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Evaluation of an on-line solid-phase extraction method for determination of almokalant, an antiarrhythmic drug, by liquid chromatography

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Abstract

A fully automated liquid chromatographic method based on a Prospekt solid-phase extraction unit is described for determination of the antiarrhythmic drug almokalant in plasma. The assay comprises solid-phase extraction on a C_2 phase and separation on a C_{18} column with fluorometric detection. In the original procedure 40 samples a day could be run unattended but by modifying the sequence in the solid-phase extraction process it was possible to increase this number to 70. The method gives an absolute recovery of 92% and a repeatability (C.V.) of 2.9% at 75 nmol/l of plasma. The limit of quantitation is 2 nmol/l of plasma (C.V. <20%). As regards accuracy and precision the performance of the method is as good as the manual method based on liquid–liquid extraction. The Prospekt method is, above all, faster and requires far less manual effort.

Keywords: Almokalant

1. Introduction

Almokalant, 4 - (3 - ethyl - 3 - [(propylsulphinyl) - propyl]amino-2-hydroxypropoxy)benzonitrile, is an antiarrhythmic drug substance which has been under investigation in early clinical phase. To support this and preclinical phase studies the substance has been determined in plasma for a couple of years using HPLC methods with liquid–liquid extraction (LLE). Later on we have turned to solid-phase extraction in order to automate the analyses.

Solid-phase extraction has been widely used to automate sample preparation. There are two different strategies available for automation: off-line, for example ASPEC and ZYMARK [1–4], and on-line, Prospekt and OSP-2. We have preferred the on-line approach and the Prospekt system. Owing to the uniform flow of solvents through the cartridge no problems occur with clogged cartridges and very reproducible extractions are obtained. The Prospekt system was first described in 1987 by Nielen et al. [5] and so far a couple of papers regarding applications using this system have been published [6–9]. In this study we have used the Prospekt system for determination of almokalant and the evaluation of the on-line solid-phase method developed is de-

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scribed. We also discuss benefits and differences relative to the method based on LLE.

2. Experimental

2.1. Chemicals and reagents

Almokalant and the internal standard H 222/98 (see Fig. 1) were synthesized at Medicinal Chemistry, Astra Hässle AB (Mölndal, Sweden). Acetonitrile, 2-propanol, methanol, hexane and dichloromethane were of HPLC grade (Rathburn, UK). Citric acid, orthophosphoric acid, NaH_2PO_4, Na_2HPO_4, Na_3PO_4 and NaOH (Titrisol) were of analytical grade (E. Merck, Darmstadt, Germany). N,N-Dimetyl-*n*-octylamine (DMOA) was obtained from ICN Pharmaceuticals, USA. Water was purified through a Milli-Q reagent grade water system (Millipore, MA, USA).

2.2. Chromatographic system for solid-phase extraction

A schematic representation of the chromatographic system is shown in Fig. 2. It was composed of two LKB 2150-pumps (Bromma, Sweden), a Marathon autosampler (Spark Holland, Emmen, Netherlands) and a Spark Holland Prospekt module. The Prospekt

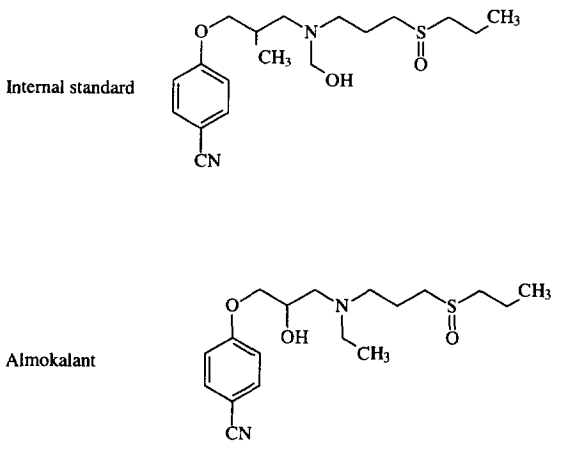

Fig. 1. Chemical structures for almokalant and internal standard (H 222/98).

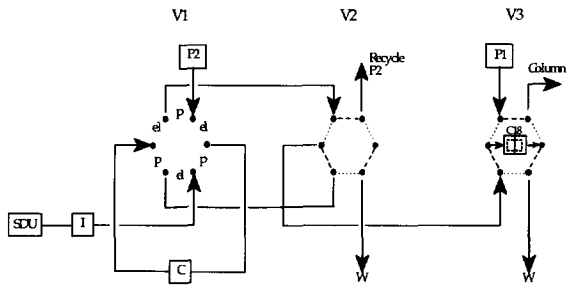

Fig. 2. Schematic representation of the solid-phase extraction system. V1, V2, V3=valves belonging to the Prospekt unit; SDU=solvent delivery unit; I=injector; C=extraction cartridge; P1=pump 1; P2=pump 2; W=waste; C_{18}=preconcentration and guard column. See also Table 1.

module comprised a microprocessor, a cartridge transport system, three six-port valves and a solvent delivery unit (SDU) with the capability of delivering up to six solvents. Eppendorf tubes (1.8 ml) were used. The solid-phase extraction cartridges (10×2 mm) contained 20 mg of Bakerbond C_2, 30–40 μm (Spark Holland).

The guard and pre-concentration column was a 10×4.3 mm Machery-Nagel C_{18}, 5 μm from Upchurch Scientific (Oak Harbor, UK). The analytical column was a 100×4.6 mm Microspher C_{18}, 3 μm (Chrompack, Middelburg, Netherlands). For the guard and preconcentration column the mobile phase was 2 m*M* DMOA in phosphate buffer pH 3.1, (I=0.05) and for the analytical column the mobile phase contained 5% acetonitrile, 1.7% 2-propanol and 2 m*M* DMOA in phosphate buffer pH 3.1 (I=0.05). The four solvents used for conditioning and loading of the extraction cartridge were (1) 100% methanol, (2) 40% methanol in water (v/v), (3) water, and (4) phosphate buffer pH 10, I=0.1. Before use the solvents and the mobile phases were ultrasonicated and then bubbled with helium. The flow-rate through the guard column and the analytical column was 1.0 ml/min and the eluent was monitored with a Shimadzu Model RF-535 fluorescence detector (Kyoto, Japan), using an excitation wavelength of 248 nm and emission at 305 nm. In certain experiments a Kratos Spektraflow 783 UV detector (NJ, USA) was used. Data was collected and processed by a Multichrom chromatographic data system (VG Data Systems, Altrincham, UK).

2.3. Solid-phase extraction procedure

The frozen plasma was allowed to thaw at room temperature, was vortex mixed and centrifuged. A 500-μl volume of plasma was pipetted into a 1.8-ml Eppendorf tube, to which 100 μl of internal standard solution (4 μmol/l of H 222/98 in citrate buffer pH 5.0, I=0.01), 100 μl of citrate buffer (pH 5.0, I=0.01), 50 μl NaOH 0.3 mol/l and 50 μl phosphate buffer (pH 10.0, I=0.5) had been added. The plasma standards (500 μl of blank plasma and 100 μl of standard solution) were treated as the samples except for the citrate buffer. The samples and the plasma standards were placed in the Marathon.

Prospekt changes cartridge, and orders the Marathon to load sample. Marathon flushes the lines with 57 μl of wash solution (20% methanol in water) and with 95 μl of sample. Marathon loads 650 μl of sample into a loop of 1100 μl and gives a go-signal to the Prospekt. Prospekt conditions the cartridge with 2 ml solvent (1), 0.5 ml solvent (2) and 2 ml solvent (4). Prospekt gives injection order to the Marathon and the sample is transferred to the extraction cartridge. Prospekt washes the extraction cartridge with 3 ml solvent (4). Prospekt changes valve 1, and almokalant and the internal standard are transferred onto the pre-concentration column. The transfer is accomplished by 2.5 ml mobile phase (pump 2) at 0.5 ml/min. Prospekt switches valve 3, and almokalant and the internal standard are injected onto the analytical column. Finally Prospekt fills the lines with solvent 1 (see Table 1).

2.4. Liquid–liquid extraction method

A 1.00-ml volume of plasma and 100 μl NaOH (1 mol/l) are extracted with 5.00 ml of a mixture of hexane–dichloromethane (1:1, v/v). The aqueous phase is frozen and the organic phase is transferred to a conical tub and evaporated. The residue is dissolved in 300 μl phosphate buffer pH 6.5, I= 0.01. A 125-μl aliquot of the extract is injected onto the analytical column. The mobile phase contained phosphate buffer pH 6.5 with acetonitrile as organic modifier and with DMOA to suppress peak tailing. Instrumentation was the same as described for solid-phase extraction (Section 2.3).

3. Results and discussion

3.1. Method development

Almokalant has a pK_a of 7.8 and the plasma samples were buffered to pH 10.0 prior to extraction. The extraction recovery from plasma was studied by

Table 1
Solid-phase extraction procedure

Time	Valve	Solvent	Flow-rate (ml/min)	Comment
00:00		1	1.0	Change of cartridge; Activation with 100% methanol
00:45	2; - - -			Wash of pre-concentration column
	3; - - -			with 100% methanol
01:15		2		40% methanol in water
01:30		4		Conditioning with water
02:00		3		Conditioning with phosphate buffer
02:30	2; ...			Protecting pre-concentration column from pH 10
04:30				Injection sample
04:40			0.5	Adjusting flow-rate for cartridge loading
09:00		4	0.9	Water rinse
09:30		2		40% methanol in water
09:45		1		Filling tubes with methanol
10:00	1; el			Eluting sample from cartridge (0.5 ml/min pump 2)
15:00	1; p			Injection of sample from pre-concentration to analytical column
	3; ...			(valve 3) and fills capillaries and clamp with methanol (valve 1)
15:30				End

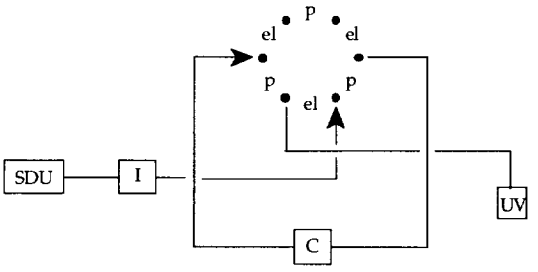

Fig. 3. Set-up for evaluation of extraction recovery. SDU=solvent delivery unit; I=injector; C=extraction cartridge; UV=UV detector.

using an on-line setup (see Fig. 3) where a UV detector was connected directly to the solid-phase extraction column. The UV detector was used in order to see interfering components more clearly. The materials studied were silica and CN, C_2, C_8 and C_{18} bonded material from Bakerbond. The idea with this set-up was to check the sequence of conditioning, the wash step and the elution, at the same time and in a way similar to the unattended method.

The solvent delivery unit (SDU) conditions the extraction column with methanol followed by a buffer solution. The autosampler injects a plasma sample while the SDU is pumping the conditioning buffer followed by a sequence of eluents. The strength of these is increased in small steps, and the test is ended by a liquid phase known to elute the analytes very fast (see Table 2). The chromatogram from the UV detector will show a big plasma front and then, preferably a clean baseline after which the analytes will appear (Fig. 4).

C_2, C_8 and C_{18} extraction columns retained the analytes efficiently. To elute them from the C_8 and C_{18} columns we had to use strong eluents, but for the C_2 column a plain phosphate buffer of pH 3 was

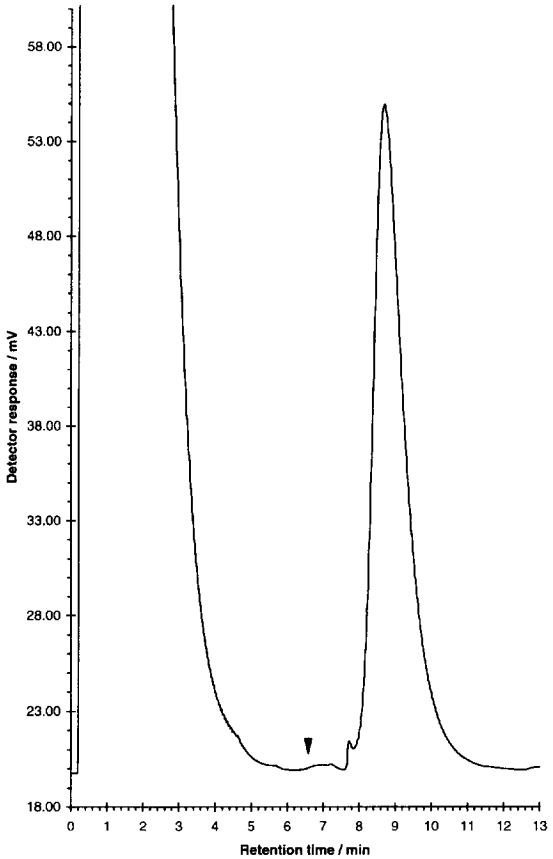

Fig. 4. Chromatogram showing elution of plasma at pH 10 of almokalant and internal standard after a change to phosphate buffer pH 3.1 with DMOA. The arrow indicates when the change was performed.

Table 2
Example of solid-phase extraction procedure

Time (min)	Valve	Solvent	Flow-rate (ml/min)	Comment
00:00		1	1.0–2.5	Conditioning of extraction column CH_3CN or MeOH
01:20		2		~60% solvent in water to decrease mixing heat
01:30		3		Water
02:00		4		Buffer of choice
04:00	autosampler			Injection of sample
06:00		5		another pH or buffer+organic solvent
08:00		6		None organic solvent or another pH
10:00		7		As above
12:00		8		CH_3CN or MeOH

sufficient. As expected all tested eluents gave broad peaks but this problem was solved by a pre-concentration column. When the analytes are eluted with a weak eluent it is possible to concentrate them on a column which retains them stronger. This pre-concentration column, in this case a C_{18}, also acts as a guard for the analytical column.

3.2. Mobile phase

The pH of the mobile phase for the LLE method was 6.5 and gave optimal separation from co-extracted metabolites. The pH of the mobile phase for the SPE method was 3.1. DMOA is needed to get a good peak shape of the analytes on this analytical column and its concentration was varied between 1 and 5 mmol/l. At lower pH less DMOA is necessary. For the LLE method the only organic solvent needed was acetonitrile but for the SPE method a combination of 2-propanol and acetonitrile was preferable.

3.3. Performance of the automated method

The recoveries of almokalant from plasma were calculated by comparing the peak areas of almokalant and internal standard with those obtained for the same amount injected directly onto the analytical column. The recoveries were 92 and 98%, respectively. The within-day variability was determined by performing replicate analyses of plasma samples containing 146 nmol/l of almokalant and was found to be 1.2% ($n=23$). The between-day variability and accuracy were calculated by performing replicate analyses of quality control samples with added almokalant (75 and 11 nmol/l) every analysis day. The between-day variability was found to be 2.9% ($n=22$) and 4.8% ($n=7$) and the accuracy was found to be 94.4% and 99.4%, respectively. The total capacity of the method was 40 authentic samples per day and 4 such series of analysis per week. More than 3000 authentic samples have been analyzed with this method, quantitation being performed using peak-height measurement (Fig. 5). The routine calibration performed was based on one standard concentration and at least 6 replicates. The linearity of the method was confirmed by assay of a full standard curve, 1.2 nmol/l to 2500 nmol/l (8 levels), once every month (see Table 3). Based on data from these standard curves the LOQ was set to 2 nmol/l (see also Figs. 6 and 7).

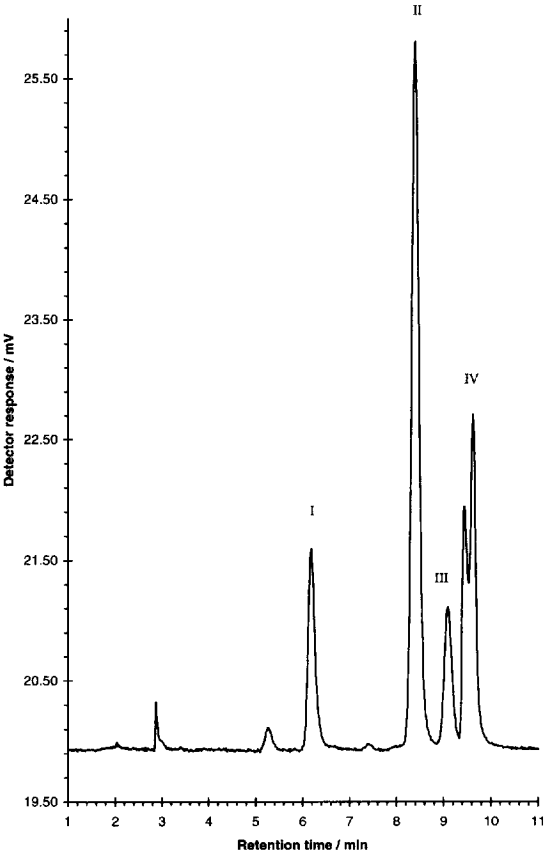

Fig. 5. Chromatogram of a human plasma sample taken 3 h after dose with the SPE-method. Peaks: I=almokalant (63 n*M*); II=internal standard (1700 n*M*); III=metabolite M18A; IV=metabolite M18B.

3.4. Further improvements of SPE method

The time for the solid-phase extraction step was recently shortened from 20 to 12.5 min by letting the injector work under remote control instead of serial interface. This permits the Prospekt to do the SPE-cartridge conditioning while Marathon takes up sample. The flow-rate of conditioning, loading and cleaning was also increased. With this faster method one analyst can analyze about 70 authentic samples per day.

Table 3
Quality data from standard curves

LLE results				SPE results			
Nominal concentration (nmol/l)	Accuracy (%)	C.V. (%)	*n*	Nominal concentration (nmol/l)	Accuracy (%)	C.V. (%)	*n*
1.24	114.3	7.9	24	1.20	102.8	16.1	18
48.9	98.4	1.9	9	58.8	100.7	4.9	9
1940	97.8	2.7	9	1140	98.4	1.6	9

3.5. Comparison

Hundred and ninety samples that previously had been analyzed with the LLE method, were assayed with this automated method. The results show an excellent agreement between the two methods. The mean of the difference between the SPE method and the LLE method results divided with the LLE method results was 0.024 (S.D. 0.054, $n=144$, range 10–100 nmol/l) (Fig. 8).

The instrumentation for the LLE method is very easy to handle and it takes about 0.5 h to prepare it for analysis. Manual preparation is simple but time-consuming and it takes about 5 h to prepare 40

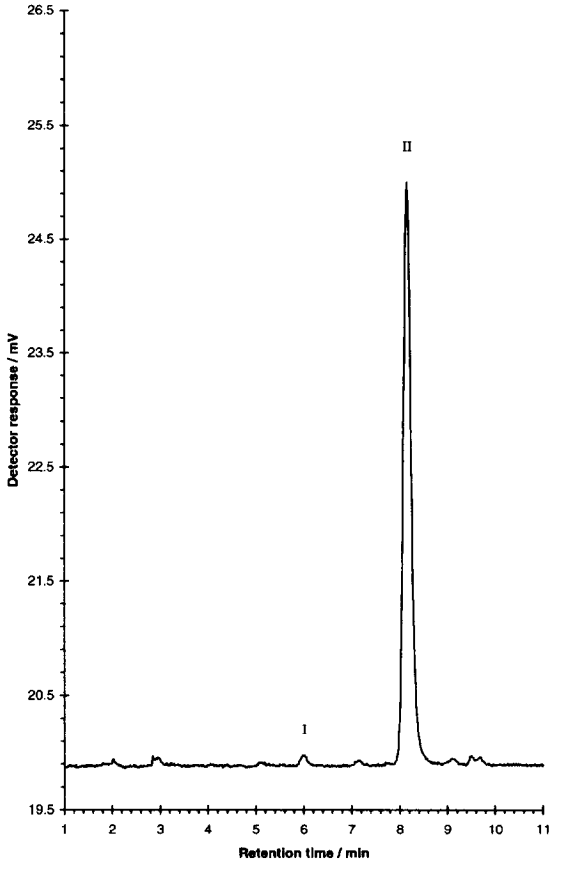

Fig. 6. Chromatogram of a human plasma sample taken 32 h after dose with the SPE-method. Peaks: I=almokalant (3.7 n*M*); II= internal standard (1700 n*M*).

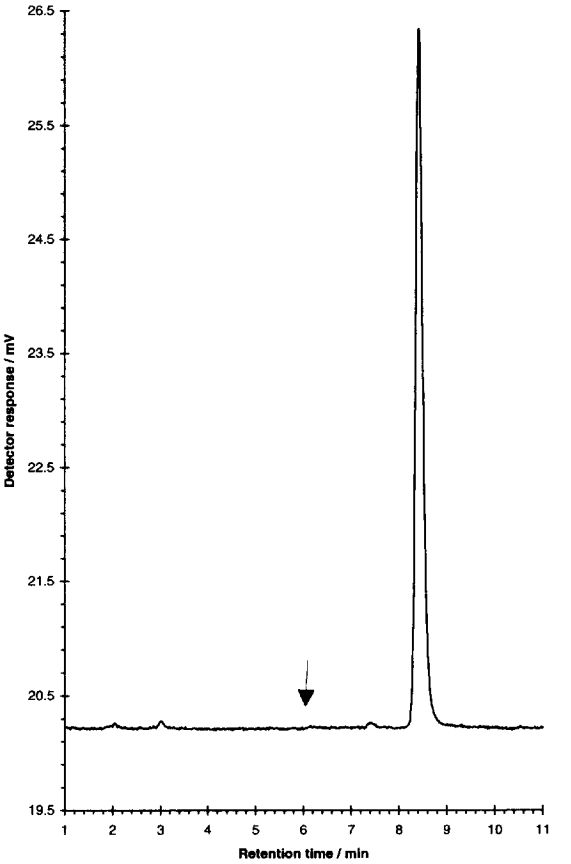

Fig. 7. Chromatogram of a predose human plasma sample with the SPE method. The arrow indicates where almokalant would elute.

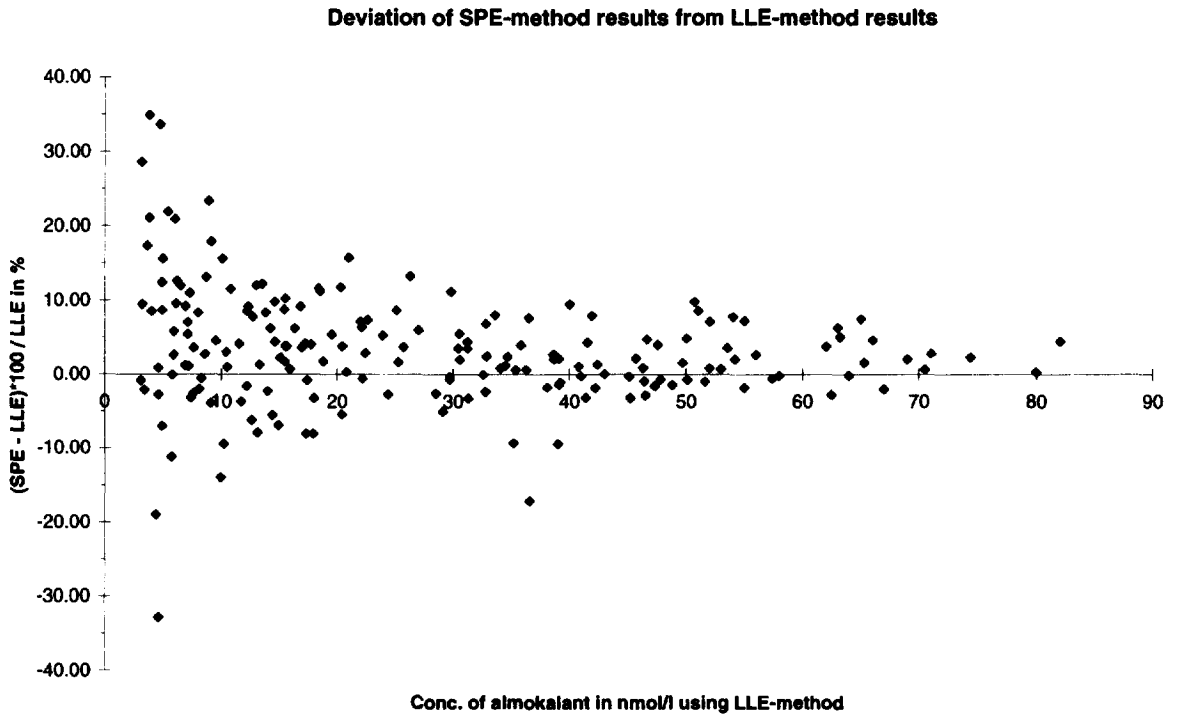

Fig. 8. Comparison between results obtained with the LLE method and results obtained with the SPE method, deviations from equality.

samples for analysis. The instrumentation for the SPE method is more complicated but takes only about 2 h to prepare for analysis. The fast SPE method is clearly superior to the LLE method when large numbers of samples are to be analyzed. When the sample load is low, the methods are equally good.

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