivity. However, when dealing with *in vitro* CYP inhibition assay, such a level of sensitivity and selectivity is generally not required and this solution may be quite expensive to apply. As an alternative, a different strategy was proposed, which consists in optimizing as much as possible the chromatographic step to have sufficient selectivity, and to combine this optimized chromatographic method with simple, easy-to-use and relatively

Table 1
Physico-chemical properties and MS conditions for optimal detection of the 8 substrates and 8 metabolites in UHPLC and UHPSFC.

CYP450 isoform	Substrate CYP-specific metabolite	Physico-chemical properties[a]					MS		UHPLC–MS	UHPSFC–MS
		Acidic//basic pKa	LogP	LogD (pH=3)	LogD (pH=7)	LogD (pH=9)	SIR (m/z)	ESI polarity (+/−)	Cone voltage (V)	
1A2	Phenacetin	−//1.4	1.6	1.6	1.6	1.6	180.1	ESI+	10	10
	Acetaminophen	9.9//1.7	0.3	0.3	0.3	0.3	152.1	ESI+	10	5
2A6	Coumarin	–	1.4	1.4	1.4	1.4	147.0	ESI+	15	5
	7-Hydroxycoumarin	7.9//−	1.6	1.6	1.5	0.4	163.0	ESI+	10	5
2B6	Bupropion	−//7.2	3.5	0.4	3.0	3.5	240.1	ESI+	10	10
	Hydroxybupropion	11.9//7.7	2.6	−0.5	1.8	2.6	238.1 (—H_2O)	ESI+	20	25
2C9	Flurbiprofen	4.1//−	4.1	4.1	1.4	0.4	199.1 (—COO)	ESI−	30	25
	4′-Hydroxyflurbiprofen	4.2, 9.6//−	3.3	3.3	0.5	−0.5	215.1 (—COO)	ESI−	25	25
2C19	Omeprazole	8.7//1.6, 4.4	2.2	0.4	2.2	1.9	346.1	ESI+	5	5
	5-Hydroxyomeprazole	8.7,13.4//1.6, 4.1	0.5	0.4	1.6	1.1	362.1	ESI+	10	10
2D6	Dextromethorphan	−//9.1	4.1	1.0	2.0	3.8	272.2	ESI+	15	25
	Dextrorphan	10.1//9.1	3.5	0.4	1.5	3.1	258.2	ESI+	20	30
2E1	Chlorzoxazone	8.5//−	2.2	2.2	2.2	1.6	168.0	ESI−	20	10
	6-Hydroxychlorzoxazone	7.8,10.5//−	1.6	1.6	1.4	−0.1	184.0	ESI−	15	15
3A subfamily	Midazolam	−//6.0	3.9	1.6	3.9	3.9	326.1	ESI+	20	30
	1′-Hydroxymidazolam	13.6//3.6	3.1	2.2	3.1	3.1	342.1	ESI+	15	25

[a] ACD/Labs predicted values.

"cheap" single quadrupole instrument, to achieve the requested sensitivity.

In the present work, UHPLC and UHPSFC methods were developed for the separation of 8 probe substrates and 8 CYP-specific metabolites and the single quadrupole detector was also used to assist and fasten the chromatographic method development procedure (peak tracking).

Due to the limited spectral resolution of the single quadrupole mass spectrometer (m/z 0.7 FWHM), distinguishing between different compounds in MS is not always straightforward. Indeed, various peaks can be observed on one given SIR channel, due to (i) the isotopic abundance of Cl^{35}/Cl^{37} occurring for several compounds, (ii) a small m/z difference for several analytes (*e.g.*, hydroxybupropion, m/z 256 *versus* dextrorphan, m/z 258), and (iii) the in-source fragmentation of metabolites (*e.g.*, loss of water for hydroxybupropion, m/z 256 and thermal degradation of phenacetin into acetaminophen, m/z 152), yielding two identical peaks within the same SIR trace of the corresponding substrates (*e.g.*, bupropion, m/z 240 and phenacetin, m/z 180). For these reasons, a baseline resolution of the 16 compounds in both UHPLC and UHPSFC conditions was required prior to detection when using a single quadrupole analyzer.

3.1. Method development in UHPLC–MS

To develop a powerful UHPLC–MS method for the analysis of the mixture containing 8 substrates and 8 metabolites, a sequential two-step procedure was applied, including a generic screening of UHPLC conditions and a chromatographic optimization method using modelling software.

3.1.1. Screening procedure

As described in Table 2, a generic screening procedure was initially applied to the mixture of 16 standard compounds. Four different UHPLC columns of identical dimensions (50 mm × 2.1 mm, 1.7 μm) were used. In terms of column chemistries, three hybrid silica phases bonded with C18, phenyl, and C18 with polar embedded groups were considered. Because most of the selected substances were ionizable, a charged surface hybrid stationary phase (CSH C18) was also chosen to modify selectivity. Because analytical compounds possess a wide range of physico-chemical properties, three different volatile buffers (*i.e.*, pH 3, 6 and 9) were investigated. Finally, experiments were conducted using the two organic modifiers commonly used in RPLC mode, methanol (MeOH) and acetonitrile (ACN). This screening procedure was similar to the one presented in [14].

A generic gradient from 2% to 90% over 4 min was applied with all the possible combinations of stationary phases, mobile-phase pH, and organic modifiers (corresponding to 24 different conditions). Following these experiments, various chromatographic and MS responses were evaluated, including the retention of the compounds, chromatographic selectivity between the peaks, and sensitivity (background noise and signal).

When applying columns packed with sub-2 μm particles, one of the most important constraints is the elevated pressure drop related to the use of MeOH in the mobile phase. Because MeOH/water mixtures are almost twice as viscous as ACN/water mixtures, ACN is generally considered as the first choice in UHPLC for high-throughput separations at elevated flow rates. Apart from speed of analysis, MS sensitivity also has to be considered to select the most appropriate organic modifier. Similarly to previous findings [15], ionization was enhanced by an average factor 2–5 with MeOH *versus* ACN for the designated set of compounds, suggesting that enhanced sensitivity and lower limits of detection are achieved with polar protic solvents. Because the sensitivities achieved with a miniaturized single quadrupole detector may be limited for *in vitro* metabolism study, and because peak shapes of all of the compounds were comparable regardless of the organic modifier, MeOH was selected to reach the highest possible sensitivity. On the other hand, it is important to consider that the mobile phase flow rate will be limited with such a highly viscous mobile phase, thus increasing analysis time. In this study, a flow rate of 500 μL/min induced a maximal pressure drop of approximately 800 bar was applied with a MeOH/water mobile phase.

The influence of the mobile-phase pH (acidic, neutral and basic) was also evaluated. The neutral pH was not useful due to a lack of sensitivity for many ions. This lack of sensitivity was attributed to the limited number of compounds that were fully ionized at pH 6, as illustrated in Table 1. Indeed, only flurbiprofen, 4′-hydroxyflurbiprofen, dextromethorphan and dextrorphan were ionized under these pH conditions. Other analytes were only partially ionized, and their retentions were moderate. Most of them were eluted with a limited proportion of the organic modifier, which was detrimental for the desolvation of ions in ESI. Because both ionization and desolvation were sub-optimal under neutral pH conditions, sensitivity was systematically too limited. On the contrary, the achieved sensitivities were enhanced at pH 3 and 9. Acidic compounds were completely ionized at basic pH, but the

Table 2
Summary of the initial screening conditions for UHPLC–MS and UHPSFC–MS.

UHPLC			UHPSFC		
Column	pH	Organic modifier	Column	Organic modifier	Mobile phase additives
BEH C18	3	Acetonitrile	BEH	Methanol	Ammonium formate 10 mM
BEH C18	3	Methanol	BEH	Methanol	Ammonium formate 10 mM + 2% water
BEH C18	6	Acetonitrile	BEH	Isopropanol	Ammonium formate 10 mM
BEH C18	6	Methanol	BEH	Isopropanol	Ammonium formate 10 mM + 2% water
BEH C18	9	Acetonitrile	BEH 2-EP	Methanol	Ammonium formate 10 mM
BEH C18	9	Methanol	BEH 2-EP	Methanol	Ammonium formate 10 mM + 2% water
BEH phenyl	3	Acetonitrile	BEH 2-EP	Isopropanol	Ammonium formate 10 mM
BEH phenyl	3	Methanol	BEH 2-EP	Isopropanol	Ammonium formate 10 mM + 2% water
BEH phenyl	6	Acetonitrile	CSH fluoro-phenyl	Methanol	Ammonium formate 10 mM
BEH phenyl	6	Methanol	CSH fluoro-phenyl	Methanol	Ammonium formate 10 mM + 2% water
BEH phenyl	9	Acetonitrile	CSH fluoro-phenyl	Isopropanol	Ammonium formate 10 mM
BEH phenyl	9	Methanol	CSH fluoro-phenyl	Isopropanol	Ammonium formate 10 mM + 2% water
CSH C18	3	Acetonitrile	HSS C18 SB	Methanol	Ammonium formate 10 mM
CSH C18	3	Methanol	HSS C18 SB	Methanol	Ammonium formate 10 mM + 2% water
CSH C18	6	Acetonitrile	HSS C18 SB	Isopropanol	Ammonium formate 10 mM
CSH C18	6	Methanol	HSS C18 SB	Isopropanol	Ammonium formate 10 mM + 2% water
CSH C18	9	Acetonitrile			
CSH C18	9	Methanol			
Shield RP18	3	Acetonitrile			
Shield RP18	3	Methanol			
Shield RP18	6	Acetonitrile			
Shield RP18	6	Methanol			
Shield RP18	9	Acetonitrile			
Shield RP18	9	Methanol			

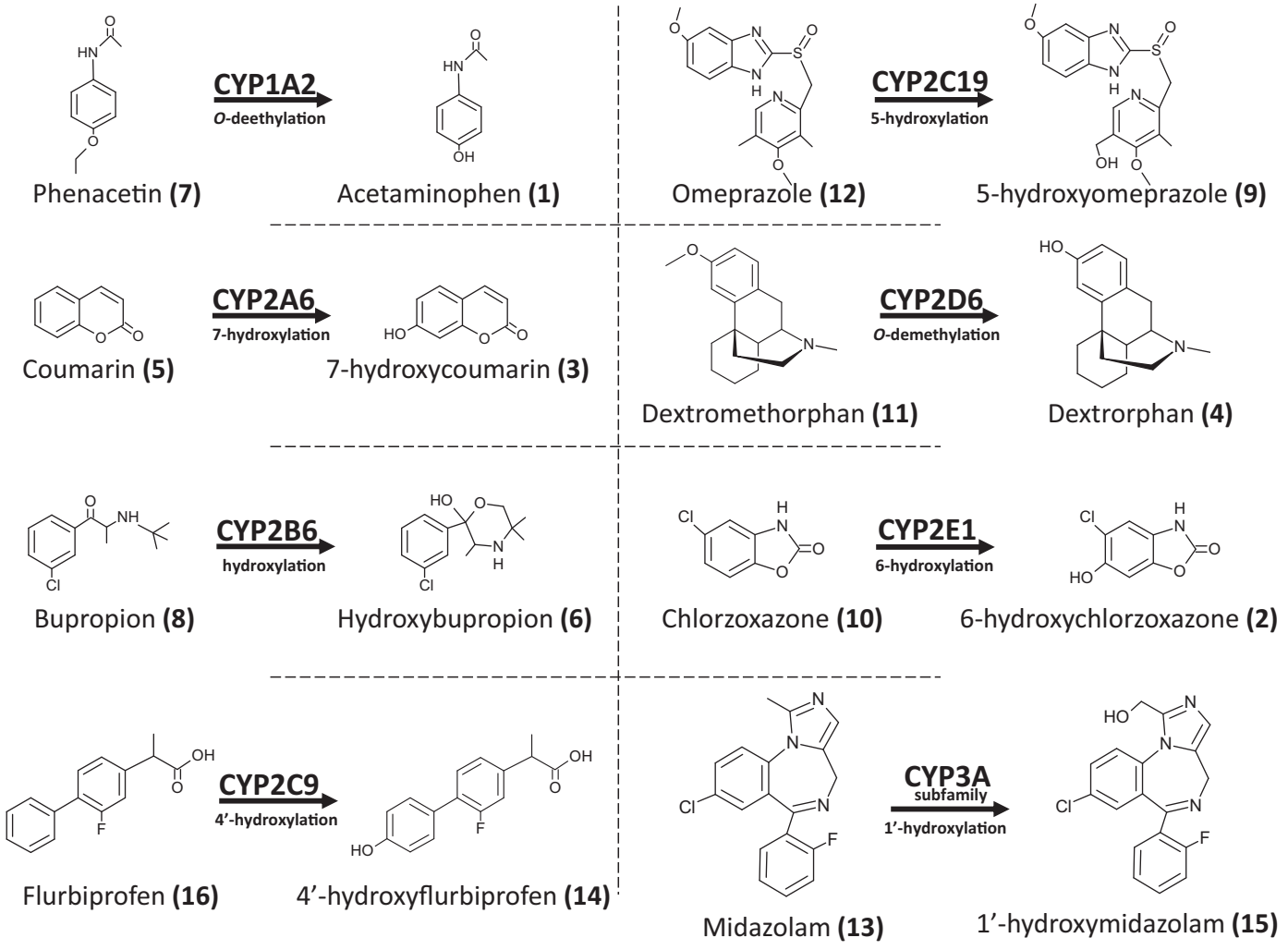


Fig. 1. Chemical structures of the substrates and CYP-specific metabolites.

desolvation of ions was quite limited, as the acidic substrates and metabolites were eluted with a limited proportion of the organic modifier. The situation was exactly the opposite with basic substances; the ionization at pH 9 was poor, but these substances were eluted with a higher proportion of organic modifier, improving the desolvation of ions in ESI. In acidic conditions, either the ionization or desolvation was enhanced compared to neutral pH, for basic and acidic compounds, respectively [16]. From a chromatographic point of view, pH 9 was beneficial for retention and selectivity, but distorted peaks (*i.e.*, tailing, fronting, shouldering and peak splitting) were often observed. For these reasons, the rest of the study was carried out exclusively under acidic pH.

Regarding the selection of stationary phase, the differences in retention and selectivity were not significant. Indeed, no phase outperformed the other ones, regardless of the pH and the organic modifier. All of the compounds were sufficiently retained with the generic gradient, for all of the stationary phases, with differences in the nature and number of critical pairs. As expected, the MS sensitivity was also not a relevant criterion to select the best column, because the sensitivities achieved with the four stationary phases were similar. Therefore, the most versatile, robust and chemically stable stationary phase, the Acquity BEH C18, was selected.

For the screening step, it was valuable to have a compact MS device to unambiguously identify the individual compounds in each condition because it was possible to quickly determine which peaks were poorly detected, distorted or badly resolved. Then, considering the analytical conditions and physico-chemical properties of the problematic compounds, some reliable explanations could be found.

3.1.2. Method optimization procedure

Next to the initial screening procedure, the gradient profile was further optimized using the most promising combination of stationary phase, mobile-phase pH and organic modifier, on the basis of two generic gradients that only differed in slope (7.3% and 22%/min). This gradient optimization was simultaneously performed with an optimization of the mobile phase temperature in the range 30–60 °C within a narrower range of acidic pH (pH 3–4). Overall, the mixture of 16 compounds was injected under 12 different conditions, including two gradients (2–90% in 4 and 12 min) at two temperatures (30 and 60 °C) and three pH conditions (3.0, 3.5 and 4.0). For data manipulation, a linear solvent strength (LSS)-based HPLC modelling software was employed. The most recent version of computer-assisted method development software allows a simultaneous modelling of the effect of three variables for a given separation. In this study, gradient steepness (t_G), temperature (T), and mobile-phase pH were selected as model variables to create a cube resolution map with the three factors [17–19].

However, for a successful method optimization on the basis of LSS based retention models, it is necessary to individual track each peak in each of the different analytical conditions. This task is often tedious and time-consuming. Here, the ability of the simple MS detector to identify the retention times of all of the compounds within the mixture was employed. This method considerably speeds up the method development procedure, in comparison with peak tracking performed with UV detection.

Fig. 2 shows the corresponding 3D surface responses generated by the modelling software. As depicted in this cube representation, the best condition correspond to a linear gradient from 2% to 90% MeOH over 8 min at a temperature of 35 °C and a pH of 3.7. The simulated chromatogram, calculated for an average column efficiency of 7000 plates (*i.e.*, a reasonable plate count for a 50 mm × 2.1 mm, 1.7 μm column) is also provided in Fig. 2. On this simulated chromatogram, the minimal resolution (Rs_{min}) was 2.89 and the most critical peak pair was phenacetin and hydroxybupropion (m/z of 180 and 238), both detected in positive ESI mode.

The experimental chromatogram obtained under optimal conditions is provided in Fig. 3. As shown in Table 3, the discrepancies between simulated and observed retention times were in the range of 0–5.4%, which is acceptable. A baseline separation of all 16 substrates and metabolites was ultimately achieved in approximately 7 min under the optimal UHPLC–MS conditions.

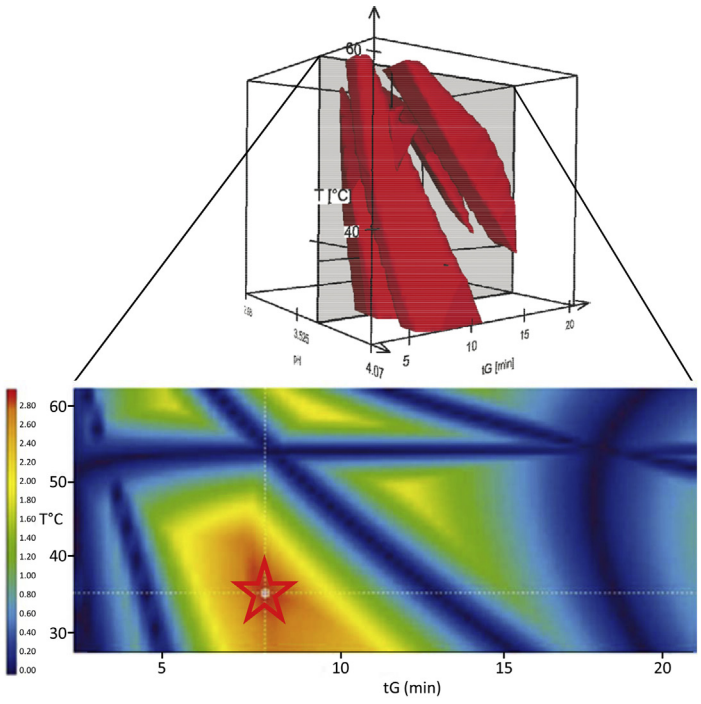
3.2. Method development in UHPSFC–MS

3.2.1. Screening procedure

Similarly to the methodology used for UHPLC–MS, an initial screening procedure was applied in UHPSFC–MS to first find out the best combination of a stationary phase, an organic modifier and an additive. For the stationary phases, columns of 100 mm × 3 mm were exclusively employed to achieve fast kinetic performance. Shorter and/or narrower columns were not compatible with the selected UHPSFC systems because the extra-column band broadening of our instrument was estimated at ~85 μL^2 *versus* only 8 μL^2 for the UHPLC system. Then, severe loss in performance may have been observed with 50 mm × 2.1 mm columns under UHPSFC conditions, as demonstrated elsewhere [20]. Despite an upper pressure limit of only 400 bar, columns packed with 1.7 μm particles were successfully employed due to the limited viscosity of the CO_2-based mobile phase [21]. Four different stationary phase chemistries were selected, including a hybrid silica phase (BEH), a hybrid silica bonded with a 2-ethylpyridine moiety (BEH 2-EP), a silica phase bonded with C18 alkyl chains (HSS C18 SB) and a charged surface hybrid silica particle bonded with a fluorophenyl moiety (CSH FP). As shown in a recent study by Khater et al., these four phases offer a high degree of orthogonality [22]. In addition, the four phases were available with dimensions of 100 mm × 3 mm, 1.7 μm (and 1.8 μm for the HSS C18 phase). Based on our previous experience, only alcohols were tested as organic modifiers, because the performance achieved with acetonitrile, tetrahydrofuran and other types of organic solvents was disappointing with pharmaceutical compounds [23]. Therefore, methanol and isopropanol were considered as organic modifiers for the initial screening step. Because the mixture of substrates and metabolites to be analyzed contains numerous acidic and basic substances, 10 mM ammonium formate was systematically added to the mobile phase [24]. This additive was particularly appropriate because it is volatile and therefore compatible with MS detection. Furthermore, it allows obtaining symmetrical and narrow peaks with both acidic and basic substances. Finally, the screening experiments were carried out in the absence and in presence of 2% water in the mobile phase, as it has been shown that a small addition of water can contribute to enhancing peak shapes, reduce retention times of most polar substances, improve MS sensitivity and modify selectivity under SFC conditions [25].

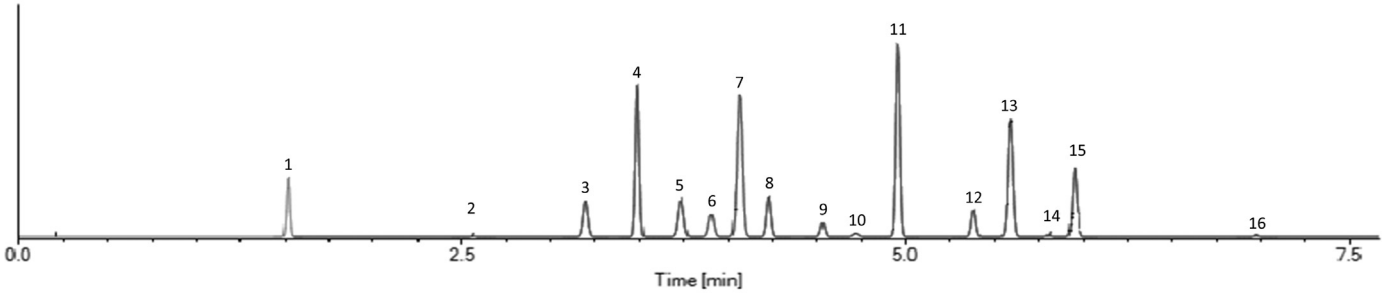
A generic method consisting of a 0.5 min initial isocratic step at 2% followed by a gradient from 2% to 30% in 4 min was applied with all possible combinations of stationary phases, mobile phase additives and organic modifiers (corresponding to 16 different conditions). Following these experiments, the retention of the compounds, the peak shapes, the chromatographic selectivity between peaks and the sensitivity (background noise and signal) were evaluated to find out the best analytical conditions. The single quadrupole device was successfully used to identify the 16 different analytes in all screening conditions.

Among the four stationary phases, the CSH FP performed the worst. Even if all of the compounds of interest were eluted during the generic gradient on this column, the peaks were distorted (tailing and broadening) and most significantly, a strong background noise was observed on numerous SIR channels. This behaviour



Selected working point	
T°	35°C
pH	3.7
plate number	~ 7000
Critical resolution	2.89

Gradient Table	
Time (min)	%MeOH
0.00	2.00
8.00	90.00



Simulated chromatogram

Fig. 2. 2D and 3D surface responses and corresponding simulated chromatogram obtained with modelling software. Conditions: column Acquity BEH C18 (50 mm × 2.1 mm, 1.7 μm), phase A: buffer pH 3.7, phase B: methanol, gradient from 2 to 90% B in 8.00 min, flow rate: 0.5 mL/min, temperature: 35 °C. Peak identification: (1) acetaminophen, (2) 6-hydroxychlorzoxazone, (3) 7-hydroxycoumarin, (4) dextrorphan, (5) coumarin, (6) hydroxybupropion, (7) phenacetin, (8) bupropion, (9) 5-hydroxyomeprazole, (10) chlorzoxazone, (11) dextromethorphan, (12) omeprazole, (13) midazolam, (14) 4′-hydroxyflurbiprofen, (15) 1′-hydroxymidazolam, (16) flurbiprofen.

could be related to bleeding phenomena, related to the presence of a positive charge of an unknown nature at the surface of the hybrid silica particles, to the bonding chemistry of the fluorophenyl group itself, or to presence of residues (metal catalysts) remaining from the bonding chemistry. One of the most important issues observed with the BEH phase was related to the very low retention and unacceptable peak shape of coumarin, which was eluted too early during the initial isocratic step. Various modifications were evaluated to improve its retention, such as reducing the backpressure or decreasing the initial percentage of organic modifier down to 1%, but none of them were successful. Finally, all of the compounds were eluted with adequate retention on the BEH 2-EP and HSS C18 SB phases. However, the peak shape and overall selectivity was enhanced on the BEH 2-EP. For example, there was a triplet of three peaks (m/z of 238, 342 and 326) with a resolution close to zero on the HSS C18 SB phases. Thus, the BEH 2-EP phase was selected for further optimization.

Regarding the choice of organic modifier, the elution strength of isopropanol was lower than that of MeOH, due to a lower H-bond acidity and basicity for isopropanol. This lower elution strength was problematic because a few compounds, such as dextromethorphan, were not eluted on the BEH column even at 30% isopropanol, probably because this compound was the most basic among our set of substrates and metabolites. In addition, the peaks corresponding to basic substances, such as hydroxybupropion on the BEH 2-EP phase, were much broader and tailed. Finally, the sensitivity was also reduced when MeOH was replaced by isopropanol. These observations confirm the superiority of MeOH over isopropanol as an organic modifier for our particular application.

The screening analyses were carried out in the absence and in presence of 2% water within the mobile phase. Contrary to our expectations, the effect of water on the peak shape and selectivity was negligible (especially when MeOH was used as organic modifier), regardless of the stationary phase. However, water was found to increase sensitivity in positive ESI mode, while its sensitivity reduction in negative ESI mode was moderate. Thus, 2% water was systematically added to the mobile phase for the rest of the study.

3.2.2. *Method optimization procedure*

In UHPSFC, computer-assisted method development software cannot be used, because it has been demonstrated that the linear-solvent-strength theory (LSS) was not valid (*i.e.*, that a linear relationship exists between $\log k$ and %MeOH) under SFC conditions [26,27]. In addition, the van't Hoff equation ($\log k = f(1/T)$) has never

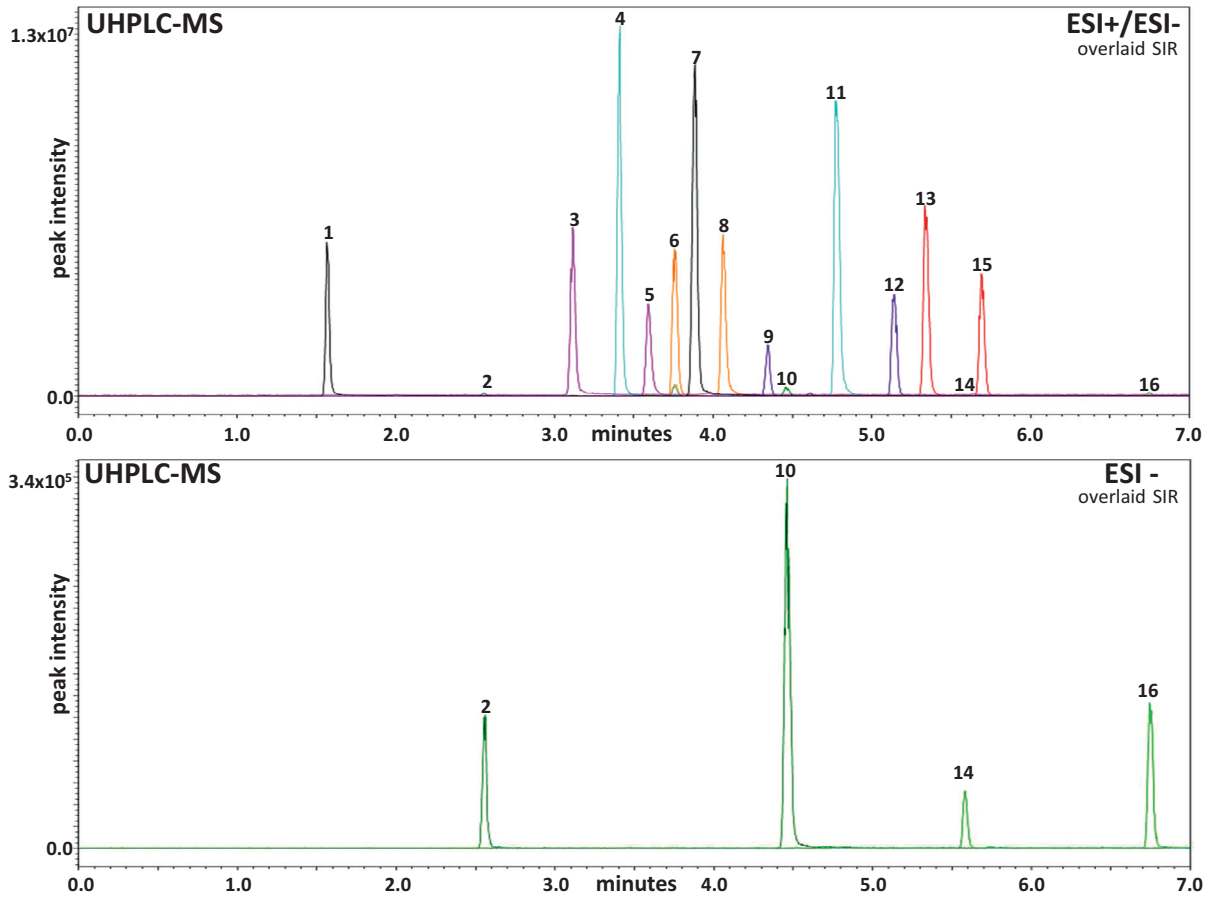


Fig. 3. Optimal chromatograms in UHPLC–MS. Conditions: column Acquity BEH C18 (50 mm × 2.1 mm, 1.7 μm), phase A: buffer pH 3.7 water with 10 mM ammonium formate, phase B: methanol, gradient from 2 to 90% B in 8.00 min, flow rate: 0.5 mL/min, temperature: 35 °C, injection volume: 2 μL. Peak identification similar to Fig. 2.

been employed with supercritical or subcritical fluids and polar stationary phases. Therefore, a simple univariate approach was carried out to improve the separation achieved using the BEH 2-EP phase with the generic gradient previously described. With the generic conditions, the separations between acetaminophen and omeprazole (*m*/*z* of 152 and 346) and between phenacetin, flurbiprofen and midazolam (*m*/*z* of 180, 199 and 326) were insufficient, with a partial co-elution of these species. Therefore, various parameters were investigated to tune the selectivity and improve the separation of the most critical pairs. The effect of the mobile phase temperature in the range 35–45 °C was found to be negligible, most likely because the most critical compounds were eluted with a relatively high proportion of organic modifier, limiting the impact of the temperature on the mobile phase density. Because both critical peak pairs were composed of substances with diverse acidic and basic properties, the concentration of ammonium formate was modified in the range of 5–20 mM, without any effect on selectivity. Finally, the only parameter that had a significant effect on the selectivity was the slope of the gradient. The slope of the gradient was varied between 7.0% and 1.9%/min, and a baseline separation of the 16 compounds was ultimately achieved. To further decrease analysis time, a multistep gradient was applied to improve resolution without

Table 3
Comparison between predicted retention time and experimental retention time for the 16 compounds.

CYP isoform	Substrate CYP-specific metabolite	Predicted retention time (min)	Experimental retention time (min)	Difference (%)
CYP1A2	Phenacetin	4.06	3.89	−4.2
	Acetaminophen	1.52	1.58	+3.5
CYP2A6	Coumarin	3.73	3.60	−3.6
	7-hydroxycoumarin	3.19	3.12	−2.2
CYP2B6	Bupropion	4.23	4.07	−3.8
	Hydroxybupropion	3.90	3.76	−3.6
CYP2C9	Flurbiprofen	6.97	6.75	−3.1
	4′-Hydroxyflurbiprofen	5.80	5.58	−3.8
CYP2C19	Omeprazole	5.38	5.14	−4.5
	5-Hydroxyomeprazole	4.53	4.34	−4.1
CYP2D6	Dextromethorphan	4.95	4.78	−3.5
	Dextrorphan	3.49	3.41	−2.3
CYP2E1	Chlorzoxazone	4.72	4.46	−5.4
	6-Hydroxychlorzoxazone	2.56	2.56	0.0
CYP3A subfamily	Midazolam	5.59	5.34	−4.4
	1′-Hydroxymidazolam	5.95	5.69	−4.3

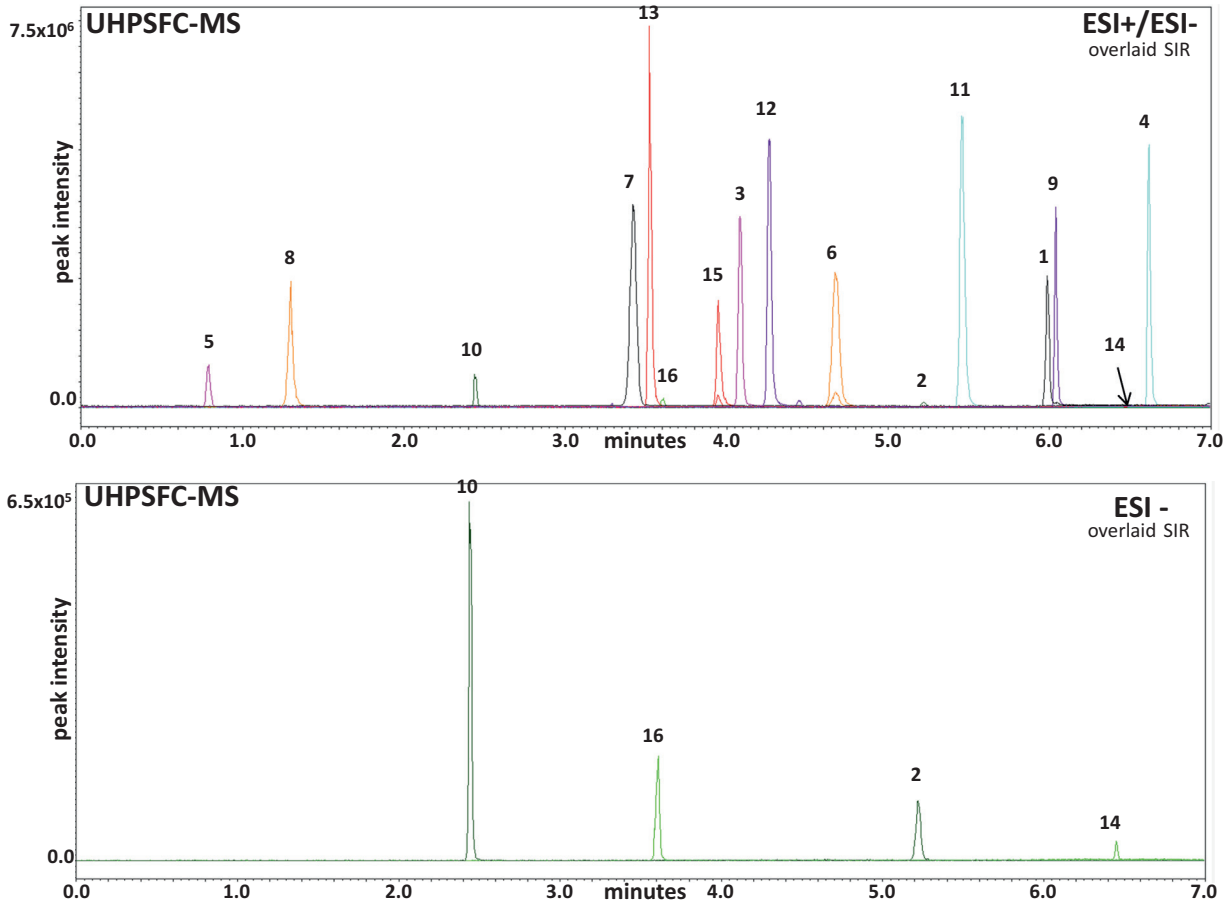


Fig. 4. Optimal chromatograms in UHPSFC–MS. Conditions: column Acquity UPC^2 BEH 2-ethylpyridine (100 mm × 3.0 mm, 1.7 μm), phase A: CO_2, phase B: methanol/water (98:2) with 10 mM ammonium formate, 1% B for 0.5 min then gradient from 1 to 9.5% between 0.5 and 5 min and 9.5 to 30% between 5 and 6.5 min, flow rate: 2 mL/min, temperature: 40 °C, backpressure: 150 bar, make-up pump: ethanol at 0.7 mL/min between 0 and 1.6 min, 0.5 mL/min between 1.6 and 6.2 min and 0.3 mL/min between 6.2 and 7 min, injection volume: 2 μL. Peak identification similar to Fig. 2.

drastically affecting throughput. The final separation shown in Fig. 4 was obtained with an initial isocratic step at 1% MeOH for 0.5 min, to sufficiently retain coumarin, followed by a linear gradient from 1% to 9.5% in 4.5 min. A steeper gradient from 9.5% to 30% was run for 1.5 min to elute the most strongly retained compounds, specifically dextromethorphan, acetaminophen, 4′-hydroxyflurbiprofen, 5-hydroxyomeprazole and dextrorphan. With this method, the 16 peaks were baseline-resolved with an analysis time of approximately 7 min.

3.3. Comparison of achieved sensitivities in UHPLC–MS and UHPSFC–MS

After the method development, the sensitivities achieved with the compact single quadrupole device for the mixture of 8 substrates and their CYP-specific metabolites were evaluated under the optimal UHPLC–MS and UHPSFC–MS conditions described above.

To reach the highest possible sensitivity, the electrospray ionization (ESI) parameters were optimized. With this particular MS detector, the number of parameters that can be adjusted is relatively limited. The probe temperature and capillary voltage were fixed at generic values because their impact on the sensitivity was found to be negligible. On the contrary, cone voltages were optimized for each analyte because its impact on the sensitivity was important. Various cone voltages between 5 and 30 V were tested, and the signal-to-noise ratio was monitored for all these conditions. The optimal cone voltages are reported in Table 1.

For the coupling of UHPSFC and MS, a double T-union interface was employed, similar to the one described in [7]. In this interface, the first zero-dead volume T-union allows the addition of an organic solvent with an external pump to enhance MS ionization and avoid analyte precipitation. The nature of the organic solvent and its flow rate were optimized because both parameters may impact sensitivity. Ethanol (EtOH) was used as a make-up solvent; it offers a good sensitivity for all of the compounds, particularly in negative ionization mode (ESI$^-$). Another benefit of ethanol is its limited environmental impact. Regarding the flow rate, the first eluted peaks (*i.e.*, coumarin and bupropion) were better detected with a high amount of EtOH, while the most strongly retained compounds (*i.e.*, 4′-hydroxyflurbiprofen and dextrorphan) required less EtOH. This behaviour appears quite logical because the proportion of protic solvent contained in the mobile phase was very limited at the beginning of the run (1% of MeOH) and much higher at the end (30% of MeOH). To achieve the best sensitivity for all substances, 0.7 mL/min of EtOH was added from 0 to 1.6 min, 0.5 mL/min of EtOH was used from 1.7 to 6.1 min and 0.3 mL/min of EtOH was employed after 6.2 min.

To evaluate the instrumental sensitivity in UHPLC–MS and UHPSFC–MS, a solution containing the 16 standard compounds was reconstituted at a concentration of 1 μg/mL in a dissolution solvent composed of a 50:50 (v/v) ACN/H_2O mixture with 25 mM HEPES buffer pH 7.4 to mimic the metabolic incubation medium. Successive dilutions of this initial solution were performed with the same diluent to reach final concentrations of 500, 200, 100, 50, 20, 10, 5, 2 and 1 ng/mL. These ten solutions were injected in both UHPLC–MS

Table 4
Limits of quantitation (LOQ, ng/mL) of the 8 substrates and 8 metabolites in UHPLC–MS and UHPSFC–MS.

CYP450 isoform	Substrate CYP-specific metabolite	UHPLC–MS	UHPSFC–MS	Sensitivity modification
1A2	Phenacetin	5	10	÷2
	Acetaminophen	20	20	=
2A6	Coumarin	5	40	÷8
	7-Hydroxycoumarin	50	10	×5
2B6	Bupropion	3	20	÷6.6
	Hydroxybupropion	2	10	÷5
2C9	Flurbiprofen	50	50	=
	4′-Hydroxyflurbiprofen	100	200	÷2
2C19	Omeprazole	3	10	÷3.3
	5-Hydroxyomeprazole	5	20	÷4
2D6	Dextromethorphan	2	2	=
	Dextrorphan	3	10	÷3.3
2E1	Chlorzoxazone	5	5	=
	6-Hydroxychlorzoxazone	30	100	÷3.3
3A	Midazolam	5	4	×1.2
subfamily	1′-Hydroxymidazolam	5	20	÷4

and UHPSFC–MS, and signal-to-noise values were measured for all compounds. The limit of detection (LOD) and limit of quantitation (LOQ) were estimated as signal-to-noise values of 3 and 10, respectively. The LOQ values were reported in Table 4. The LOD values achieved in UHPLC–MS ranged from 1 to 50 ng/mL, while they were between 1 and 75 ng/mL for UHPSFC–MS. The LOQ values were between 2 and 100 ng/mL in UHPLC–MS, while they ranged from 2 to 200 ng/mL in UHPSFC–MS. In agreement with previous findings, the LOD and LOQ were generally worse in ESI^- compared to the positive ionization mode (ESI^+), and the two most acidic compounds (*i.e.*, flurbiprofen and 4′-hydroxyflurbiprofen) had the highest LOD and LOQ values. Based on the concentrations employed for the *in vitro* CYP-dependent metabolism assay (see Section 2.3), the LOQ achieved in both UHPLC–MS and UHPSFC–MS were found to be sufficient. This result confirms that the single quadrupole detector used in this study may be of interest for such an application.

As shown in Table 4, the sensitivities achieved in the UHPSFC–MS were, on average, 3-fold lower than in UHPLC–MS. The 7-hydroxycoumarin was the only compound with a better LOQ (5-times lower) in UHPSFC–MS. For a few compounds (*i.e.*, coumarin, bupropion and hydroxybupropion), sensitivity was 5- to 8-fold better in UHPLC–MS compared with UHPSFC–MS. For all of the other compounds, the change in sensitivity between the two chromatographic modes was more limited and varies between 0.8 and 4.

In a previous study [7], the sensitivity differences between UHPLC–MS and UHPSFC–MS, attributed to the interfacing method and the nature of the solvent entering the ESI/MS were discussed in details. In [7], it was demonstrated that sensitivity was enhanced by a factor of up to 10 in UHPSFC–MS compared to UHPLC–MS, due to a better desolvation of CO_2/MeOH *versus* ACN/H_2O mobile phase. This result contradicts the findings of this new study, and the following explanations could be taken into account. First, the compounds were obviously not identical to the ones employed in [7], and the substrate/metabolite pairs selected here cover a wider range of physico-chemical properties. Indeed, some of the compounds were poorly retained (eluted with 1% MeOH), while some other compounds were eluted with up to 30% of MeOH in UHPSFC conditions. In addition, acidic compounds detected in the ESI^- mode were not evaluated in the previous study. Second, MeOH was used as an organic modifier in UHPLC, while ACN was used in [7]. As discussed in Section 3.1.1, the sensitivity was enhanced by a factor 3–5 when using MeOH rather than ACN. Third, it is important to consider that the injected volumes in UHPLC and UHPSFC were not scaled in proportion to the column volumes. Indeed, the column volume in UHPSFC was 4 times higher than in UHPLC (100 mm × 3 mm *versus* 50 mm × 2.1 mm). In the present case, the injected volume was held constant at 2 μL to limit peak shape issues caused by a large injected volume in SFC [28]. A further increase in detection sensitivity in UHPSFC–MS by a factor of 4 might thus be expected if the injection volume could be proportionally adjusted. Fourth, the ionization source design was different between the triple quadrupole employed in [7] and the modern single quadrupole employed in the present study. As demonstrated in [29], which describes a comparison of sensitivity achieved in RPLC–MS and HILIC-MS with several MS brands/types, modern MS devices offer enhanced evaporation efficiency, which is important for RPLC mobile phases containing high proportions of water. Therefore, it is highly probable that a better desolvation and thus better sensitivity was achieved in UHPLC–MS with the single quadrupole employed in this study, compared with the triple quadrupole employed in [7]. Last but not least, a single quadrupole is much less selective than a triple quadrupole, and the background noise was not identical between UHPLC–MS and UHPSFC–MS conditions, while it was the same with the triple quadrupole employed in [7].

3.4. Application to in vitro CYP-mediated metabolism testing

The cocktail methodology is an attractive approach used to simultaneously monitor the enzymatic activity of the most important cytochromes P450 (CYPs) in clinical or pre-clinical studies. This approach allows the easy characterization of the phase I metabolism of biological systems and also the evaluation of the inhibition and induction properties of xenobiotics [4]. In the present study, the cocktail approach was employed to demonstrate the *in vitro* impact of two phytochemicals (*i.e.*, yohimbine and resveratrol) towards CYP probe reactions, using the UHPLC–MS and UHPSFC–MS methods previously developed. For this purpose, numerous microsomal incubations were performed in triplicates ($k = 3$) and injected in triplicates ($n = 3$) in both analytical platforms. The reaction medium was relatively complex and contained the mixture of 8 CYP probe substrates and their metabolites, 25 mM HEPES buffer at pH 7.4, 0.25 mg/mL of proteins (HLMs), an excess of NADPH as a co-factor, and acetonitrile as a stopping agent for the microsomal reaction. Even if a precipitation of proteins and centrifugation was performed as described in [13], the presence of remaining proteins in the sample injection should be considered. In the case of UHPSFC conditions, both proteins and oligonucleotides (NADPH) might be weakly eluted from the stationary phase and could induce a modification of the surface chemistry, leading to undesired changes in chromatographic properties (*i.e.*, retention, selectivity and peak width). To evaluate this issue, the retention times were monitored during the injection of the incubated solutions. Three individual incubations in the absence of an inhibitor,

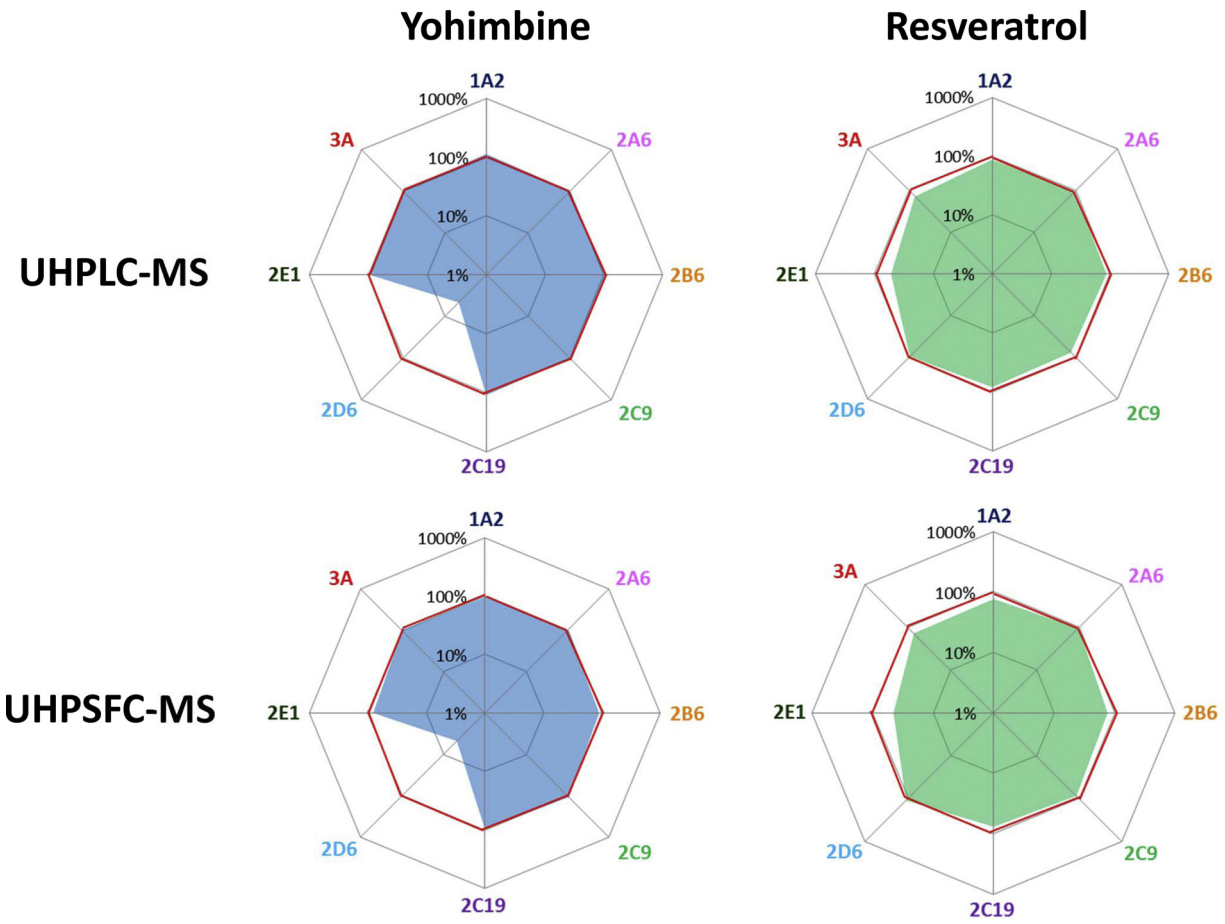


Fig. 5. Determination of the *in vitro* inhibitory potential of two phytochemicals, *i.e.*, yohimbine and resveratrol (both incubated at 10 μM), on the CYP activities with the cocktail approach and using UHPLC–MS and UHPSFC–MS. Microsomal CYP metabolic ratios obtained in presence of phytochemicals are expressed as percentage of the metabolic ratios obtained without inhibitors (100%).

in the presence of yohimbine and in the presence of resveratrol were injected in triplicates. Following the course of these 27 injections, the average RSD values for the retention times of the 16 compounds were 0.14% and 0.15% in UHPLC–MS and UHPSFC–MS, respectively. All the RSD values were between 0.0 and 0.2%, except acetaminophen in UHPLC (0.8%), coumarin in UHPSFC (0.6%) and bupropion in UHPSFC (0.6%). However, this behaviour can be easily explained because these 3 compounds were the least strongly retained compounds. These observations tend to demonstrate that the presence of proteins and oligonucleotides was not critical for either of the two analytical techniques. Apart from the presence of "critical" substances within the injection mixture, it is also important to consider that the final reaction medium was composed of 50% of water and 50% of acetonitrile. This highly aqueous sample diluent may be critical for the first eluted peaks in UHPSFC–MS, but surprisingly no peak distortion was observed, as illustrated in Fig. 4. This observation confirms that UHPSFC can be considered as a viable strategy for *in vitro* metabolism studies.

After the triplicate injections of all of the incubation media, the peak areas were reported for all of the substrates and CYP specific metabolites to assess the inhibition properties of the two phytochemicals. The analytical repeatability for the peak areas was evaluated in both UHPLC–MS and UHPSFC–MS. The average inter-injection repeatability for the peak areas for the 16 compounds was estimated at 7.7% and 10.5% for UHPLC–MS and UHPSFC–MS, respectively, while the inter-incubation variability for the peak areas was 12.4 and 14.5% in UHPLC–MS and UHPSFC–MS, respectively. In all of the cases, the overall variability was found to be acceptable (less than 20%), considering the fact that (i) biological samples were analyzed and (ii) MS detection was employed without any correction by a deuterated or chemically related internal standard, generally included in the quenching solution. The variability achieved with UHPSFC–MS was always slightly superior to the variability with UHPLC–MS, but this difference was not critical for determining the inhibition properties.

To evaluate the impact of the selected phytochemicals on microsomal CYP activities, incubations including the tested compounds at 10 μM were compared to control incubations (*i.e.*, with no inhibitors), as described by Spaggiari et al. [13]. Briefly, the metabolic ratios for all the CYPs, *i.e.*, the ratios of the metabolite to the substrate peaks, were obtained in the presence of yohimbine and resveratrol and expressed as a percentage of the metabolic ratios obtained in the control incubations. To better visualize the activity of all the major CYPs, percentages were represented using an octagonal radar plot representation of CYP phenotypic profiles, where each axis corresponds to the relative activity of the CYP reported in a logarithmic scale. As depicted in Fig. 5, the radar plots obtained for yohimbine and resveratrol were quite similar, confirming that UHPLC–MS and UHPSFC–MS were both able to characterize the *in vitro* inhibition properties of xenobiotics. In the case of yohimbine, the activities of all of the CYPs were similar within 20%, except for CYP2D6. The chromatographic peaks of dextromethorphan (increasing) and its metabolite dextrorphan (decreasing) were strongly affected; corresponding to a reduction of CYP2D6 activity by approximately 20-fold and confirming that yohimbine is indeed a strong selective inhibitor of this isoform [30]. For resveratrol, the activity of CYP2E1 (6-hydroxychlorzoxazone/chlorzoxazone ratio) was reduced by

approximately a factor of 2, and the activity of the members of the CYP3A subfamily (1′-hydroxymidazolam/midazolam ratio) and CYP1A2 (acetaminophen/phenacetin ratio) were diminished by more than 20%. These observations demonstrate that resveratrol moderately inhibits CYP2E1 activity and weakly inhibits CYP1A2 and CYP3A subfamily activities [31].

Finally, the sensitivity achieved with the MS device employed in this study was sufficient to draw reliable conclusions from the cocktail approach. However, it is important to keep in mind that if strong inhibitors of CYP2C9 were tested, it is highly probable that the sensitivity of this particular MS detector would become insufficient to discriminate the level of inhibition, because the amount of produced 4′-hydroxyflurbiprofen would be very low, and the LOQ of this metabolite is relatively high, especially for UHPSFC–MS.

4. Conclusion

A new strategy involving the combination of fully optimized chromatographic method with a simple, compact and cheap single quadrupole MS detector was proposed for a simple *in vitro* CYP inhibition assay using the cocktail approach. This MS device was used with both UHPLC and UHPSFC, and optimized methods were developed to baseline resolve a mixture of 8 substrates and their 8 respective CYP-specific metabolites.

The interest of this new compact MS was first highlighted for the method development step. The use of such an MS device was found particularly attractive for unambiguously identifying and tracking peaks during the method development and allows speeding up this procedure. In the case of UHPLC, a two-step strategy was applied. In the first stage, a screening procedure was applied to determine the best combination of a column, an organic modifier and a mobile-phase pH. Once the best combination was selected, the selectivity was further improved by adjusting the gradient profile, temperature and pH using HPLC modelling software. Ultimately, a baseline resolution of the 16 compounds was achieved, and the analysis time was less than 7 min. A similar procedure was applied to develop a method for UHPSFC. In this case, the optimization of the separation was carried out without the help of modelling software because the retention models have not yet been described under the SFC conditions. However, due to the efficient peak tracking provided by the MS detector, a baseline separation was rapidly obtained, and the analysis time was again less than 7 min.

Following the method development, the sensitivity achieved with this MS detector was evaluated under both UHPLC and UHPSFC conditions for the 16 analytes of interest. The LOQs were between 2 and 100 ng/mL in UHPLC–MS, while they ranged from 2 to 200 ng/mL in UHPSFC–MS. As expected, the sensitivity in the negative ESI mode was systematically lower than in the positive mode. However, based on the concentrations generally employed for current *in vitro* phase I metabolism assays, these LOQs were found to be sufficient.

Finally, the two developed analytical methods, UHPLC–MS and UHPSFC–MS, were applied to an *in vitro* metabolism study using the cocktail approach, where the aim was to evaluate the inhibition properties of two phytochemicals, yohimbine and resveratrol. The same conclusions were drawn with both UHPLC–MS and UHPSFC–MS, that yohimbine was a strong selective inhibitor of the CYP2D6 subfamily, while resveratrol was a moderate inhibitor of the CYP1A2, CYP2E1 and CYP3A subfamily. These observations were in line with previously published data.

In conclusion, this study confirms that the combination of fully optimized chromatographic methods (both in LC and SFC modes), together with single quadrupole detector may be a suitable strategy for *in vitro* CYP inhibition assay. Compared with the gold standard which consists in using generic LC conditions together with state-of-the-art MS/MS devices, our methods may be easier to use for a technician operating in a routine environment. In addition, the instrumental cost is significantly reduced and finally, appropriate levels of selectivity and sensitivity achieved with this approach were demonstrated.

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