ELSEVIER

Journal of Chromatography A, 794 (1998) 253–262

JOURNAL OF
CHROMATOGRAPHY A

Evaluation of particle beam high-performance liquid chromatography–mass spectrometry for analysis of cholesterol oxides

M. Careri*, D. Ferretti, P. Manini, M. Musci

Dipartimento di Chimica Generale ed Inorganica, Chimica Analitica, Chimica Fisica, Università di Parma, Viale delle Scienze, I-43100 Parma, Italy

Abstract

The capabilities of using particle beam (PB) liquid chromatography–mass spectrometry (LC–MS) was investigated for the analysis of cholesterol and oxycholesterols; normal-phase (NP) and reversed-phase (RP) chromatographic methods making use of narrow-bore columns were set up for the simultaneous separation of these compounds. The influence of PB interface parameters, LC conditions (solvent composition and flow-rate) and MS conditions was explored. PB mass spectra were recorded in electron impact (EI), positive-ion chemical ionization (PCI) and negative-ion chemical ionization (NCI) modes and the corresponding spectral data discussed. Better performance in terms of ion abundance were observed in EI mode both under NP and RP conditions. Using EI source and operating in selected-ion monitoring (SIM) mode, linearity, sensitivity and precision of the analysis were explored. The experimental data fitted a quadratic model in the overall range studied. Using the RP-LC–PB-MS method detection limits at the low-ng level were achieved for all the analytes; in addition, repeatability ranging from 3% for triol to 9% for cholesterol and 7-ketocholesterol at the 4 ng level was obtained. © 1998 Elsevier Science B.V.

Keywords: Interfaces, LC–MS; Cholesterol oxides

1. Introduction

Cholesterol undergoes autoxidation by a free-radical mechanism leading to the formation of hydroperoxides and then to a number of oxidation products, the so-called "oxycholesterols" [1]. Among the many derivatives obtained by further degradation of hydroperoxides in cholesterol-containing foods, 25-hydroxycholesterol (25-OH), cholestan-3β-5α-6β-triol (triol), 5,6α-epoxy-cholesterol (5,6α-EP), 5,6β-epoxy-cholesterol (5,6β-EP), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 7-keto-cholesterol (7-keto) and cholesta-4,6-diene-3-one (4,6-diene-3-one) have been identified as major products. Because of the sensitivity of cholesterol to oxidation, increasing concern has been expressed by food scientists that certain prominent oxycholesterols have been implicated in adverse human health effects [1–3] and that these oxidation products repeatedly have been demonstrated to be present in cholesterol-containing foods which had been processed or stored under oxidizing conditions [4–10]. Cholesterol oxidation products (COPs) are found in many common foods and have been shown to be atherogenic, cytotoxic, mutagenic and possibly carcinogenic [11–13]. Among these compounds, 25-OH, 5,6α-EP and triol have been shown to be the most toxic to cultured cells and experimental animals [11,14,15].

*Corresponding author.

0021-9673/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII S0021-9673(97)00764-4

A comprehensive review of the occurrence and biological effects of oxysterols has recently been published [16]. The possible health implications of dietary cholesterol oxides has stimulated the need for identification and determination of these substances in foods.

Due to the presence of low concentrations (ppm or ppb) of COPs in foods [16], isolation procedures which separate the sterol oxidation products from other interfering substances in foods and quantitation of oxycholesterols are a difficult analytical problem. Analytical procedures for the determination of the extent of cholesterol oxidation in foods, especially in heated and/or stored foods having high cholesterol content, include capillary gas chromatography (GC) [6,17–21], GC–mass spectrometry (MS) [6,10,18] and high-performance liquid chromatography (HPLC) [4,5,7–10,22–25]. Taking into account that HPLC simplifies greatly the quantitation procedure and introduces fewer artifacts, normal-phase (NP) [4,7–10,22–25] and reversed-phase (RP) [5,10,23,24] HPLC methods have been applied to the separation of cholesterol oxides by several researchers. In general, NP-liquid chromatography (LC) systems proved more effective than RP, but full resolution over the entire polarity range of oxidation products was not achieved with the use of isocratic solvent systems. In some cases separation and quantitation were achieved for narrowly defined groups of oxidation products such as C-7-substituted cholesterols [7,8,10] or the isomeric cholesterol-5,6-epoxides [5]. Some workers devised HPLC routine methods for the determination of 7-keto, which is considered as a tracer of the oxidative process since it is formed predominantly and it is one of the oxides found in the highest amounts [7,10]. Unspecific detectors such as UV and refraction index types are used; UV detection has to be performed at short wavelength (206 nm), where some COPs exhibit also a poor absorption, so that there are limitations concerning the solvents and gradients which can be used; in particular, 5,6α-EP and triol do not possess adequate UV absorption characteristics. Laser light-scattering detection has been reported as a reliable tool in the HPLC of COPs by Kermasha et al. [25].

Taking into account that cholesterol in most foods occurs at low levels and thus only traces of COPs may be formed and considering the large number of these derivatives with close structural similarities to each other, in many cases reliable separation and identification is difficult to achieve. Much more, these analytes do not possess suitable chromophores for easy detection by conventional methods and the amount of compounds to be detected is in most cases too small for the sensitivity of the detector. The application of the mass spectrometer as a specific detector may improve the analytical possibilities and leads to high sensitivity in such examination. In a few instances electron impact (EI) MS using a direct probe has been reported for the analysis of some oxycholesterols after collecting peak materials from several HPLC injections [4,7,8]. These off-line methods require preliminary isolation and purification of the compounds. The developments of the technique HPLC–MS allows MS analysis to be coupled on-line with analytical HPLC separation, the main advantage of LC–MS being its ability to provide qualitative information on the detected peaks. To date applications of this coupled technique to the analysis of COPs are completely lacking.

This paper describes the first application of LC–MS with particle beam (PB) interface to the analysis of cholesterol and its oxidation products. The goal of the work was to develop and to validate the analytical method by using NP chromatography on a cyano-bonded column and RP chromatography on a C_{18} column; in both cases, narrow-bore columns were chosen to obtain optimum sensitivity for PB experiments. The influence of PB interface parameters, LC conditions (solvent composition and flow-rate) and MS conditions were investigated. When operated under optimum instrument parameters, the calibration graphs of the analytes were calculated and the sensitivity of the mass spectrometer towards the compounds of interest was studied under both NP and RP conditions. PB mass spectra were recorded in EI, positive-ion chemical ionization (PCI) and negative-ion chemical ionization (NCI) modes and the corresponding spectral data discussed.

2. Experimental

2.1. Chemicals

Cholesterol, 25-OH, 7β-OH, 7-keto, triol, 5,6α-EP

were purchased from Sigma (Milan, Italy). All organic solvents were HPLC grade. Heptane, propan-2-ol, methanol and acetonitrile were obtained from Lab-Scan (Dublin, Ireland) and all other solvents were obtained from Carlo Erba (Milan, Italy).

2.2. Standard preparation

Standard solutions containing approximately 1 mg/ml were prepared in either diethyl ether or acetone and diluted to the working concentration levels required for the study of analytical characteristic of the LC–PB-MS system. These stock solutions are stable for at least 2–3 weeks if stored under nitrogen in the dark at 4°C.

Experiments for the evaluation of the ''optimum'' set of operational parameters were performed on standard solutions of all the analytes at 200 μg/ml levels.

A mixture was prepared to contain cholesterol and cholesterol oxide standards (200 μg/ml of each) by diluting each stock solution with diethyl ether, immediately before use and kept in the dark.

2.3. LC–MS

LC–MS was carried out with a Hewlett-Packard Model HP 59980A PB interface, fitted on a Hewlett-Packard Model HP 5989A quadrupole mass spectrometer.

The nebulizing gas was high-purity helium (inlet pressure 35 p.s.i.; 1 p.s.i.=6894.76 Pa); the temperature of the desolvation chamber was 30°C.

The mass spectrometer was operated in both EI and chemical ionization (CI) modes: positive and negative ion CI mass spectra were obtained with methane as a reagent gas (ion source manifold pressure $1.4 \cdot 10^{-4}$ Torr; 1 Torr=133.322 Pa). The ion source and the quadrupole temperatures were 250° and 100°C respectively, the electron energy was 70 eV in EI mode and 230 eV in CI mode. Using EI and CI sources, the mass ranges m/z 105–425 and 105–460 were scanned at a rate of 1.16 s/scan, respectively.

Reference mass spectra of 5,6α-EP and triol were obtained using a Finnigan MAT SSQ710 spectrometer equipped with an EI/CI source, a direct inlet system and a quadrupole mass analyzer. The CI source was used with methane as reagent gas, methane ionization energy being 70 eV. Source and quadrupole temperatures were 220°C and 140°C, respectively.

In order to identify the optimum set of parameters for operation of PB interface, a series of preliminary experiments were carried out in flow injection (FIA) mode, by injecting 200 ng of each of the analytes. The eluents used for NP and RP experiments were a heptane–propan-2-ol (94:6) mixture and a methanol–acetonitrile (90:10) mixture, respectively, at a flow-rate of 0.3 ml/min. The nebulizer capillary position, the helium pressure, the desolvation chamber temperature and the MS source temperature were considered as factors.

The LC system consisted of a Hewlett-Packard Model HP 1090 liquid chromatograph equipped with an HP 1050 autosampler and a Nucleosil 5-CN column (250 mm×2.0 mm, 5 μm) (Macherey-Nagel, Düren, Germany); a mobile phase of heptane–propan-2-ol (94:6) was used at a flow-rate of 0.3 ml/min. RP separations were carried out on an Ultracarb ODS (20) column (250 mm×2.0 mm, 5 μm) (Phenomenex, Torrance, CA, USA) with methanol–acetonitrile (90:10) as eluent at a flow-rate of 0.3 ml/min.

Linearity and detection limits were determined by using selected-ion monitoring (SIM). When the SIM technique was applied, the following ions using the dwell times enclosed in parentheses were monitored: m/z 107 (100), 145 (200), 275 (200), 301 (200), 386 (100) for cholesterol, m/z 107 (300), 145 (200), 213 (200), 273 (200), 384 (100) for 25-OH, m/z 107 (100), 161 (100), 384 (800) for 7β-OH, m/z 131 (400), 173 (500), 384 (100) for 5,6α-EP, m/z 135 (200), 161 (200), 174 (400), 192 (100), 400 (100) for 7-keto, m/z 109 (300), 123 (200), 229 (200), 247 (200), 269 (100), 402 (100) for triol. For the determination of the linear dynamic range the 2–1000 ng range was explored. A quadratic regression fitting ($y=a+bx+cx^2$) was done on all the calibration data. The calculations of the detection limits for the compounds studied were based on a signal-to-noise ratio of 3, throughout this work. The instrumental precision was calculated by considering the repeatability of four measurements of chromatographic peak areas at two amount levels for each column (16 ng and 250 ng in the case of the CN

column and 4 ng and 250 ng in the case of the ODS column).

Data were acquired by the HP MS 59940A Chem Station (HP-UX series).

3. Results and discussion

3.1. Optimization of the PB interface parameters

In order to explore the PB-LC–MS technique for its ability to analyze cholesterol and oxycholesterols, preliminary studies were carried out under FIA conditions in full scan mode. The effects of various PB variables, LC conditions and MS source temperature on overall sensitivity were investigated for these analytes. The main parameters in the interface were the position of the fused-silica capillary in the nebulizer, the nebulizing gas pressure and the desolvation chamber temperature. These factors were evaluated with a low-polar solvent (heptane–propan-2-ol, 94:6) and with a high-polar solvent (methanol–acetonitrile, 90:10); these eluents were specifically chosen because they are suitable for LC separations of cholesterol and the COPs considered in NP and RP mode, respectively.

To optimize aerosol formation, the optimal nebulizer position was chosen in such a way that the capillary is retracted about 0.5 mm from the nebulizer tip, even though when the fused-silica tube protrudes about 3 mm the response for some of the analytes considered (cholesterol, 5,6α-EP and triol) is maximized as well. Changing the helium pressure from 25 to 60 p.s.i. resulted in decreased sensitivity, better performance being at 35 p.s.i. for both solvent systems. As expected, in general the interface was found to operate best at low desolvation temperatures (30°C) when the low-polar LC eluent was used, except for cholesterol and triol, for which signal intensity was maximized at 70°C. Operating with high percentages of methanol in the eluent, an high desolvation temperature (70°C) was required to avoid condensation in the chamber and thus to ensure an efficient transmission of the compounds. As already observed by other authors [26], the maximum response of all the analytes was observed when using methanol as the eluent, however the low percentage of acetonitrile (10%) required for chromatography did not cause an appreciable loss in sensitivity.

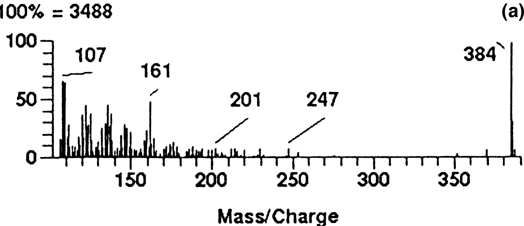
As for the LC conditions, in addition to the solvent composition, the LC flow-rate was explored in the 0.2–0.4 ml/min range because of the use of the narrow-bore columns. A weak maximum in response was observed at 0.3 ml/min with both the eluents.

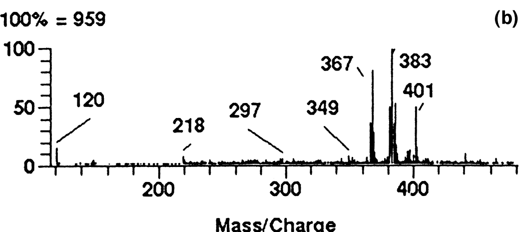
The influence of the ion source temperature was investigated at 200, 250 and 300°C in full scan mode under EI conditions; even though the best total ion current for all the analytes was acquired at 300°C, an extensive fragmentation was observed at this temperature. Instead, mass spectra recorded at 200°C showed more abundant molecular ion current for all the compounds except for triol, but a decrease in response. The difference in spectral quality was less pronounced for triol, since for this compound the molecular ion was not detected in the EI mass spectrum and signal abundance was only 2-fold higher at 300°C than at 200°C. Therefore all the experiments were performed at temperature of 250°C, which was chosen as a compromise between a more extensive fragmentation obtained at higher temperatures and a lower response observed at 200°C.

3.2. Mass spectrometric data

Amounts on the order of 10 ng of oxysterols were needed to obtain good quality EI mass spectra. The use of organic solvents limited the acquisition mass range, so that it was impossible to record below 100 u when using the heptane–propan-2-ol mixture. As an example, the EI, PCI and NCI mass spectra of 7β-OH are shown in Fig. 1.

In Table 1 the MS data for the compounds examined are summarized; structural assignment and relative abundance of the fragment ions are contained in the Table. Valuable structural information was obtained from PB mass spectra. In general, the compounds exhibit a complex fragmentation pattern resulting from cleavages of any four rings and of the C-17 side chain (Scheme 1). The loss of C-15, C-16, C-17 together with their substituents appears to be one of the most frequent fragmentation for the oxycholesterols. In addition, EI mass spectra of all the analytes are characterized by ions corresponding





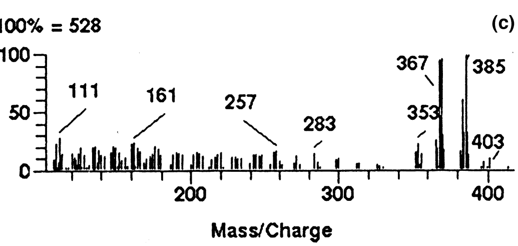


Fig. 1. PB-MS mass spectra of 7β-hydroxycholesterol (molecular mass 402) obtained in (a) EI mode, (b) NCI mode and (c) PCI mode at a source temperature of 250°C.

to the release of water and to the loss of methyl groups. Cleavage of the ring B between C-9, C-10 and C-6, C-7 was observed for all compounds.

In general, the predominant ions in the PCI mass spectra of COPs resulted from protonation of molecular ion followed by loss of one or two molecules of water; in contrast, the base peak in the PCI spectrum of 5,6α-EP and of triol appeared at m/z 111, and was assigned to the fragment indicated in Table 1.

Few fragments were detected in the NCI spectra of these compounds, the deprotonated molecular ion being the base peak in the spectrum of cholesterol and of 25-OH; the loss of an hydroxy group was predominant in the spectrum of 5,6α-EP, whereas the simultaneous elimination of water and of an hydroxy group was observed in the NCI spectrum of triol at m/z 385.

The PB EI and CI mass spectra of 5,6α-EP and triol were compared with those obtained by direct probe introduction. As expected, as the ion source used with the PB-MS system is the classical EI/CI ion source, similar fragmentation pattern was obtained and only negligible differences in terms of ion abundances were observed. These differences could be attributed to the different ion source temperature used, since in the case of the direct inlet analysis it was impossible to operate at a temperature greater than 220°C, whereas the PB mass spectra discussed were recorded at 250°C.

3.3. LC–PB-MS analyses of COPs in NP and in RP mode

After the optimization of the PB interface parameters under FIA conditions, LC–MS experiments were performed with the CN column in NP mode. Using the three ionization modes, a mixture of cholesterol and COP standards was injected, obtaining the LC-PB–MS chromatograms illustrated in Fig. 2. In general, the peak broadening caused by the PB interface was not particularly noticeable. From examination of the three ion-current profiles, it can be stated that EI should be the first choice in the analysis of oxycholesterols because of the most intense signals achieved for all the analytes; using CI source and monitoring positive-ion signals, the most abundant signal was that of 25-OH, whereas under NCI conditions response of 7-keto increases relatively to that of other compounds. The elution order obtained was in agreement with that reported in literature for the same stationary phase [9].

Modification of the chromatographic conditions with a C_{18} column and an eluent made up of 10% acetonitrile in methanol led to an improvement in response of the analytes studied under EI and CI conditions (Fig. 3). Also operating under these RP conditions it was possible to obtain the separation of six cholesterol-related compounds within 17 min and except for 25-OH and triol, for which a resolution factor $R_s=1.0$ corresponding to a 10% overlapping was obtained, the other compounds were baseline separated (Fig. 3); it is noteworthy that in these conditions retention times are considerably reduced with respect to those obtained on C_{18} column by other authors [23,24]. This chromatographic mode was found particularly useful for PB-MS detection of

Table 1
PB-EI and methane CI mass spectra of cholesterol and oxycholesterols[1]

Compound[b]	EI			PCI			NCI		
	m/z	R.A.[c]	Identity	*m/z*	R.A.[c]	Identity	*m/z*	R.A.[c]	Identity
Cholesterol (M.W.=386)	386	15	$[M]^+$	385	35	$[M-1]^+$	385	100	$[M-H]^-$
	368	13	$[M-H_2O]^+$	369	100	$[MH-H_2O]^+$	367	8	$[M-H-H_2O]^-$
	353	13	$[M-H_2O-CH_3]^+$						
	301	22							
	275	28	$[m/z\ 273+2H]^+$						
	231	18							
	213	35	$[m/z\ 231-H_2O]^+$						
	107	100	$[m/z\ 125-H_2O]^+$						
5,6α-EP (M.W.=402)	175	15	$[m/z\ 195-2H-H_2O]^+$	385	32	$[MH-H_2O]^+$	401	85	$[M-H]^-$
	161	32	$[m/z\ 195-H_2O+2H]^+$	368	33	$[MH_2-2H_2O]^+$	385	100	$[M-OH]^-$
				367	28	$[MH-2H_2O]^+$	383	79	$[M-H-H_2O]^-$
	123	92	$[m/z\ 138-CH_3]^+$	111	100	$[m/z\ 107+4H]^+$	367	28	$[M-2H-H_2O-CH_3]^-$
	107	100	$[m/z\ 140-2H_2O+3H]^+$						
25-OH (M.W.=402)	402	4	$[M]^+$	402	6	$[M]^+$	401	100	$[M-H]^-$
	384	17	$[M-H_2O]^+$	385	37	$[MH-H_2O]^+$	383	32	$[M-H-H_2O]^-$
	369	11	$[M-H_2O-CH_3]^+$	367	100	$[MH-2H_2O]^+$			
	273	26	$[M-m/z\ 129]^+$						
	213	31	$[m/z\ 231-H_2O]^+$						
	107	100	$[m/z\ 125-H_2O]^+$						

Table 1. Continued

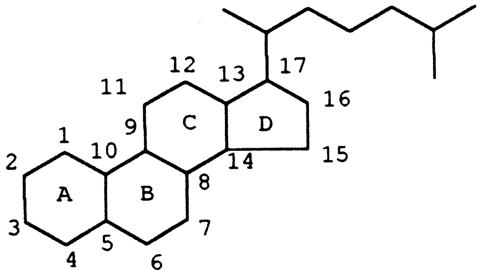
Compound[b]	EI			PCI			NCI		
	m/z	R.A.[c]	Identity	m/z	R.A.[c]	Identity	m/z	R.A.[c]	Identity
7-Keto (M.W.=400)	402	4	$[MH_2]^+$	383	100	$[MH-H_2O]^+$	381	100	$[M-H-H_2O]^-$
	400	12	$[M]^+$	367	26	$[M-H_2O-CH_3]^+$	365	6	$[M-2H-H_2O-CH_3]^-$
	384	4	$[MH_2-H_2O]^+$						
	382	11	$[M-H_2O]^+$						
	367	13	$[M-H_2O-CH_3]^+$						
	174	100	$[m/z\ 193-H_2O-H]^+$						
	161	65	$[m/z\ 193+4H-2H_2O]^+$						
	107	30	$[m/z\ 125-H_2O]^+$						
7β-OH (M.W.=402)	384	100	$[M-H_2O]^+$	385	100	$[MH-H_2O]^+$	401	55	$[M-H]^-$
	369	6	$[M-H_2O-CH_3]^+$	367	93	$[MH-2H_2O]^+$	383	100	$[M-H-H_2O]^-$
	161	48	$[m/z\ 195-2H_2O+2H]^+$				367	78	$[M-2H-H_2O-CH_3]^-$
	107	66	$[m/z\ 125-H_2O]^+$						
Triol (M.W.=420)	402	18	$[M-H_2O]^+$	385	46	$[MH-2H_2O]^+$	419	31	$[M-H]^-$
	384	22	$[M-2H_2O]^+$	369	34	$[M-2H_2O-CH_3]^+$	401	85	$[M-H-H_2O]^-$
	369	14	$[M-2H_2O-CH_3]^+$	367	29	$[M-H_2O-CH_3-2H]^+$	398	100	$[m/z\ 401-H_2-H]^-$
	247	45	$[m/z\ 266-H-H_2O]^+$	111	100	$[m/z\ 109+2H]^+$	383	70	$[M-H-2H_2O]^-$
	229	55	$[m/z\ 247-H_2O]^+$						
	123	88	$[m/z\ 138-CH_3]^+$						
	109	100	$[m/z\ 158-2H_2O-CH_3+2H]^+$						

[a] Compounds listed on basis of their elution order in normal-phase mode.
[b] Fragmentation indicated in the skeleton refers to EI ionization.
[c] R.A.: Relative abundance.

triol, 7-OH and 7-keto, whereas response of 25-OH and cholesterol decreased relatively to that of other substances. Greater signal abundance was observed for 5,6α-EP in RP mode than in NP mode.

3.4. Linearity, precision and sensitivity of the NP-LC–PB-MS and the RP-LC–PB-MS methods

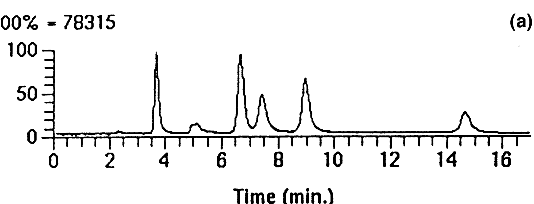
The results obtained attest a better performance of

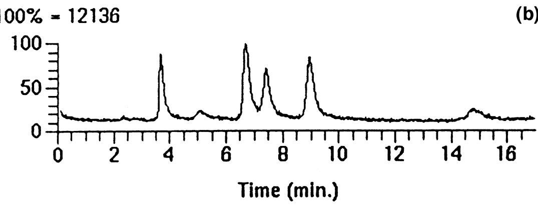


Scheme 1. Skeleton of cholesterol-related compounds.

the LC–PB-MS system under EI conditions, so that further studies on linearity, sensitivity and precision of the LC–PB-MS methods were performed using EI source. Tables 2 and 3 summarize the EI data concerning the linearity and the detection limits obtained using the CN and C_{18} chromatographic columns. In both cases the linear regression failed, since the data at the low level (10–62 ng) gave rise to a sensitivity significantly lower than those at high level (125–1000 ng). Non-linearity effects of the LC–PB-MS system at low concentrations due to a scarce transfer of the analytes through the interface at low concentrations have been discussed in various papers [27–29] and by Creaser and Stygall [30] in a review dealing with the PB interface for LC–MS.

The experimental data fitted a quadratic model corresponding to the equation $y=a+bx+cx^2$, as is shown in the Tables. It is interesting to note that the RP method applied for COPs resulted in improved sensitivity for all the compounds investigated; in fact, the sensitivity values, expressed for a quadratic dependence as dy/dx, x being the concentration at the mean point of the concentration range consid-





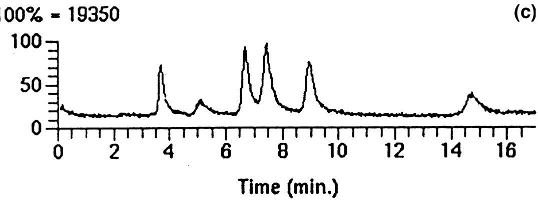
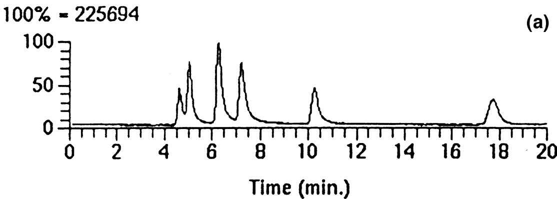
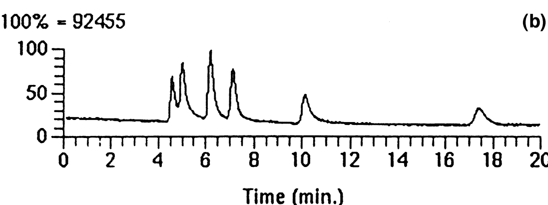


Fig. 2. LC–PB-MS total ion chromatograms of a mixture of cholesterol and oxycholesterols obtained using a CN bonded-silica column (250×2.0 mm, 5 μm). Eluent: heptane–propan-2-ol (94:6); flow-rate, 0.3 ml/min. MS conditions: (a) EI mode, (b) PCI mode, (c) NCI mode. Elution order: 1 cholesterol, 2 5,6α-EP, 3 25-OH, 4 7-keto, 5 7β-OH, 6 triol.





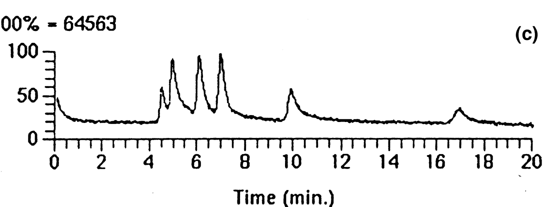


Fig. 3. LC–PB-MS total ion chromatograms of a mixture of cholesterol and oxycholesterols obtained using an ODS column (250×2.0 mm, 5 μm). Eluent: methanol–acetonitrile (90:10); flow-rate, 0.3 ml/min. MS conditions: (a) EI mode, (b) PCI mode, (c) NCI mode. Elution order: 1 25-OH, 2 triol, 3 7β-OH, 4 7-keto, 5 5,6α-EP, 6 cholesterol.

Table 2
Calibration graph results for NP-LC–PB-MS analysis of cholesterol and COPs[a,b]

Compound[c]	Range (ng)	$a\cdot10^{-7}$	$b\cdot10^{-6}$	$c\cdot10^{-2}$	r^2	n	LOD[d]
Cholesterol	2–1000	−1.84 (±0.63)	1.45 (±0.05)	1.50 (±0.58)	0.9964	44	2
5,6α-EP	16–1000	–	0.31 (±0.04)	3.62 (±0.45)	0.9953	32	16
25-OH	16–750	–	0.06 (±0.02)	5.87 (±0.29)	0.9964	28	10
7-keto	16–750	–	–	27.41 (±0.75)	0.9982	28	10
7β-OH	4–750	−1.38 (±0.42)	0.86 (±0.06)	1.46 (±1.36)	0.9892	32	4
Triol	16–1000	−0.92 (±0.25)	0.32 (±0.01)	3.54 (±0.16)	0.9989	32	10

[a] Calibration fitting: $y=a+bx+cx^2$.
[b] ± values are confidence intervals for 95% probability level.
[c] Compounds listed on the basis of their elution order in normal-phase mode.
[d] Limit of detection ($S/N=3$) in ng under EI-SIM conditions.

ered, are from 2- to 10-times higher depending on the analyte than those calculated by plotting the data obtained using the CN column.

The minimum detectable amount ranged from 2 ng for cholesterol to 16 ng for 5,6α-EP when the CN column was used. When operating with the C_{18} column, all the compounds examined provided remarkably similar detection limits (2–3 ng) and thus generally lower than those obtained in NP mode. These detection limits were comparable with those found using UV detection at 206 nm for the analytes detectable with this system, i.e., 7-keto, 7β-OH, 25-OH and cholesterol [8,23], and lower than those reported for laser light-scattering detection [25].

Precision of the PB-LC–MS system was evaluated by performing 4 repetitive injections of 16 and 250 ng of the analytes into the CN column; relative standard deviations (R.S.D.s) range from 1.4% for 25-OH to 13% for 5,6a-EP at the 16 ng level, whereas multiple injections at the 250 ng level showed an R.S.D. between 1.4% for cholesterol and 7% for 7β-OH. When RP conditions were chosen, the following results in terms of repeatability were obtained: from 3% for triol to 9% for cholesterol and 7-keto at the 4 ng level, from 3% to 8% for four 250 ng on-column injections of cholesterol and 7-keto respectively. Day-to-day repeatability ranging from 4 to 18% and from 5 to 12% was observed under NP and RP conditions, respectively.

These results attest a satisfactory repeatability of the method, whereas the minimum detectable amounts obtained in RP mode indicate an adequate sensitivity of PB–MS detection for the assay of COPs in foods.

4. Conclusions

Qualitative information including molecular mass and fragmentation pattern on the detected peaks are

Table 3
Calibration graph results for RP-LC–PB-MS analysis of cholesterol and COPs[a,b]

Compound[c]	Range (ng)	$a\cdot10^{-7}$	$b\cdot10^{-6}$	$c\cdot10^{-2}$	r^2	n	LOD[d]
25-OH	2–250	–	2.15 (±0.21)	0.58 (±0.08)	0.9936	32	2
Triol	4–1000	–	0.66 (±0.05)	0.11 (±0.05)	0.9983	40	3
7β-OH	4–1000	−3.56 (±1.23)	1.14 (±0.10)	0.08 (±0.01)	0.9933	40	2
7-keto	2–1000	−9.53 (±3.86)	5.41 (±0.32)	0.22 (±0.03)	0.9942	44	2
5,6α-EP	4–1000	−1.05 (±0.36)	0.27 (±0.03)	0.09 (±0.003)	0.9985	40	3
Cholesterol	2–1000	−10.3 (±3.10)	8.22 (±0.86)	1.02 (±0.34)	0.9979	44	2

[a] Calibration fitting: $y=a+bx+cx^2$.
[b] ± values are confidence intervals for 95% probability level.
[c] Compounds listed on the basis of their elution order in reversed-phase mode.
[d] Limit of detection ($S/N=3$) in ng under EI-SIM conditions.

provided by the LC–PB-MS technique. In addition, when all the ionization parameters are optimized, low-detection limits, comparable to those obtained by HPLC-UV for those analytes detectable with this system, are obtained. The major advantage is the capability of the MS detection to identify analytes even in the case of incomplete separation. Study of the PB parameters which affect sensitivity indicated that the nebulization and desolvation steps in the PB interface and the LC eluent composition play a role in obtaining optimal response also for the analytes investigated. For this class of substances, the RP-LC system based on the use of a narrow-bore C_{18} column and methanol–acetonitrile (90:10) mixture at low flow-rate proved successful for LC–PB-MS analysis of cholesterol and its oxides. Since the sensitivity appears to be adequate, the method validated in this work will be applied to real samples having different cholesterol content and thus different amounts of COPs.

Acknowledgements

This work was financially supported by Ministero dell' Università e della Ricerca Scientifica e Tecnologica, Italy.

References

[1] L.L. Smith, in Cholesterol Autoxidation, Plenum Press, New York, 1981.
[2] P.B. Addis, A.S. Csallany and S.E. Kingdom, in J.W. Finley and D.E. Schwass (Editors), ACS Symposium Series No. 234, American Chemical Society, Washington, DC, 1983, pp. 85–98.
[3] S.K. Peng, C.B. Taylor, J.C. Hill, R.J. Morin, Atherosclerosis 54 (1985) 121.
[4] I.-L. Kou, R.P. Holmes, J. Chromatogr. 330 (1985) 339.
[5] K. Sugino, J. Terao, H. Murakami, S. Matsushita, J. Agric. Food Chem. 34 (1986) 36.
[6] S.W. Park, P.B. Addis, J. Agric. Food Chem. 34 (1986) 653.
[7] V.R. De Vore, J. Food Sci. 53 (1988) 1058.
[8] A.S. Csallany, S.E. Kingdom, P.B. Addis, J.-H. Lee, Lipids 24 (1989) 645.
[9] Y.C. Chen, C.P. Chiu, B.H. Chen, Food Chem. 50 (1994) 53.
[10] G. Penazzi, M.F. Caboni, P. Zunin, F. Evangelisti, E. Tiscornia, T. Gallina Toschi, G. Lercker, J. Am. Oil Chem. Soc. 72 (1995) 1523.
[11] S.K. Peng, P. Tham, C.B. Taylor, B. Mikkelson, Am. J. Clin. Nutr. 32 (1979) 1033.
[12] P.B. Addis, Food Chem. Toxicol. 24 (1986) 1021.
[13] P.B. Addis and S.W. Park, in R.A. Scanlon and S.L. Taylor (Editors), Food Toxicology: A Perspective on the Relative Risks (Proceedings, 1988 IFT-IUFOST Basic Symposium), Marcel Dekker, New York, 1989, Ch. 11.
[14] M.J. Kelsey, R.J. Pienta, J. Cancer Lett. 6 (1979) 143.
[15] W.B. Zhang, P.B. Addis, T.P. Krick, J. Food Sci. 56 (1991) 716.
[16] P. Paniangvait, A.J. King, A.D. Jones, B.G. German, J. Food Sci. 60 (1995) 1159.
[17] S.W. Park, P.B. Addis, Anal. Biochem. 149 (1985) 275.
[18] J. Nourooz-Zadeh, L.-Å. Appelqvist, J. Food Sci. 52 (1987) 57.
[19] F.J. Monahan, J.I. Gray, A.M. Booren, E.R. Miller, D.J. Buckley, P.A. Morrissey, E.A. Goman, J. Agric. Food Chem. 40 (1992) 1310.
[20] C.B. Johnson, J. Chromatogr. A 736 (1996) 205.
[21] K. Granelli, P. Fäldt, L.-Å. Appelqvist, B. Bergenståhl, J. Sci. Food Agric. 71 (1996) 75.
[22] G. Maerker, E.H. Nungesser, I.M. Zulak, J. Agric. Food Chem. 36 (1988) 61.
[23] C. Sallin, E. Baumann, U. Bütikofer, R. Sieber, J.O. Bosset, Trav. Chim. Alim. Hyg. 84 (1993) 141.
[24] B.H. Chen, Y.C. Chen, J. Chromatogr. A 661 (1994) 127.
[25] S. Kermasha, S. Kubow, M. Goetghebeur, J. Chromatogr. A 685 (1994) 229.
[26] W.V. Ligon Jr., S.B. Dorn, Anal. Chem. 62 (1990) 2573.
[27] A. Apffel, M.L. Perry, J. Chromatogr. 554 (1991) 103.
[28] F.R. Browner, W.M. Draper, Biol. Mass Spectrom. 20 (1991) 515.
[29] J.S. Ho, T.D. Behymer, W.L. Budde, T.A. Bellar, J. Am. Soc. Mass Spectrom. 3 (1992) 662.
[30] C.S. Creaser, J.W. Stygall, Analyst 118 (1993) 1467.