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Journal of Chromatography A, 1033 (2004) 321–331
JOURNAL OF CHROMATOGRAPHY A
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Normal-phase high-performance liquid chromatographic separations using ethoxynonafluorobutane as hexane alternative II. Liquid chromatography–atmospheric pressure chemical ionization–mass spectrometry applications with methanol gradients

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Received 13 October 2003; received in revised form 23 January 2004; accepted 26 January 2004

Abstract

We have reported recently that high-speed normal-phase (NP) HPLC separations of a broad range of organic compounds can be performed on cyano columns using gradients of methanol in hexane-like solvent—ethoxynonafluorobutane (ENFB), available commercially. In this communication, we demonstrate that atmospheric pressure chemical ionization (APCI) in combination with mass spectrometry (MS) can be effectively used for detection in such separations. The efficiency of APCI under conditions studied has also been compared to the efficiency of traditional electrospray ionization (ESI) in combination with MS for reversed-phase (RP) HPLC of the same compounds. The compounds included in this study were steroids, benzodiazepines, and other central nervous system-active substances, nonsteroidal anti-inflammatory drugs, tricyclic antidepressants, and β-adrenergic blocking agents. Non-polar compounds were found to respond stronger when APCI–MS technique was used, whereas APCI and ESI ionization efficiencies were comparable when polar substances were studied. The combination of normal-phase HPLC separation conditions with mass spectral detection may expand the range of LC–MS applications traditionally associated with reversed-phase HPLC and ESI–MS detection.

Keywords: Gradient elution; Mobile phase composition; Ethoxynonafluorobutane; Steroids; Benzodiazepines; β-Blockers; Nonsteroidal anti-inflammatory drugs; Antidepressants

1. Introduction

Modern liquid chromatography combined with selective and sensitive mass spectrometry (LC–MS) plays a vital role in the development of new drugs. It is used at the discovery stage to help analyze and purify new molecular entities, determine their physico-chemical properties, evaluate pharmacokinetics, and identify major metabolites of potential drug candidates. Medicinal synthetic chemistry has benefited from the application of modern separation (HPLC) and detection technologies [atmospheric pressure electrospray ionization (ESI) and chemical ionization (APCI)] to the analysis of intermediates and final products for numerous research programs. Rapid structure–activity relationship studies are often facilitated by combinatorial and parallel synthetic efforts within a specific discovery program. Despite numerous advances in combinatorial methodology, the isolation and/or purification of synthesized products remains a bottleneck of the process. The use of commercially available automated systems for UV- and mass-directed semi-preparative and HPLC purification, in most cases, relies upon reversed-phase (RP) HPLC methods and UV and MS detectors (mainly with ESI) triggering the fraction collection [1–3]. The main features of the RP approach include the use of short columns and generic gradients of acetonitrile or methanol in acidified aqueous media and usually have good selectivity for sample components. Mass-directed fraction collection takes advantage of the ability of many organic molecules to produce ions with minimal fragmentation in the ESI source under aqueous conditions. An APCI source is usually employed in

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doi:10.1016/j.chroma.2004.01.042

RP-HPLC to improve detection characteristics and sensitivity of a particular analytical method (e.g., [4–10]).

Normal-phase (NP) chromatographic methods using organic solvents can provide a practical alternative to aqueous conditions by utilizing better solubility, selectivity different from RP mobile phases, and ease of solvent removal from fractions isolated, while generation of significant amounts of toxic and flammable waste is considered among its disadvantages [11].To address the industry needs for a simple, robust, fast, reproducible, and efficient separation approach for a wide variety of organic compounds, we have demonstrated recently that a majority of such substances can be chromatographed on cyano columns using gradients of methanol in a hexane-like solvent—ethoxynonafluorobutane (ENFB), available commercially [12]. This non-toxic, non-flammable, environmentally friendly, recyclable, relatively cheap, and non-polar solvent may effectively replace hexane in a number of normal-phase applications.

Recently, we have conducted a series of experiments to evaluate the ability of an APCI–MS interface to serve as a detector for normal-phase HPLC when such conditions were used to separate various (mainly, drug-like) organic compounds. We report here the results on the ability of different solutes to be ionized in the APCI source and subsequently analyzed by a mass spectrometer. LC–MS response intensity and solutes' fragmentation under APCI conditions have also been compared to data obtained when conventional RPLC–MS with an ESI interface was employed for the same group of compounds.

2. Experimental

2.1. Reagents and solvents

All compounds used in this study were purchased from Sigma–Aldrich (St. Louis, MO, USA) or obtained from the company's compound bank. Their structures can be found on the Sigma–Aldrich web page (www.sigmaaldrich.com) or in the Merck Index [13]. Structures of paroxetine and ebastine are shown in Fig. 1.

Ethoxynonafluorobutane was purchased as Novec Engineered Fluid HFE-7200 from 3M Co. (St. Paul, MN, USA). Its chromatographic properties are summarized in [12]. All other solvents were of HPLC grade and obtained from EM Science (Gibbstown, NJ, USA).

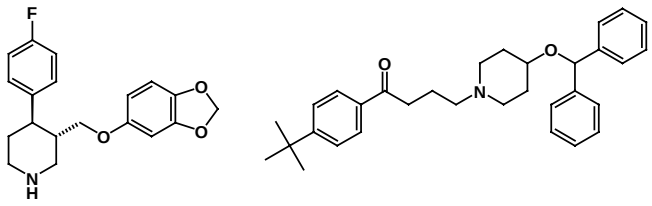

Fig. 1. Structures of paroxetine and ebastine.

2.2. HPLC and MS instrumentation

An 1100 Series LC–mass-selective detector (VL model, Agilent Technologies, CA, USA) detector equipped with APCI source and combined with Agilent's 1100 Series autosampler (model G1329A), thermostatted column compartment (G1316A), and diode-array detector (G1315A) was used for normal-phase LC–MS separations. The instrument was also equipped with four-solvent (model G1312A with additional two-way low-pressure solvent switching valve) and two-solvent (G1312A) gradient pumps. Both pumps were connected to the flow path using a T-connector. Such a set-up allowed for the use of six solvents in three HPLC methods applied.

Another LC–mass-selective detector equipped with ESI source and combined with Agilent's 1100 Series autosampler (model G1329A), thermostatted column compartment (G1316A), diode-array detector (G1315A), and binary gradient pump (G1312A) was used for gradient RPLC–MS.

2.3. Columns and mobile phases

NP-LC–MS separations were carried out at 35 °C on 5, 10, and 15 cm Luna CN columns (Phenomenex, Torrance, CA, USA) with 0.2 cm i.d. packed with 3 μm particles. Neutral compounds were chromatographed on a 5 cm column using a linear gradient of 1–40% of methanol in ENFB in 5 min (∼20 column volumes), the column was then washed with methanol for 2.5 min and re-equilibrated with starting solvent for 2.5 min (Tables 1 and 2).

The separation of a mixture of eight progestins and corticosteroids was achieved on a longer (10 cm) column with linear gradient of 3–5% of MeOH in ENFB for 10 min, then 5–30% from 10 to 20 min. A mixture of three benzodiazepins was resolved on a 15 cm cyano column using linear gradient of 3–40% of MeOH in ENFB for 10 min and flow rate 0.4 ml/min.

A linear gradient (5–50%) of MeOH containing 0.1% ammonia (prepared from 2 M solution of ammonia in methanol, Sigma–Aldrich) in pure ENFB and a 5 cm × 0.2 cm column were used for NPLC–MS of basic compounds (Table 3).

The separation of mixtures of six tricyclic antidepressants and four β-blockers were carried out on a 10 cm × 0.2 cm column using 3–50 and 5–50% gradients, respectively, of MeOH (0.1% NH_3) in ENFB in 10 min and at 0.4 ml/min flow rate.

Acidic compounds were chromatographed on a 5 cm × 0.2 cm column using MeOH gradient (1–20% in 5 min) with 0.1% of acetic acid added to both solvents (Table 4).

The separation of a mixture of six profens was carried out on a longer column (10 cm × 0.2 cm) under the same gradient conditions but extending the gradient to 10 min.

A Luna C_{18} (3 cm × 0.2 cm) column packed with 5 μm particles (Phenomenex) was used for RP-HPLC at 35 °C and 0.6 ml/min flow rate with a linear gradient (∼10 column

Table 1
LC–MS of steroids[a]

Compound name	t_R in NPLC–MS[b] (min)	M_r	Main ion in the spectrum, *m/z*, NPLC–MS	Other ions in the spectrum, *m/z*[c], NPLC–MS	Main ion in the spectrum *m/z*, RPLC–MS[d]	Other ions in the spectrum, *m/z*[c], RPLC–MS	S/N in NPLC–MS, TIC	S/N in RPLC–MS, TIC
11-Pregnen-3,20-dione	1.18	314	315	297 (40)	315	297 (20)	175	10
5-Pregnen-3β,20β-diol	2.77	318	283	301 (60)	283	301 (40)	186	5
5β-Pregnane-3α-ol-20-one	2.13	318	301	283 (53)	301	283 (80)	176	7
5β-Pregnane-3α,17α-diol-20-one	2.68	334	299	317 (20), 281 (20), 271 (30)	299		180	7
5α-Pregnane-3β,20α-diol	2.81	320	285	303 (20)	285		170	5
5α-Pregnane-3,20-dione	1.09	316	317	299 (53), 633 (20)	317	299 (25)	182	7
5β-Pregnane-3,20-dione	1.11	316	299	317 (80), 281 (25)	299	281 (70), 317 (23)	177	8
5β-Pregnane-3α,11β,17α,20β-tetrol	3.17	352	299	273 (60), 255 (40), 317 (30), 281 (20)	299	281 (35), 256 (23), 275 (20), 376 (20)	176	9
5β-Pregnane-3α,20α-diol	2.76	320	285		285		176	9
5β-Pregnane-3β-ol-20-one	2.01	318	301	283 (50)	301	283 (75)	180	14
5α-Pregnane-17α-ol-3,20-dione	2.34	332	315	333 (40), 287 (25)	333	315 (20), 355 (18)	179	8
5β-Pregnane-17α-ol-3,20-dione	2.35	332	315	297 (45), 283 (40)	315	298 (70)	180	8
5α-Androstane-3β-ol-16-one	2.24	290	291	273 (85), 319 (40), 215 (33), 255 (30)	291	273 (90), 215 (60), 255 (40), 233 (35)	81	32
5β-Androst-3α,17β-diol diacetate	0.64	376	257	317 (37)	257	399 (30)	77	26
5α-Androstane-3α,17β-diol	2.72	292	257	275 (38)	257	275 (40)	81	21
5β-Androstane-3α,17β-diol	2.76	292	257	275 (85)	257	275 (70)	83	33
5α-Androst-3α,17β-diol 3-acetate	1.79	334	257	275 (97)	257	275 (70)	81	27
Dexamethasone	3.52	392	373	313 (38), 355 (35), 333 (30), 295 (30), 393 (18)	373	355 (45), 382 (25)	173	21
Progesterone	1.36	314	315		315		151	5
17α-Hydroxy-progesterone	2.49	330	271	253 (45)	331		170	21
20β-Hydroxy-pregn-4-ene-3-one	2.36	316	317	299 (20)	317		83	79
20α-Hydroxy-pregn-4-ene-3-one	2.42	316	317	299 (20)	317		84	81
11β-Hydroxyprogesterone	2.67	330	331	313 (30)	331		84	46
11α-Hydroxyprogesterone	2.82	330	331		331		83	82
Corticosterone	3.21	346	347	329 (30)	347		84	66
Hydrocortisone	3.52	362	363	303 (40)	363		175	25
20α-Dihydrocortisole	4.03	364	365	347 (25)	365		66	55
Estrone	2.51	270	271	253 (45)	271	253 (65)	90	12

[a] See data and discussion in [4–10] for comparison of ESI–MS and APCI–MS responses under reversed-phased conditions.
[b] Luna CN, 2 mm × 50 mm; 0.6 ml/min; gradient 1–40% MeOH in ethoxynonafluorobutane in 5 min; (+) APCI–MS.
[c] Number in parenthesis represents ion's relative intensity.
[d] Luna C_{18}, 2 mm × 30 mm; 0.6 ml/min; gradient 20–100% acetonitrile in water (0.1% HCOOH) in 3 min; (+) and (−) ESI–MS.

Table 2
LC–MS of neutral compounds

Compound name	t_R in NP LC/MS (min)	M_r	Main ion in the spectrum, *m/z*, NPLC–MS	Other ions in the spectrum, *m/z*[a] NPLC–MS	Main ion in the spectrum *m/z* RPLC–MS	Other ions in the spectrum, *m/z*[a], RPLC–MS	S/N in NPLC–MS, TIC	S/N in RPLC–MS, TIC
β-Carotene	0.65	536	537	457 (5)		No response	66	No response
α-Tocopherol	0.93	430	429	461 (50)	429	452 (35)	75	4
Naphthalene	0.51	128	129			No response	5	
Triphenylene	0.64	228	229			No response	22	
Mieschler ketone	0.94	178	161	179 (55), 137 (40)	161	137 (35), 179 (20)	82	15
Guaphenesine	2.47	198	125	151 (75), 163 (75), 137 (25), 199 (20)	163	125 (95), 152 (73), 135 (50), 221 (25)	52	7
2-Phenylcyclohexanone	0.62	174	175	107 (40), 157 (30)	144		42	15
Trans-stilbene oxide	0.57	196	197		106	197 (85)	81	8
Benzoin	1.23	212	167	195 (85)	167	195 (40)	63	43
Theophylline	3.11	180	181		181		31	12
2-Naphthol	1.86	144	145	155 (22)	145		29	8
Metronidazole	2.71	171	128	172 (95)	128	172 (20)	176	22
Antipyrin	2.47	188	189		189		167	39
Coumarin	0.75	146	147		147		77	11
Sulfamoxol	3.63	267	268	156 (33)	268		51	26
Diazepam	1.74	284	285		285		142	38
Clobazam	2.71	300	301	259 (22)	301	259 (28)	77	85
Temazepam	2.09[b]	300	301	283 (20)	301	283 (30), 255 (25)	147	34
Clonazepam	3.05	315	316		316		116	26
Lorazepam	6.74	320	321	303 (80)	321		120	28
Alprazolam	3.38	308	309		309		179	31

Experimental conditions as in Table 1.
[a] Number in parenthesis represents ion's relative intensity.
[b] Chromatographic conditions as in Table 4.

volumes) of 20–100% acetonitrile in water containing 0.1% formic acid.

2.4. Ionization and MS acquisition conditions

The column eluent was introduced into the APCI source operated under the following set of conditions: drying gas flow rate, 4 l/min; nebulizer gas pressure, 55 psi(1 psi = 6894.76 Pa); drying gas temperature, 300 °C; vaporizer temperature, 350 °C; capillary voltage, 6000 V; corona current, 10 μA. Mass spectra were measured in a scan mode (100–1000 u) with the fragmentor set at 80, gain at 1, threshold at 250, step size at 0.1, peak width at 0.2 min, and cycle time at 1.39 s/cycle.

Gradient RPLC–MS was carried out using the following ionization conditions: drying gas flow rate, 15 l/min; nebulizer gas pressure, 30 psi; drying gas temperature, 350 °C; capillary voltage, 4000 V. Mass spectral acquisition parameters were the same as for the normal-phase instrument.

3. Results and discussion

Hyphenation of liquid chromatography with mass spectrometry allows one to combine modern powerful separation methods with sensitive and selective detection technique. We have shown recently that highly efficient chromatographic separations can be carried out on cyano columns using fast gradients of methanol in ENFB [12]. Given the broad applicability of such a technique for separations, it was promising to study whether ionization under atmospheric pressure and MS analysis could be performed under the NP chromatographic conditions described and employing APCI interface for ionization. If successful, such a combination may provide new tools for a wide variety of analytical, preparative, chiral and bioanalytical HPLC applications.

Despite the broad use of ESI for LC–MS, its preferred application range includes polar compounds of a 500–10 000 u molecular mass range, whereas APCI favors non-polar and medium-polarity molecules with masses between 100 and 1000 u [14]. Such compounds represent traditional targets for a medicinal chemist seeking biologically active substances with sufficient bioavailability.

A critical step in any attempt to combine NP chromatographic conditions with mass spectral analysis would be to develop experimental conditions where solutes produce ions in the presence of mobile phase solvents. It was shown earlier [15] that a neutral hydrophobic steroid progesterone and three methylthiohydantoin derivatives of amino acids formed protonated molecular ions inside of the APCI source when chromatographed on a silica column in chloroform containing alcohols. The source was also able to tolerate other organic solvents. The mechanism of positive ionization included a charge transfer from the high voltage source to the alcohol and then to the solute. Similarly, acidic compounds (trinitrophenol and trinitrotoluene) were able to produce

Table 3
LC–MS of basic compounds

Compound name	t_R in NPLC–Ms[a] (min)	M_r	Main ion in the spectrum, *m/z*, NPLC–MS	Other ions in the spectrum, *m/z*[b], NPLC–MS	Main ion in the spectrum *m/z*, RPLC–MS[c]	Other ions in the spectrum, *m/z*[b], RPLC–MS	S/N in NPLC–MS, TIC	S/N in RPLC–MS, TIC
Propranolol	2.89	259	260		260		35	156
Atenolol	4.02	266	267		267		33	151
Acetobutolol	3.82	336	337		337		39	156
Alprenolol	2.55	249	250		250		31	153
Oxprenolol	2.89	265	266		266		28	155
Metoprolol	2.92	267	268		268		35	153
Amitriptyline	0.81	277	278		278	233 (20)	43	170
Doxepin	0.91	279	280		280		81	113
Clomipramine	0.97	314	315		315		42	85
Imipramine	0.98	280	281		281		41	85
Nortriptyline	3.02	263	264	233 (35)	264	233 (45)	26	170
Nordoxepin	3.13	265	266	235 (20)	266	235 (20)	25	113
Desipramine	3.57	266	267		267		14	83
Pyrimethamine	2.13	248	249		249		50	59
Astemizole	2.21	458	459		326	459 (40)	36	21
Haloperidol	2.22	375	376		376		38	22
Terfenadine	2.01	471	472		472		50	89
8-Hydroxyquinoline		145		Not ionized	146			27
Perphenazine	2.12	403	404		404	233 (40)	25	40
Domperidone	2.83	425	426		426		23	16
Sulfametizole	3.21	270	271	156 (85)	156	271 (50)	16	10
Mequitazine	3.81	322	323		323		18	11
Reserpine	2.65	608	609		609		16	26
Diltiazem	1.75	414	373		373	178 (25)	20	30
Thioridazine	1.94	370	371		371		25	31
Pyrilamine	1.26	285	286	241 (50), 121 (75)	121	242 (20)	22	17
Verapamil	1.48	454	455		455		21	39
Clonidine	2.52	229	230		230		14	21
Pindolol	3.99	248	249		249		26	29
Troger's base	1.03	250	251		251		53	41
Cimetidine	3.14	252	253	159 (65), 117 (46)	159	253 (87), 117 (57)	17	88
Sulpiride	3.22	341	342		342		20	33
Terbutaline	4.23	225	226	152 (50)	152	226 (25)	11	31
Fluoxetine	2.36	309	310		310	148 (55)	5	7
Hydroxyzine	1.14	374	375	201 (30)	201	375 (70)	43	106
Lidocaine	0.59	234	235		235		28	43
Acylguanosine	3.39	225	152	226 (40)	152		9	14
Sotalol	3.88	272	255	273 (40)	255	213 (25)	32	33
Sulconazole	2.15	396	397	329 (50), 125 (35), 183 (25)	125	397 (55), 331 (53), 183 (35)	43	107
Brompheniramine	1.35	318	274	319 (60)	276	319 (55), 247 (45), 168 (28)	24	53

[a] Luna CN, 50 mm × 2 mm; 0.6 ml/min; gradient 5–50% MeOH (0.1% ammonia) in ethoxynonafluorobutane in 5 min; (+) APCI–MS.
[b] Number in parenthesis represents ion's relative intensity.
[c] Luna C_{18}, 30 mm × 2 mm; 0.6 ml/min; gradient 20–100% acetonitrile in water (0.1% HCOOH) in 3 min; (+) and (−) ESI–MS.

$(M - H)^-$ ions [15]. The NP positive APCI–MS technique was successfully used to analyze tocopherol and its derivatives using silica gel columns and isooctane–diisopropyl ether–dioxane as a mobile phase [16] and gradients of isopropanol and MeOH in hexane—to separate and identify oxidation products of chlorophyll *a* on a silica gel column [17].

In order to evaluate the ability of various organic compounds to form ions under NP conditions we used fast gradients of MeOH in fluorinated solvent ENFB and a short cyano column coupled to an APCI source installed on a single quad mass spectrometric detector. The system was equipped with two gradient pumps allowing the use of six solvents and three LC–MS methods: for neutral, basic, and acidic compounds (see Section 2). Hexane-like ENFB was chosen because it is miscible with the majority of other HPLC solvents, it is non-flammable, environmentally safe, and has good selectivity for a broad range of organic compounds [12]. It has a boiling point and viscosity higher than hexane, which helps to maintain stable pump pressure and reproducibility of gradient formation (see discussion in [16]). Using the set of experimental parameters described, we expected mobile phase and solute molecules to be vaporized in the APCI source, ENFB to be removed with the stream of nitrogen and MeOH to serve as a chemical ionization agent

Table 4
LC–MS of acidic compounds

Compound name	t_R in NPLC–Ms[a] (min)	M_r	Main ion in the spectrum, *m/z*, NPLC–MS	Other ions in the spectrum, *m/z*[b], NPLC–MS	Main ion in the spectrum *m/z*, RPLC–MS[c]	Other ions in the spectrum, *m/z*[b], RPLC–MS	S/N in NPLC–MS, TIC	S/N in RPLC–MS, TIC
Ibuprofen	0.94	206	161	119 (25)	161	119 (60)	43	9
Flurbiprofen	1.51	244	199		199		12	7
Indoprofen	2.91	281	282		282		53	21
Naproxen	1.66	230	185	231 (40)	185	231 (20)	29	15
Indomethacin	2.62	357	358	139 (95), 174 (40)	358	139 (73), 380 (20)	49	33
Tolmetin	2.43	257	258	214 (48), 119 (97)	119	258 (50)	51	34
Fenoprofen	1.41	242	197		197		20	19
Warfarin	2.52	308	309	163 (86), 251 (42), 147 (34)	164	251 (75), 309 (70), 147 (40), 331 (20)	166	50
Albendazole	2.47	265	266	234 (60)	266	237 (35)	42	45
Salicylic acid	1.40	138	121	139 (20), 158 (20)	121		11	5
Acetyl salicylic acid	1.94	180	121	153 (20)	121		25	10
Salicyl alcohol	2.34	124	107	213 (55), 119 (38), 235 (20)	119	213 (70)	50	38
Sulfasalazine	4.12	398	Not ionized		399			16
Furosemide	4.15	330	Not ionized		329	285 (45)	3	27

[a] Luna CN, 50 mm × 2 mm; 0.6 ml/min; gradient 1–40% MeOH (0.1% acetic acid) in ethoxynonafluorobutane in 5 min; (+) APCI–MS.
[b] Number in parenthesis represents ion's relative intensity.
[c] Luna C_{18}, 30 mm × 2 mm; 0.6 ml/min; gradient 20–100% acetonitrile in water (0.1% HCOOH) in 3 min; (+) and (−) ESI–MS.

facilitating the positive charge transfer to solute molecules and their subsequent MS analysis.

3.1. Normal-phase LC–MS of the neutral compounds with APCI

To establish the feasibility of the technique we used progestines, pregnanes, androstanes, and corticosteroids as examples of lipophilic compounds with varying degrees of polarity and structural diversity. The compounds were dissolved in methanol (concentration ~1 mg/ml), 1 μg of the solution was injected onto a column, eluted with a methanol gradient in ENFB, chromatographic peaks were registered in a scan mode and the signal-to-noise ratio was measured. We found that excellent response was achieved with the majority of compounds tested when both capillary voltage and corona current were set at their maximum values allowed by the instrument manual. The results are shown in Table 1. These acquisition parameters (see Section 2) served as a basis for all three normal-phase LC–MS methods used.

All steroids used in this study were able to form ions in the presence of methanol and ENFB (Table 1). Some of them exhibited $[M + H]^+$ ions as the most intense in the spectrum, but, in general, $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$ ions dominated the spectra of steroids, similar to data reported previously [18]. The ability of steroids to ionize under NPLC–APCI–MS conditions may provide new opportunities for fast and sensitive analyses of these metabolically important molecules in various biological tissues and fluids, especially for non-UV-active steroids like pregnanes and androstanes. For example, a group of eight progesterone derivatives was separated in one chromatographic run (Fig. 2). Mass spectra of chromatographic peaks are shown in Fig. 3. The quality of chromatographic separation was

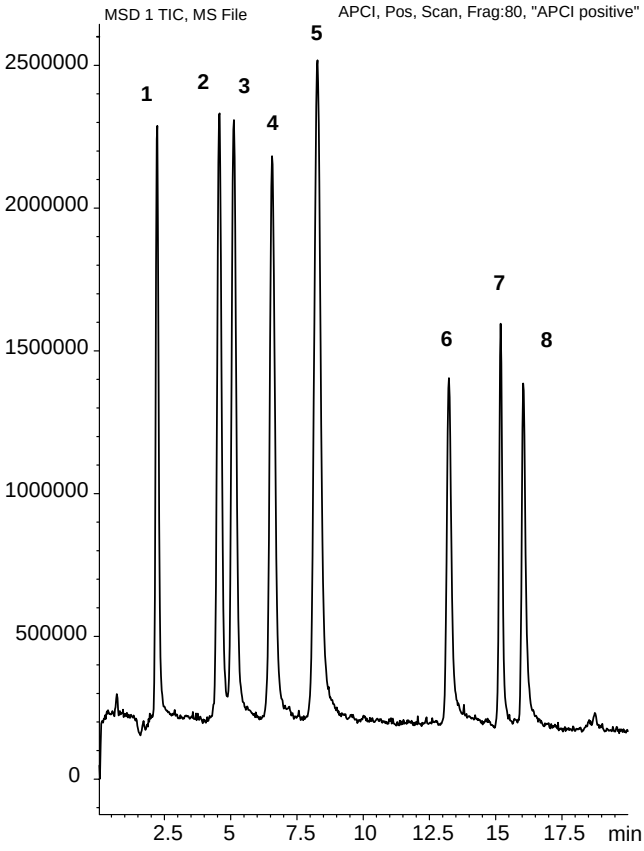

Fig. 2. Normal-phase LC–MS of a mixture of eight progestines and corticosteroids. (see Section 2 for chromatographic and MS acquisition conditions). Peaks: (1) progesterone, (2) 20β-hydroxy-pregn-4-ene-3-one, (3) 20α-hydroxy-pregn-4-ene-3-one, (4) 11β-hydroxyprogesterone, (5) 11α-hydroxyprogesterone, (6) corticosterone, 7–dexamethasone, and (8) 20α-dihydrocortisole (peaks 1–8 with molecular masses 314, 316, 316, 330, 330, 346, 393, and 364, respectively). Concentration: ~1 μg in peak.

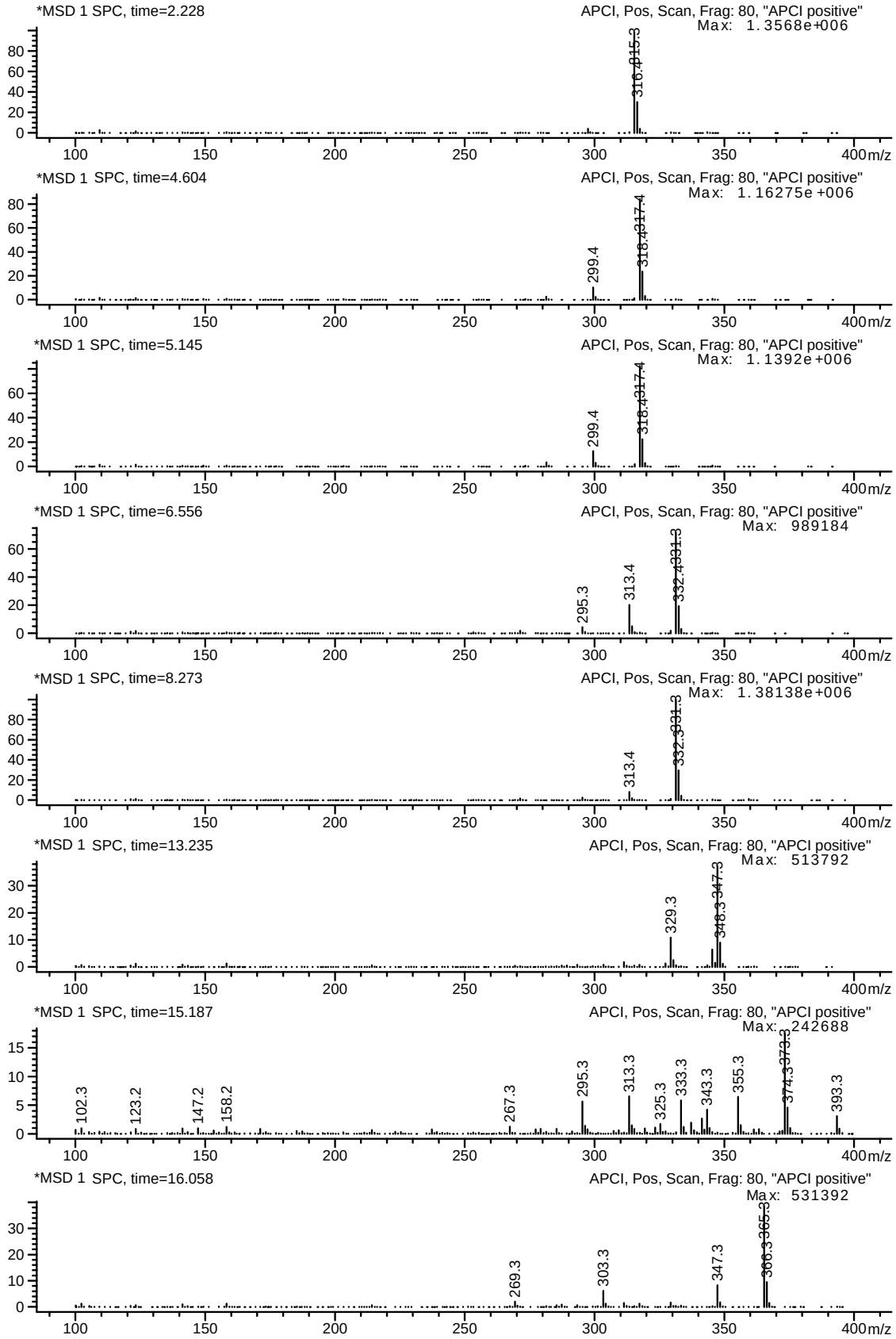

Fig. 3. Mass spectra of steroids separated by normal-phase LC–MS.

similar to previously reported, with good peak shape and selectivity [12]. Even with a broad scan range from 100 to 1000 u the total ion current (TIC) response was adequate, with a limit of detection being around 50–100 ng per peak. The sensitivity for individual components of the mixture could be improved by optimizing acquisition parameters (e.g., by narrowing the scan range to 300–400 u) and utilizing selective ion monitoring (SIM) to achieve a limit of detection of ∼1 ng per chromatographic peak. It is reasonable to expect even better sensitivity if LC–MS–MS technique is employed for mass spectral detection.

Separation of three central nervous system (CNS)-active benzodiazepins with APCI–MS detection is shown in Fig. 4. All components of the mixture responded strongly and with minimal fragmentation, while the ESI–MS response for benzodiazepines in general was somewhat weaker (Table 2).

Neutral molecules responded strongly under positive APCI conditions, producing a signal with good signal-to-noise ratio and exhibited very mild, if any, fragmentation (Table 2). Clearly, methanol served as a chemical ionization agent, while the presence of ENFB did not appear to interfere with the outcome of the ionization process. Under the conditions used, APCI seems to be a soft and effective ionization technique. Out of 20 non-steroid neutral compounds ∼80% exhibited protonated molecular ions as the most intense ion peak in the mass spectrum. Lipophilic molecules such as β-carotene and triphenylene have shown good responses with the $[M + H]^+$ ion being the predominant or the only ion in the spectrum. α-Tocopherol responded strongly, as described in [16], while ionization of naphthalene was very weak. When traditional LC–ESI–MS was employed, we detected a weak response from all steroids and α-tocopherol and no ions were formed when samples of β-carotene, naphthalene and triphenylene were injected onto a RP column (Tables 1 and 2).

3.2. Normal-phase LC–MS of the basic compounds with APCI

We have found previously that basic compounds could be efficiently resolved using methanol gradients in ENFB on a cyano column when basic modifying agents (e.g., triethylamine, diethyamine) were added to the mobile phase in order to reduce peak tailing and improve chromatographic efficiency [12]. Unfortunately, the use of the same conditions (0.1% of modifying agent in mobile phase) with an APCI source resulted in total signal suppression, especially for weaker basic compounds (as pointed out in [19]). While trying to solve this problem, we found that the addition of

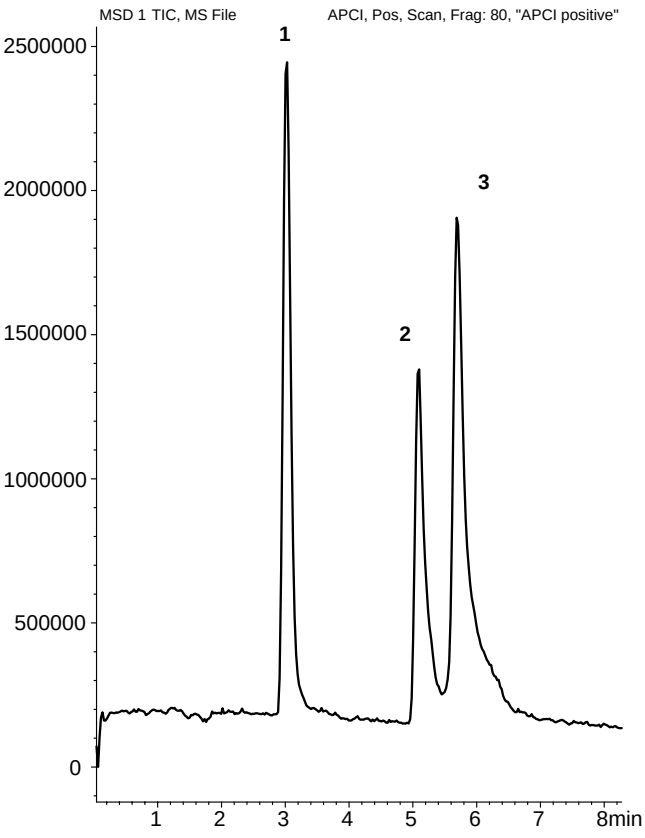

Fig. 4. Normal-phase LC–MS of a mixture of three benzodiazepins. Peaks: (1) diazepam, (2) clonazepam, and (3) alprazolam. Concentration: ∼1 μg in peak.

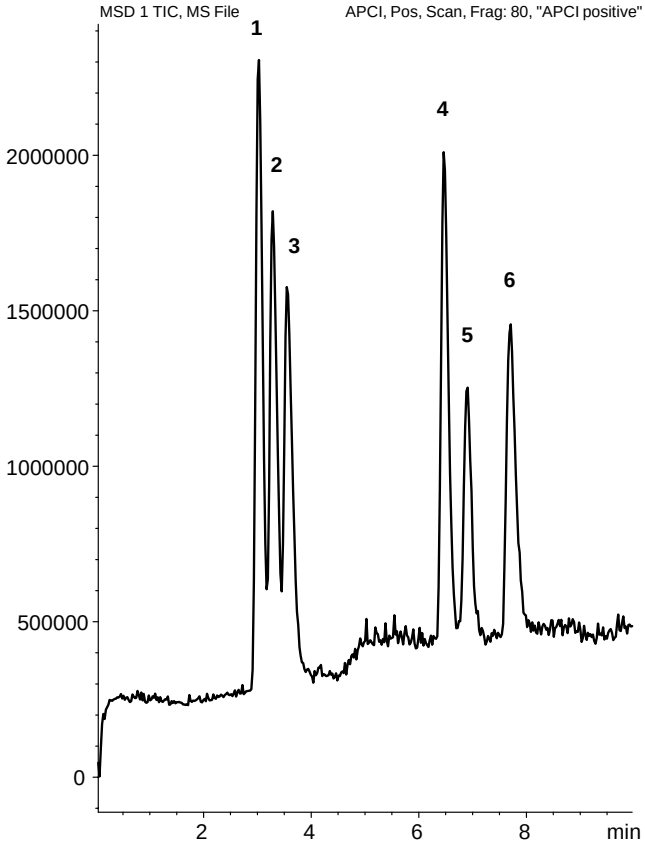

Fig. 5. Normal-phase LC–MS of a mixture of six tricyclic antidepressants. Peaks: (1) amitryptiline, (2) doxepin, (3) imipramine, (4) nortriptyline, (5) nordoxepin, and (6) desipramine. Concentration: ∼1 μg in peak.

0.1% of ammonia to MeOH and the use of 5% MeOH in ENFB as a starting solvent for LC–MS resulted in excellent chomatographic behavior and good ionization efficiency for the most of basic compounds studied (Table 3). Clearly, the ammonia was strong enough base to suppress peak tailing during chromatographic separation and not strong enough to compete with solute molecules for proton transfer and positive ions formation.

Many basic compounds used in this study are commercially available as corresponding salts with various acids. Data shown in Table 3 were obtained when free bases were used for the experiments. This was done by treating the salts with saturated solution of sodium hydrogen carbonate and subsequent extraction with *n*-butanol. While effective, this step is less convenient than direct introduction of the salt as it's solution in a polar solvent (e.g., MeOH). In a separate series of experiments, we compared LC–MS responses for both free base and salt forms of a randomly selected group of basic compounds and found them to be practically the same (Table 5).

This observation was rather unexpected, since NP-HPLC is carried out in non-aqueous medium—mixture of ENFB and methanol (containing NH_3)—where traditional dissociation is hardly probable in contrast to aqueous RP-HPLC. It is possible that the acidic portion of the salt molecule is neutralized by NH_3 present in the mobile phase and the resulting inorganic salt is removed from the column during the MeOH wash. Thus, basic compounds can be successfully analyzed by NPLC–MS with APCI–MS detection in either salt or free base form.

Out of 50 compounds of basic nature, the majority (∼92%) of them produced $[M+H]^+$ ion as the most intense ion peak in the spectrum with very little fragmentation, with ∼5% of the compounds having a protonated molecular ion as the second intense ion in the spectrum (Tables 3 and 5).

LC–MS separations of mixtures of several tricyclic antidepressants (Fig. 5, mass spectral data of chromatographic peaks are shown in Table 3) and β-adrenal blockers (Fig. 6; mass spectral data in Table 3) were carried out on a cyano column using gradients of MeOH (0.1% ammonia) in ENFB with positive APCI and TIC detection (100–1000 u scan) (see Section 2). Both chromatograms demonstrate good separation efficiency and selectivity for the mixture components. The sensitivity and signal-to-noise ratio could be significantly improved by narrowing the scan range or using SIM mode of detection, with a limit of detection being estimated to be ∼5–10 ng per peak.

3.3. Normal-phase LC–MS of acidic compounds with APCI

Compounds of acidic nature can be successfully resolved in a gradient of MeOH in ENFB when 0.1% of acidic modifier (e.g., trifluoroacetic and formic acids) is present to ensure the adequate chromatographic performance [12]. We

Table 5
Comparison of LC–APCI–MS and LC–ESI–MS response from salts and corresponding bases

Compound name	t_R in NP LC/MS (min)	M_r	Main ion in the spectrum, *m/z*, NPLC–MS	Other ions in the spectrum, *m/z*[a], NPLC–MS	Main ion in the spectrum *m/z*, RPLC–MS	Other ions in the spectrum, *m/z*[a], RPLC–MS	S/N in NPLC–MS, TIC	S/N in RPLC–MS, TIC
Doxylamine, base	1.2	270	182	271 (60), 183 (25)	182	167 (20)	19	9
Doxylamine succinate, salt	1.2	270	182	271 (55), 183 (23)	182		21	10
Pirenzepine, base	2.5	351	352	113 (25)	352	113 (50)	23	11
Pirenzepine 2HCl, salt	2.6	351	352	113 (20)	352	113 (45)	17	11
Flupentixol, base	1.5	434	435		435	218 (30)	20	13
Flupentixol 2HCl, salt	1.5	434	435		435		16	11
Harmine, base	1.7	212	213		213		24	19
Harmine HCl, salt	1.7	212	213		213		19	20
Promethazine, base	0.9	284	285		285	240 (20), 198 (45)	28	26
Promethazine HCl, salt	0.92	284	285		285	198 (25)	16	23
Carbinoxamine, base	1.31	290	202	291 (97), 204 (45)	202	291 (40), 204 (50), 167 (70)	30	46
Carbinoxamine maleate, salt	1.25	290	202	291 (92), 204 (40)	202	291 (45), 204 (65), 167 (65)	26	27
Ebastine, base	1.11	469	470		470		33	33
Ebastine fumarate, salt	1.11	469	470		470		26	40
Paxil, base	3.51	329	330		330		34	46
Paxil HCl, salt	3.49	329	330		330		20	40
Loperamide, base	2.81	476	477		477		75	61
Loperamide HCl, salt	2.82	476	477		477		50	58
Naltrexone, base	1.80	341	342		342		15	58
Naltrexone HCl, salt	1.82	341	342		342		22	35

Experimental conditions as in Table 3.
[a] Number in parenthesis represents ion's relative intensity.

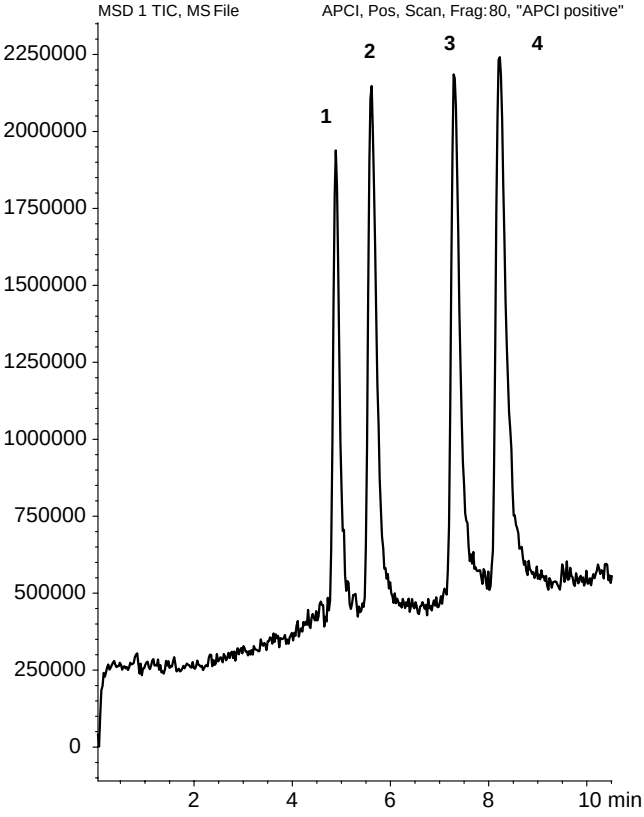

Fig. 6. Normal-phase LC–MS of a mixture of β-blockers. Peaks: (1) alprenolol, (2) propranolol, (3) acetobutolol, and (4) atenolol. Concentration: ∼1 μg in peak.

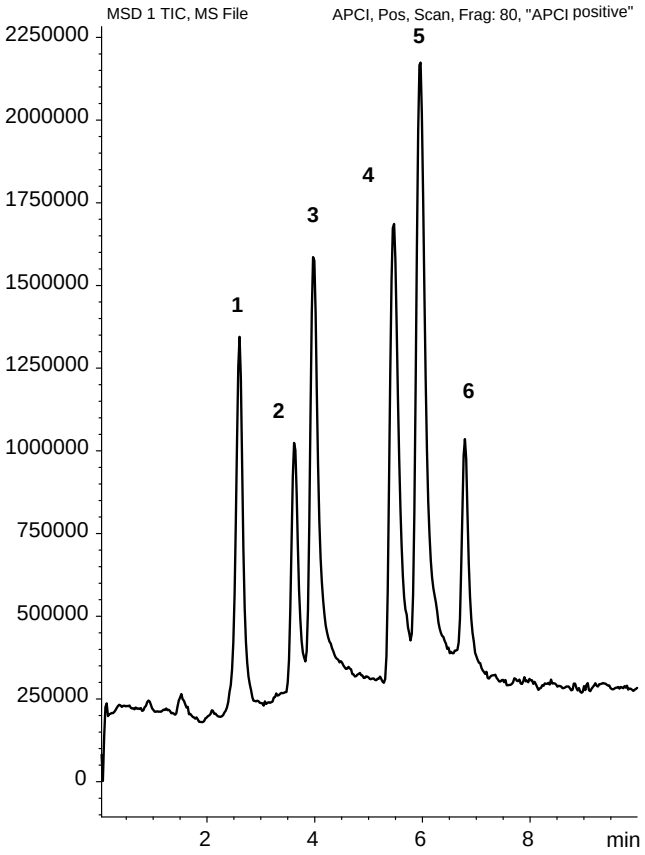

Fig. 7. Normal-phase LC–MS of a mixture of six profens. Peaks: (1) ibuprofen, (2) fenoprofen, (3) naproxen, (4) tolmetin, (5) indomethacin, and (6) indoprofen. Concentration: ∼1 μg in peak.

found that when positive APCI was employed under such conditions, the acidic compounds failed to produce any ions. Both trifluoroacetic acid (TFA) and formic acid suppressed the signal when used as modifiers for LC–MS. Acetic acid, on the contrary, was strong enough to suppress peak tailing and ensured a good MS response when a mixture of six profens was separated on a cyano column using a gradient of methanol in ENFB (Fig. 7) (see Section 2). Ions resulting from the loss of water and decarboxylation were the most intense ones in profens' mass spectra (Table 4). The ability of an acidic molecule to undergo fragmentation seemed to be diminished with an increase in its polarity (manifested by increased chromatographic retention) with $[M + H]^+$ ions becoming the predominant ions in the tolmetin, indoprofen, warfarin, and albendazole mass spectra.

3.4. NP-HPLC–APCI–MS and RP-HPLC–ESI–MS response comparison

One of the main goals of this study was to create a generic approach that would allow ionization of a broad range of organic compounds under atmospheric pressure and the use of a mass spectrometer as a "universal" detector to analyze them. It was important to establish the limits of applicability and overall efficiency for each technique being considered for such an approach.

Generic non-optimized atmospheric pressure ionization and mass spectral detection parameters were used for the experiments. The column size, gradient volume, and it's slope were also made as generic as possible. The flow rate used was three times higher then conventional without any detrimental effects on the quality of the separation, as we have shown in [12]. The amount of material injected was the same (1 μg per chromatographic peak) in each experiment. The efficiency of ionization was evaluated by comparing the signal-to-noise ratios in the NP and RP modes (with APCI and ESI source, respectively) for each compound. Conclusions that can be drawn from data in Tables 1–5 do not intend to imply advantages of one technique over another, nor do the results indicate that a certain technique is optimal for any given class of organic compounds. Our goal was rather to demonstrate the feasibility of ionization in normal-phase in addition to the well-established reverse-phase
one.

Overall, APCI–MS with methanol and basic and acidic modifiers such as ammonia and acetic acid in the presence of ENFB is a viable ionization technique for hydrophobic compounds and compounds of medium polarity. Very polar

molecules appeared to be better analyzed by ESI–MS. The overall response seemed to be stronger with normal-phase APCI–MS for the majority of neutral substances and comparable with reversed-phase ESI–MS response for basic (with the exception of tricyclic antidepressants favoring ESI) and acidic compounds.

4. Conclusions

Normal-phase LC–MS using gradients of methanol with or without acidic and basic modifiers in ethoxy nonafluorobutane and coupled with an APCI source was successfully applied for fast HPLC separations with MS detection of a broad range of organic compounds including neutral hydrophobic β-carotene, α-tocopherol, medium-polarity pregnanes, androstanes, corticosteroids, benzodiazepins, acidic nonsteroidal anti-inflammatory drugs, salycilates, basic β-blockers, and tricyclic antidepressants. The ability of APCI interfaces to ionize solutes after the separation seems to be broad enough to include compounds with distinctively different polarities and functionalities, with the best response shown for non-polar and medium-polar substances. The MS response for basic compounds was comparable with the response obtained under conventional reversed-phase HPLC conditions with ESI–MS detection. Very polar compounds were better suited for reversed-phase HPLC-ESI–MS analysis. Potential applications of the technique described may include analytical and preparative HPLC, normal-phase chiral HPLC of non-UV-active compounds, isolation of combinatorial and parallel synthetic products and bioanalytical applications.

Acknowledgements

The authors thank Dr. Marshall Siegel (Wyeth Research, Pearl River, New York, USA) for valuable discussions and comments.

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