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Short communication

Separation of steroid isomers by ion mobility mass spectrometry

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ABSTRACT

Ion mobility mass spectrometry performed in a compact traveling wave cell (TWIM-MS) is shown to provide a reliable, fast and repeatable method to separate derivatized steroid isomers. Three steroid isomer pairs were analyzed in their native form and as their *p*-toluenesulfonyl isocyanate derivatives. The native steroids were separated from each other, but no separation could be attained for the isomers. The derivatized steroid isomers were, however, properly separated by TWIM-MS with peak-to-peak resolutions close to or as high as baseline resolution (R_{p-p} = 0.77–1.08).

1. Introduction

Steroid hormones are synthesized from cholesterol mainly in the adrenal glands and the gonads, and in the human feto-placental unit [1–3]. The high physiological significance of steroids is exemplified by their participation in the regulation of various central metabolic pathways, including reproductive functions, energy metabolism, water and salt balance, and behavioral and cognitive functions [1,4–7]. Additionally, some androgens, due to their positive effects on muscle strength and anabolic actions, serve as doping agents in sports [8–12]. Most steroids consist of α- and β-isomers. The "bent" steroid ring structure associated with β-isomers may have significantly different biological effects in the human body compared to the more planar ring structure in α-isomers [13,14].

The ability to characterize and properly quantitate steroids in biological fluids at trace levels has been a major and challenging analytical goal. Traditionally, steroid hormones have been measured using gas chromatography–mass spectrometry (GC–MS) or immunoassays [15–17]. The suitability of immunoassays in the analysis of complicated biological samples is, however, questionable due to matrix effects and possible cross reactions. GC–MS using electron ionization (EI) seems to alleviate such concerns by providing high selectivity and sensitivity, but the method is time-consuming, since steroid conjugates must be hydrolyzed and derivatized before analysis. Liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS) provides faster analysis of steroids than GC–MS since steroids and their conjugates can be analyzed without derivatization [15–17], but the ionization efficiency of ESI especially for less polar steroids still needs to be improved by derivatization procedures [18–24].

GC–MS and LC–MS techniques provide high specificity and sensitivity in the analysis of steroids, but the long chromatographic runs are often needed to achieve sufficient specificity and separation efficiency of steroid isomers. Therefore, the availability of faster methods in steroid analysis could significantly increase sample throughput and decrease the costs of analysis.

The techniques of ion mobility spectrometry (IMS) and ion mobility spectrometry coupled to mass spectrometry (IM–MS) have been known for decades and they have been used in numerous different applications [25–27]. IM–MS, which is becoming more popular along the development of commercial instruments, provides high sensitivity, specificity, and analysis times on millisecond timescale. The separation in IM–MS is based both on the mass and the charge of the analyte and on other parameters such as the analyte collision cross section and the molecular interactions with the drift gases of different polarizabilities [27,28]. IM techniques

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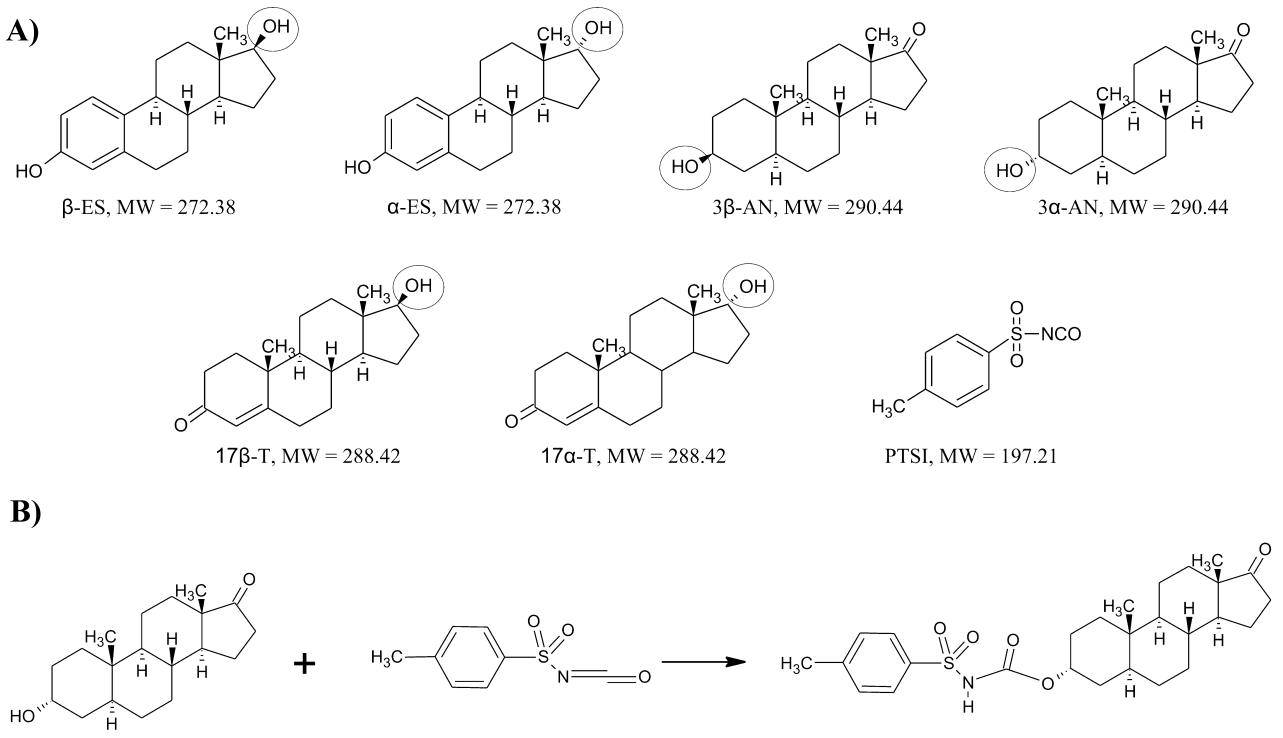

Fig. 1. (A) Structures of the analytes and the derivatization reagent. The site at derivatization is marked by a circle. For abbreviations, see Section 2.1. (B) Example of the derivatization reaction.

are also increasingly used in conjunction with LC–MS [29,30], where IM provides an additional degree of separation and therefore significantly improved peak capacity and specificity. This can be particularly beneficial for example in the analysis of complex biological samples (e.g. in metabolomics and proteomics) [30–32].

The recently introduced traveling wave ion mobility (TWIM) provides a new mode of ion propulsion (and separation) for mobility experiments [33]. The TWIM device combined to MS (TWIM-MS) has been shown to be a powerful tool in bioanalysis providing high ion transmission and good separation efficiency especially when using more polarizable (as well as more massive) drift-gases [30,34,35].

Although IMS and IM–MS are increasingly used in bioanalytics, there are only very few reports on the analysis of steroids with these techniques. Mirmahdieh et al. presented a fast and quantitative analysis method of testosterone in urine samples by atmospheric pressure chemical ionization (APCI)-IMS after sample preparation by molecular imprinted solid phase extraction [36]. Eatherton et al. demonstrated analysis of testosterone, progesterone, and estrone in a comparison of GC-IMS and capillary supercritical fluid chromatography-IMS [37]. Guddat et al. analyzed anabolic androgenic steroids (epitrenbolone, metandienone metabolite, stanozolol, 4β-OH-stanozolol, and 16β-OH-stanozolol) from urine samples by LC–high-field asymmetric waveform ion mobility spectrometry (FAIMS-MS), where FAIMS was used as a "filter" for interference removal before MS-analysis [29]. Kaur-Atwal et al. successfully analyzed the glucuronides of testosterone and epitestosterone by ESI-TWIM-MS, and by ultra performance liquid chromatography (UPLC)-ESI-TWIM-MS [30]. According to our knowledge, thus far this is the only study demonstrating the capability of IM–MS in the separation of steroid isomers.

In this work we have investigated the feasibility of ESI-TWIM-MS in the analysis of selected non-derivatized and *p*-toluenesulfonyl isocyanate derivatized steroid isomers. We also studied the effect of two different drift gases (N_2 and CO_2) at different pressures on the separation efficiency of the steroid isomers.

2. Experimental

2.1. Chemicals

HPLC grade methanol (MeOH) was purchased from J.T Baker (Mexico City, Mexico), HPLC grade acetonitrile (ACN) from Sigma–Aldrich, and analytical grade formic acid (HCOOH) and ammonium hydroxide (NH_4OH) from Acros Organics (New Jersey, United States of America). The water was purified using a Millipore Direct-Q3 UV (Millipore, Billerica, MA, United States of America) purification system.

β-Estradiol (β-ES, 1,3,5-estratriene-3,17β-diol), α-estradiol (α-ES, 1,3,5(10)-estratriene-3,17α-diol), androsterone (3α-AN, 3α-hydroxy-5α-androstan-17-one), *trans*-androsterone (3β-AN, 3β-hydroxy-5α-androstan-17-one), testosterone (17β-T, 17β-hydroxy-3-oxo-4-androstene), epitestosterone (17α-T, 17α-hydroxy-4-androsten-3-one), and the derivatization reagent *p*-toluenesulfonyl isocyanate (PTSI, 96%) were purchased from Sigma–Aldrich (St. Louis, MO, United States of America). The structures of the compounds are presented in Fig. 1A. Stock solutions (1 mg mL^{-1}) of each compound were prepared by dissolving the analytes in MeOH. Further dilutions with MeOH were made to achieve the working standard solutions of each compound.

The steroids were derivatized (Fig. 1B) according to a previously reported method [38]: 100 μL of the working standard solution of each analyte was transferred to a glass vial. The solution was evaporated to dryness under a stream of nitrogen. 100 μL of ACN and 20 μL of the derivatization reagent (PTSI) (1:10, v/v in ACN) were added to the vial. The sample mixture was vortexed for 2 min at room temperature. The derivatization reaction was stopped by adding 20 μL of water and vortexing for 30 s. After this, 860 μL of MeOH, including 0.1% NH_4OH, was added to the solution.

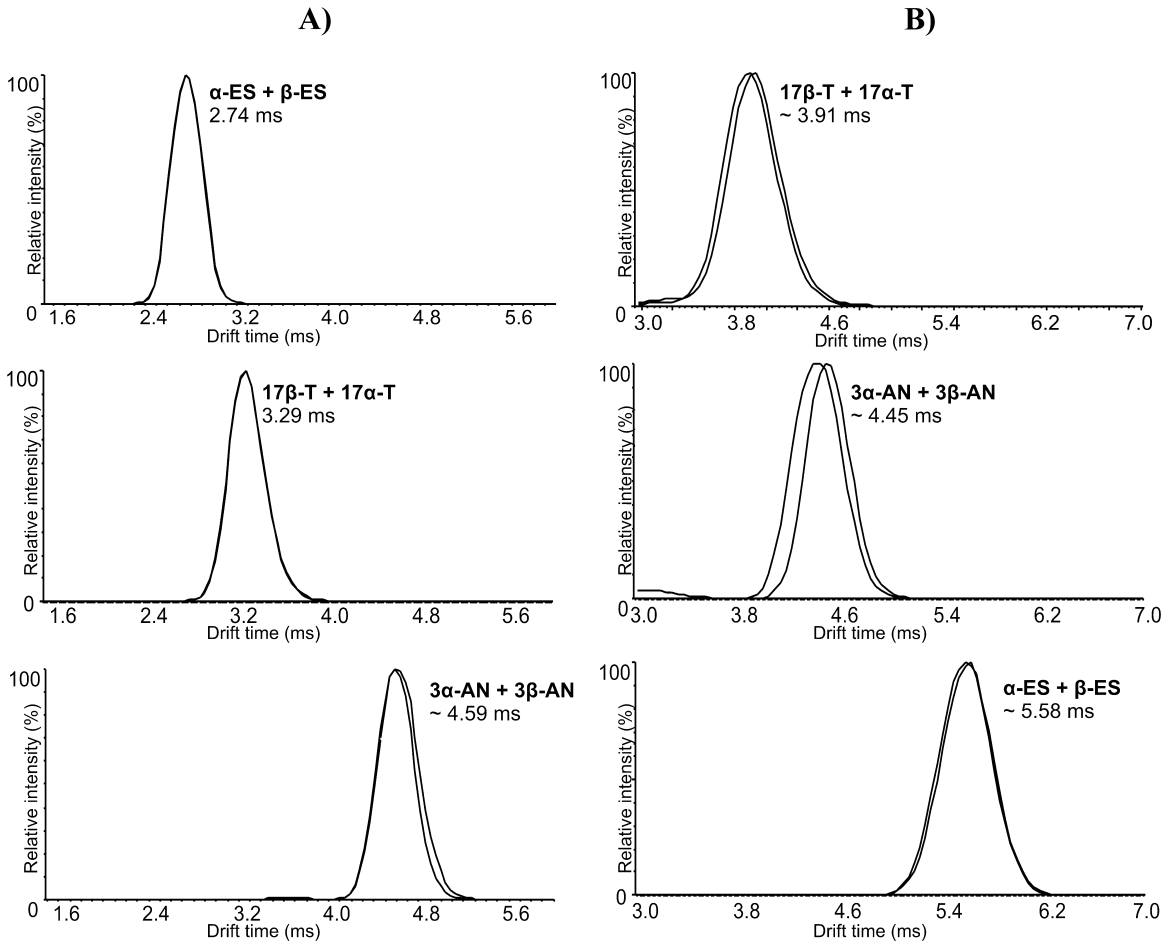

Fig. 2. Selected ion mobility responses of the native steroid isomers (10 μg mL^{-1} in MeOH + 0.1% NH_4OH or 0.1% HCOOH): (A) N_2 (2 mbar) as drift gas and (B) CO_2 (1.3 mbar for α-ES & β-ES and 17β-T & 17α-T, and 1.0 mbar for 3α-AN & 3β-AN) as drift gas. α-ES and β-ES were detected as $[M-H]^-$ in the negative ion mode, whereas 3α-AN and 3β-AN were detected as $[M+Na]^+$, and 17β-T and 17α-T as $[M+H]^+$ in positive ion mode.

2.2. Nuclear magnetic resonance

The derivatization of the hydroxyl group at position C17 of α-ES was confirmed by nuclear magnetic resonance (NMR) using ^1H, ^{13}C, COSY, HSQC and HMBC NMR analyses. In order to achieve the amount of product needed for NMR analysis, a chemical synthesis was performed in a larger scale before the actual analysis step. For more detailed information and results, see Electronic Supplementary Information 1.

2.3. Mass spectrometry and ion mobility mass spectrometry

The mass spectrometer was a Waters Synapt HDMS (Waters, Manchester, UK) with a hybrid quadrupole/traveling wave ion mobility/orthogonal acceleration time-of-flight (oa-ToF) geometry. The mass spectra were acquired using direct infusion (30 μL min^{-1}) and electrospray ionization (ESI) in either positive or negative ion mode depending on the analyte. The capillary voltage was kept at 2.5 kV throughout the studies. The software MassLynx 4.1 (Waters, Manchester, UK) was used for data acquisition. Mass spectra were measured at a scan range of *m*/*z* 200–550 and product ion spectra at *m*/*z* 50–600. For product ion spectra (Electronic Supplementary Information 2, Figs. S1 and S2) the transfer collision energies (CE) were optimized (Electronic Supplementary Information 2, Table S1).

In the TWIM-MS measurements, the steroid isomers were analyzed as native compounds (without derivatization) at a concentration of 10 μg mL^{-1}. For analyses in the positive ion mode, the dilution of stock solutions was made in MeOH + 0.1% HCOOH and for negative ion mode in MeOH + 0.1% NH_4OH. The drift gas pressure was 2.0 mbar with N_2 for all isomers. With CO_2 the drift gas pressure was 1.3 mbar for α-ES, β-ES, 17β-T, and 17α-T. For 3α-AN and 3β-AN the CO_2 pressure was 1.0 mbar. 17β-T, 17α-T, 3α-AN, and 3β-AN were analyzed in positive ion mode, while α-ES and β-ES were analyzed in negative ion mode.

The PTSI-derivatized steroid isomers were analyzed at a concentration of 10 μg mL^{-1} (in MeOH + 0.1% NH_4OH) using N_2 and CO_2 as drift gases in negative ion mode. The TWIM separation efficiency of the derivatized steroid isomers was studied using N_2 with pressures of 2.0 mbar, 2.5 mbar and 3.0 mbar, and CO_2 with pressures of 1.0 mbar and 1.3 mbar. The peak-to-peak resolutions (R_{p-p}) were calculated for each isomer pair using previously presented definitions [39]. The TWIM-MS conditions and the ionization parameters were optimized and the results are shown in Electronic Supplementary Information 2 (Tables S1 and S2, respectively).

3. Results and discussion

17β-T and 17α-T were the best ionized via ESI(+) as $[M+H]^+$, whereas $[M+Na]^+$ dominated the spectra for 3α-AN and 3β-AN. For α-ES, β-ES and all the PTSI derivatives, ESI(−) was more efficient and these molecules were therefore monitored as $[M-H]^-$. The separation efficiency of the steroid isomers in their native forms

Table 1
R_{p-p} of the TWIM-MS separation of the PTSI derivatized steroid isomers.

Isomer pair	CO_2		N_2		
	1.0 mbar	1.3 mbar	2.0 mbar	2.5 mbar	3.0 mbar
α-ES & β-ES	0	0.55	0.68	0.77	0.78
3α-AN & 3β-AN	0.90	0.98	0.92	1.08	1.24
17α-T & 17β-T	0.60	0.61	0.74	0.93	0.84

was studied using both N_2 and CO_2 as drift gases. Under optimized conditions (2 mbar N_2 or 1.0/1.3 mbar CO_2) the three different steroids ES, AN and T were successfully separated within milliseconds (Fig. 2) which shows that TWIM-MS can provide very fast analysis of steroids. The α/β steroid isomers, however, could not be separated from each other under any of the drift gas conditions tested. This fail is likely due to very similar collision cross sections of the isomers as well as similar strengths of their ion/molecule interactions with the drift gases, although the β-isomers have a more bent ring structure and were expected to be more compact and therefore to display shorter drift times than the α-isomers with more planar ring structures.

Because the steroid isomers could not be separated in their native forms (Fig. 2), they were PTSI-derivatized in order to increase the difference of either the strength of their ion/molecule interactions with the drift gas and/or the difference in shape, that is, their collision cross sections (Fig. 1B) and thus to improve the separation efficiency of the isomers. The effect of N_2 and CO_2 as drift gases at different pressures was studied in order to achieve maximum separation efficiency and sensitivity. When the pressure of N_2 was raised from 2.0 mbar to 3.0 mbar and, for CO_2, from 1.0 mbar to 1.3 mbar the R_{p-p} between the different steroid isomer pairs increased from 0.68–0.92 to 0.78–1.24 and from 0.00–0.90 to 0.55–0.98, respectively (Table 1), indicating clearly that R_{p-p} increased when the drift gas pressures were raised. The sensitivity decreased, however, about 10-fold when the pressure of N_2 was raised from 2.0 mbar to 3.0 mbar and about 2–3-fold when the pressure of CO_2 was raised from 1.0 mbar to 1.3 mbar. Based on these results, the best compromise between R_{p-p} and sensitivity was achieved with pressures of 2.5 mbar of N_2 and 1.3 mbar of CO_2. The comparison between drift gases at optimized pressures show that all three pairs of α/β isomers could be properly resolved (Fig. 3), that is, 3α-AN and 3β-AN, 17β-T and 17α-T, and α-ES and β-ES were separated with R_{p-p} of 1.08, 0.93, and 0.77, respectively, with N_2 as the drift gas at 2.5 mbar, whereas R_{p-p} with CO_2 at 1.3 mbar were 0.98, 0.61, 0.55 (Table 1). These results, as well as the resolutions measured at other pressures (N_2 at 2.0 and 3.0 mbar and CO_2 at 1.0 mbar), indicate that N_2 as the drift gas provides better separation efficiency than CO_2 for the PTSI-derivatized steroids isomers.

Limits of detections (LOD) were measured using N_2 as a drift gas at the pressure of 2.5 mbar (Table 2). The calculated LODs were below 25 ng mL^{-1} (at S/N = 3) for all isomers indicating reasonable sensitivity. Repeatability of injection was determined by five

Table 2
Limits of detection (LODs), repeatability of injection, repeatability of drift-times, and repeatability of derivatization reaction.

Steroid isomer	LOD (ng mL^{-1})	%RSD$_{peak-area}$ (injection)	%RSD$_{drift-time}$ (injection)	%RSD A (derivatization)
		100 ng mL^{-1}, N = 5		10 μg mL^{-1}, N = 4
α-ES	4	5.1	0.9	1.9
β-ES	6	6.8	0.5	6.4
3α-AN	5	12.2	1.2	3.6
3β-AN	18	4.3	1.8	3.5
17β-T	15	7.3	0.8	4.3
17α-T	22	5.8	0.8	4.8

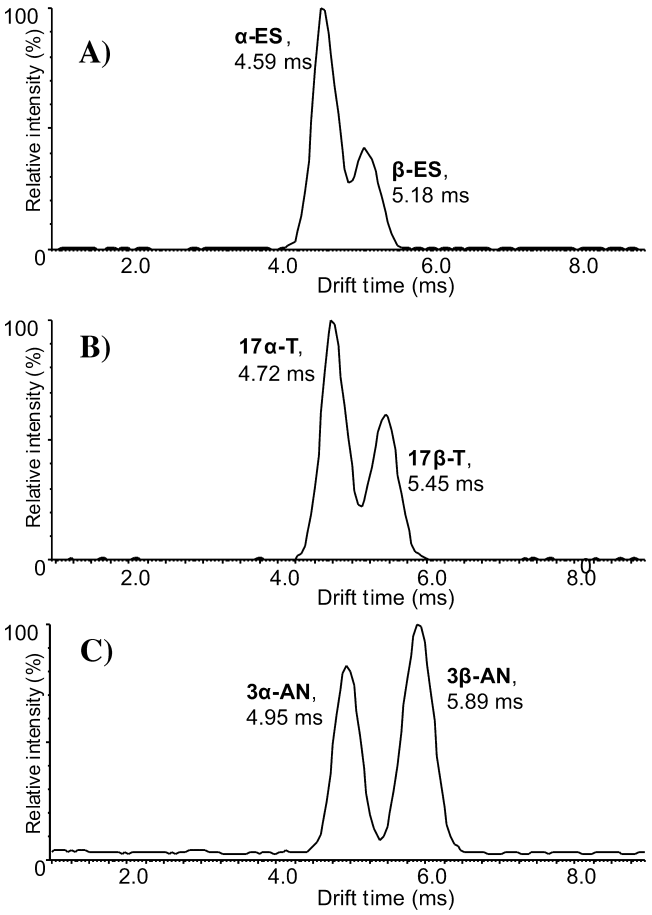

Fig. 3. Selected ion mobility responses of the PTSI-derivatized steroid isomers (10 μg mL^{-1} in MeOH + 0.1% NH_4OH) using N_2 (2.5 mbar) as the drift gas: (A) α-ES & β-ES, (B) 17β-T & 17α-T, and (C) 3α-AN & 3β-AN. The steroid isomers were detected as $[M-H]^-$ in negative ion mode.

replicate injections of the sample with a concentration of 100 ng mL^{-1}. The results show good quantitative repeatability for all steroid isomers as the relative standard deviations (%RSD) were below 13 (Table 2). Repeatability of the derivatization reaction was measured by injecting four separately prepared samples of the steroid isomers at a concentration of 10 μg mL^{-1}. The results show that the derivatization reaction is repeatable for all steroid isomers, with %RSDs below 7 (Table 2). The drift times varied between different measurements (N = 5) less than 2% indicating very good robustness of the TWIM-MS separation.

4. Conclusions

We have demonstrated the feasibility of TWIM-MS for separating steroid isomers under different drift gas conditions. The proposed method uses derivatization with *p*-toluenesulfonyl isocyanate of the steroid isomers, since the native steroid isomers appear to display very similar collision cross sections and therefore to be hard to separate by TWIM-MS. The derivatization increased the collision cross section of the isomers and/or the strength of their ion/molecule interactions with the drift gas and hence improved their separation in the ion mobility cell. As a result, the steroid isomers could be successfully separated showing almost baseline R_{p-p} resolution. This result is of great importance for steroid analysis since TWIM-MS can be performed on a millisecond scale and therefore this method can provide immediate separation, characterization and quantitation of α/β steroids. This feature can be a great advantage in routine laboratories as for instance in forensic

and doping analysis where steroid isomers need to be rapidly and reliably separated.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2013.08.056.

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