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Review

General strategies and selection of derivatization reactions for liquid chromatography and capillary electrophoresis

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Abstract

The general strategies, reasons and the different possibilities for the derivatization of biomedically important compounds are reviewed. Different approaches apply for small *versus* large analyte molecules, different advantages and disadvantages are visualized with pre- and post-column arrangements. Particular interest is focused upon solid-phase derivatization reagents.

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List of abbreviations

AFID Alkali flame ionization detector
CD Conductivity
CE Capillary electrophoresis
EC Electrochemistry
ECD Electron-capture detector
ELS Evaporative light scattering
FID Flame-ionization detector
FL Fluorescence

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FPD	Flame photometric detection
GPC	Gel permeation chromatography
HPLC	High-performance liquid chromatography
LC	Liquid chromatography
MS	Mass spectrometry
NSD	Nitrogen-selective detector
PCD	Photoconductivity
RI	Refractive index
SEC	Size exclusion chromatography
TCD	Thermal conductivity detector
TEA	Thermal-energy analyzer
UV	Ultraviolet
Vis	Visible

1. Introduction

Liquid chromatography (LC) and electrophoresis suffer as analytical techniques from relatively poor detection capabilities, which are often non-selective, non-specific, and insensitive. This is in contrast to gas chromatography (GC), where universal, completely general detectors can be used which are very sensitive and often very selective, such as flame ionization detectors (FID, AFID, FPD), thermal conductivity detectors (TCD), electron capture detectors (ECD), and so forth [1–5]. On the other hand, for LC and CE, the widely used UV absorbance detector, particularly in the scanning mode, is quite useful, at least for specific categories of compounds (*e.g.* proteins, nucleic acids, and their constituents). Though mass spectrometry (MS) is a perfectly viable detection interface for GC, HPLC, and more recently CE, in general, it is easiest to use in the GC mode, and detection limits and analyte sensitivity are generally superior in GC as well. Thus, at present there is no truly general, sensitive and selective detector for LC, *i.e.* one that will respond to all analytes, provide some structural information, and provide trace (<1 ppb) detection limits/levels. Again, MS can provide responses to virtually all organic and inorganic analytes, it can provide some structural information, often a great deal, and it can detect trace amounts of the analytes. However, it is still not used as a routine detection technique in either LC or CE (capillary electrophoresis), though this could change in the near future, as it did for GC–MS [6,7]. If the refractive index (RI) detector, which has been interfaced with HPLC already for several decades (*e.g.* SEC-RI, size exclusion chromatography), were a bit more sensitive, could provide some degree of structural information, and could provide low ppb detection limits, it might have become the universal, sensitive detector still missing for most of the LC studies. However, despite all the innovative detectors developed in the past 25–30 years, including evaporative light scattering (ELS), nitrogen-selective detection (NSD), thermal-energy analyzer (TEA), electrochemistry (EC), conductivity (CD), photoconductivity (PCD), and many others, we are still lacking a detector that is completely general, provides structural information, and offers true trace detection limits and high sensitivity.

Perhaps because of this deficiency in both LC and CE, the use of chemical, thermal, photochemical, and other physical methods have evolved to convert a non-detector-responding analyte into one or more derivatives that have enhanced chromatographic and/or detector properties [8–23]. Derivatization is basically the use of chemical reagents/reactions and/or physical methods to convert the original structure of the analyte into another molecule or mixture of reaction products. In some cases a simple photochemical ($h\nu$), acid/base, or thermal reaction will convert the original analyte structure into a product or derivative that has improved or different chromatographic and/or detector response properties. In other cases or in other chemical reactions/derivatizations, the analyte will have its structure altered by a rearrangement of bonds and atoms, and/or by the addition (tagging) of another molecule to provide the final derivative(s). In some cases, a single reaction product will be formed having vastly improved chromatographic and/or detection properties, in other cases, it may be preferable to have several such products formed at the same time and in the same reaction sample [24,25]. Thus, one can use several reaction products to improve the identification of the original analyte,

using multi-derivatives, their chromatographic properties, and their overall detection properties, to greatly improve identification and quantification of the analyte. Of particular interest are those reactions, in which the derivatizing reagent is not detectable by the method used [*e.g. o*-phthaldehyde (OPA) derivatization of amino acids] in the pre-column derivatization mode (see below). Fig. 1 summarizes, in a schematic manner, the various ways by which an original analyte molecule can be converted into one or more derivatives. Here we have to differentiate between chemical reactions that lead to derivatives without the addition of detector sensitive tags and those which lead to products containing some type of tag (UV, FL, EC, and so forth).

Derivatization procedures both in the pre- and post-column mode represent a very common step in separation protocols as can be documented by the number of papers dealing with the separation of derivatized solutes. Of the papers published in 1992 in the J. Chromatogr. Biomedical Applications, 60 papers used some type of analyte modification, not counting the derivatizations used in GC where, owing to the need to increase the volatility of the analytes, derivatization procedures are even more common (Table 1). As a matter of fact there are categories of compounds which are practically non-separable as such at all. Amino acids, carboxylic acids or carbohydrates can serve as typical examples. On the other hand there are categories of compounds which are rarely derivatized, if at all, because their physical (spectral) properties are such that derivatization is not needed. Typically nucleosides and nucleotides belong to this category.

To date use of fluorescent tags seems to be one of the most popular ways for derivatization; the particular reason is the increased sensitivity of the procedures with molecules tagged in this way (Table 2).

Solution derivatizations, homogeneous reactions, have been the most commonly performed type of derivatization, although more recently, solid-phase, also known as polymeric, reaction chemistry has been introduced with some success for both HPLC and CE [9,11,13,14,24–28]. In this introduction and overview, the way(s) by which the reactions of the analyte occur – solution *vs.* solid phase, photochemical *vs.* thermal, enzymatic *vs.* chemical, and so forth – are perhaps less important, than the question of how they can be used to improve the chromatographic/electrophoretic performance (efficiency, plate height, resolution, *etc.*) and detector response (linearity, detection limits, dual detector responses, *etc.*). Thus, it is clear that photochemical, catalytic, thermal, pH, acid/base, and more specific chemical reactions, including enzymic chemistry, can all, with varying degrees of success and depending on analyte structure, be used in LC and CE to convert the original analyte molecular structure into one or more products having properties that are desirable from a chromatographic-detector perspective.

A. Simplest imaginable derivatization scheme, no additional reagents, just light, heat, catalyst, temperature, radiolytic, and so forth, single product formation.

A ---> B

B. Several derivatives are formed at the same time from the original analyte, A, without additional chemicals or reagents involved.

A ----> B + C + D and so forth

C. A single chemical reaction between analyte A and chemical reagent B, leading to a single derivative C

A + B ---> C

D. A single chemical reaction between analyte A and chemical reagent B, leading to several derivatives, C and D

A + B -----> C + D and so forth

E. Sequential reactions on analyte A by reagent B, forming C, then sequential reaction by D, leading to E and perhaps other derivatives

A + B ---> C + D ----> E and so forth

F. Multiple reactions occurring simultaneously on analyte A by reagents B, C, and so forth, leading to multiple derivative formation

A + B + C ---> D + E and so forth

G. Tagging of analyte A with reagent B to form derivative C which contains the elements of tag B

A + B ---> C and so forth

H. All of the above chemical reactions that only convert analyte to derivative without inclusion of tag(s) could be used again now introducing a detector sensitive tag into each derivative or several tags.

Fig. 1. Summary of possible chemical derivatization schemes [13].

Table 1
Selected derivatization reactions applied for biological material analysis in 1992 as revealed by papers published in *J. Chromatogr. Biomedical Applications*

Compound assayed	Derivatization reagent	Reference
Acetylcholine (choline)	Acetylcholine esterase, choline oxidase	57
Aldehydes (formaldehyde, malondialdehyde, acetaldehyde, acetone)	2,4-Dinitrophenylhydrazine	63
Amikacin	1-Fluoro-2,4-dinitrobenzene	41
Amino acids	Phenylisothiocyanate	50
Amino acids	*o*-Phthalaldehyde-3-mercaptopropionic acid	78
Amino acids	Dimethylaminoazobenzene sulphonyl chloride	91
Amino acids	9-Fluorenylmethyl chloroformate	78
Amino acids	1-Fluoro-2,4-dinitrophenyl-5-L-alanine amide	58
Amino acids (enantiomers)	N-*tert.*-butyloxycarbonyl-L-cysteine + *o*-phthalaldehyde	85
Amino acids, D-	Dansyl chloride	93
Amino acids, N-acetyl	9-Anthryldiazomethane	67
1-Aminocyclopropane carboxylic acid	*o*-Phthalaldehyde	72
Antibiotics, macrolide (Josamucin, Rokitamycin)	Dansylhydrazine	52
Benzoylecgonine	3-Bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone	98
Butyrobetain	4′-Bromophenacyl trifluoromethane sulphonate	47
Captopril	*p*-Bromophenacyl bromide	89
Catecholamines (Epinine, dopamine)	1,2-Diphenylethylene diamine	42, 90
Chondroitin sulphates	2-Cyanoacetamide	43
Chondroitin sulphate	Dansylhydrazine	76
Corticosterone, cortisol	Sulphuric acid	82
Creatinine	Phenacyl bromide	94
Cyanide	2,3-Naphthalene dialdehyde	86
Cystathionine	1,2-Diamino-4,5-dimethoxy benzene	51
Dermatan sulphate	Dansylhydrazine	76
Diaminopimelic acid	*o*-Phthaladehyde	88
Fatty acids (hydroxy, polyunaturated	Acetic anhydride	56, 73
Fatty acids (polyunsaturated)	2-Bromoacetophenone	68
Fatty acids (non-hydroxy)	Amide dys.	61
Fulvoxamine	Dansyl chloride	44

Table 1 *(continued)*

Compound assayed	Derivatization reagent	Reference
Fumonisin	*o*-Phthalaldehyde	55
Glutathione	1-Chloro-2,4-dinitro-benzene	95
Histamine	*o*-Phthalaldehyde	48
Homocysteine	*o*-Phthalaldehyde	74
Hyaluronic acid	Dansylhydrazine	76
Hydrochloroquine (diastereomers)	(+)-Di-O-acetyl-L-tartaric anhydride	99
Iodothyronines	Dansyl chloride	69
Labetalol (stereoisomers)	(4*S*-*cis*)-2,2-diethyl-5-isothiocyanato-4-phenyl-1,3-dioxane	75
Lipopolysaccharides (*E. coli* endotoxins)	Fluorescein isothiocyanate	53
Methocarbamol (enantiomers)	(*S*)-(+)-1-(1-naphthyl)ethyl isocyanate	87
Methyl ethyl ketone	2,4-Dinitrophenyl hydrazine	79
3-Methyl histidine	*o*-Phthaladehyde	83
3-Methyl histidine	Phenylisothiocyanate	97
Metoprolol (enantiomers)	*S*-(+)-1-(1-methyl)ethyl isocyanate	80
Mexiletine (enantiomers)	*o*-Phthaladehyde-N-acetyl cysteine reagent	81
Penicillin G	1,2,4-Triazole-mercuric chloride	65
Peptides(cyclic)	Naphthalene-2,3-dicarboxaldehyde + N-acetyl-D-penicillamine	70
Polyamines (aminoxy analogues)	Ketone + *o*-phthalaldehyde	49
Polyamines	Dansyl chloride	45
Selenocysteine	N-(iodoacetylaminoethyl)-5-naphthylamine-1-sulphonic acid	64
Sialic acids	Malonitrile	77
Sialidase activity	Ninhydrin	84
l-Stercobilin	Zinc acetate	54
Sotalol (enantiomers)	*S*-(−)-alpha-methylbenzyl isocyanate	66
Taurine	Fluorescamine	59
R,*S*-Tranylcypromine (enantiomers)	*o*-Phthalaldehyde + mercaptan N-acetylcysteine	96
Tris (hydroxymethyl amino methane)	Benzoyl chloride	92
i-Urobilin	Zinc acetate	54
Valproic acid	4-Bromomethyl-7-methoxy coumarin	60
Verapamil (norverapamil, gallopamil, enantiomers)	Acetic anhydride	62
Vitamin B_6 (vitamers)	Sodium bisulphite	71
Warfarin (diastereomers)	(−)-(1*S*,2*R*,4*R*)-endo-1,4,5,6,7,7-hexachloro-bicyclo [2.2.1] hept-5-ene-2-carboxylic acid	46

Table 2
Fluoroscence labels for liquid chromatography[a]

Label	Abbreviation	Derivatised compounds[b]	Reaction conditions[c] / temp.		Wavelength		NP/RP	Ref.[d]
					λ_{ex}	λ_{em}		
N-(9-Acridinyl)maleimide	NAM	thiol	FS/25,	BC	360	435	RP	100
(d)-(1)-1-Aminoethyl-4-dimethylamino-naphthalene	DANE	carboxylic acid	S/25,	BC	320	395	NP	101
9-Aminophenanthrene		carboxylic acid	MF/70		305	375	RP	102
N-(2-Aminophenyl-6-methylbenzthiazole)-acetylohydrazine		carbonyl	S/25,	AC	345	415	RP	103
Anthracene-9-carbonylchloride		alcohol	S/25		250	460	NP	104
Anthracene-9-carboxylic acid	ACA	alcohol	F/25		360	460	NP	105
Anthracene-2,3-dicarboxaldehyde	ADA	primary amine	MF/25,	BC	380	530	NP/RP	106
Anthracene-1-isocyanate		alcohol	MF/99		255	365	NP	107
9-Anthroylnitrile		alcohol	MF/60,	BC	365	465	NP	108
p-(9-Anthroyloxy)phenacyl bromide	Panacyl Br	carboxylic acid	S/35,	BC	250	415	RP	109
9-Anthryldiazomethane	ADAM	carboxylic acid	S/25,	BC	255	415	RP	110
d-, or 1-1(1-Anthryl)-ethylamine		carboxylic acid	FS/40		260	400	NP	111
Benzoin	BZ	guanidino compounds	VF/99		325	425	RP	112
p-(2-Benzoxazolyl)-phenylmaleimide	BOPM	thiol	S/35,	pH 5	320	360	RP	113
3-Benzoyl-2-quinolinocarboxaldehyde	BCQA	p. amine	MF/25		460	555	RP	114
1-(Bromoacetyl)pyrene		carboxylic acid	MF/40,	BC	370	440	RP	115
4-Bromomethyl-7-acetoxycoumarin	Br-Mac	carboxylic acid, phenol thiol, imide	MF/50	BC	365	460	RP	116
9-(Bromomethyl)acridine		carboxylic acid	F/25,	BC	365	425	RP	117
4-Bromomethyl-6,7-dimethoxycoumarin	Br-Dmc	carboxylic acid, phenol thiol, imide	MF/70,	BC	345	425	RP	118
3-(Bromomethyl)-6,7-dimethoxy-1-methyl-2(1H)-quinazoline	Br-Dmq	carboxylic acid	F/50,	BC	370	455	RP	119
4-Bromomethyl-7-methoxycoumarin	Br-Mmc	carboxylic acid, phenol thiol, imide	MF/50		330	390	RP	120
		pyrimidin	VF/25,	BC	330	400	RP	121
5-di-*n*-Butylaminonaphtahelene-1-sulfonyl chloride	see Dns-C1							122
3-Carbonylazide-7-methoxycoumarin		alcohol	FS/25		335	405	RP	123
7-(Chlorocarbonylmethoxy)4-methyl-coumarin		alcohol	FS/25,	BC	320	390	RP	124
3-Chloroformyl-7-methoxycoumarin		alcohol	MF/99		355	400	RP	125
9-(Chloromethyl)anthracene		carboxylic acid	MF/80,	BC	365	410	RP	126
Chloromethylbenz[c,d]indol-2-(1H)-one	CMBI	carboxylic acid	F/50,	BC	365	480	NP	127
4-Chloro-7-nitrobenz-2-oxa-1,3-diazole	NBD-Cl	p./s. amine, thiol, phenol	FS/60,	pH 8	470	530	NP/RP	128
4-Chloro-7-sulfamoylbenz-2-oxa-1,3-diazole	SBD-C1	thiol	S/60,	pH 8	380	470	RP	129
1,3-Cyclohexadione	CHD	aldehyde	FS/60		305	450	RP	130
1,2-Diamino-4,5-dimethoxybenzene		thiol	MF/60,	AC	350	425	RP	131
1,2-Diamino-4,5-methylenedioxybenzene	DMB	α-dicarbonyl	F/70,	AC	355	390	RP	132
9,10-Diaminophenanthrene		carboxylic acid	F/85,	AC	255	365	RP	132

4-Diazomethyl-7-methoxycoumarin		alcohol	MF/25		325	386	RP	134
7-Diethylamino-3(4′-maleimidylphenyl-4-methylcoumarin		thiol	FS/40,	pH 8	385	465	RP	135
3,4-Dihydro-6,7-dimethoxy-4-methyl-3-oxo-quinoxaline-2-carbonylazide		alcohol	MF/60		360	440	RP	136
4,5-Dimethoxy-1,2-diaminobenzene		α-keto acid	S/99,	AC	360	450	RP	137
N-[4(6-Dimethylamino-2-benzfuranyl)-phenylmaleimide		thiol	MF/60,	BC	355	455	RP	138
N-(7-Dimethylamino-4-methylcoumarinyl)-maleimide	DMCM	thiol	VF/25,	BC	400	480	RP	139
5-Dimethylaminonaphthalene-1-(N-chloro-sulfonylamide)	Dns-NCA	thiol	VF/40		335	510	RP	140
5-Dimethylaminonaphthalene-1-ethyl-chloroformate	Dns-ECF	alcohol, amine	FS/25,	BC	335	515	RP	141
5-Dimethylaminonaphthalene-1-sulfonyl-aminoethanol	Dns-AE	carboxylic acid	S/25,	BC	330	500	RP	142
5-Dimethylaminonaphthalene-1-sulfonyl-aziridine	Dna-A	thiol	FS/60,	pH 8	330	500	RP	143
5-Dimethylaminonaphthalene-1-sulfonyl-chloride	Dns-Cl	p./s. amine	MF/45,	BC	330	500	NP/RP	144
		phenol	VF/25,	PTC	330	500	NP/RP	145
5-Dimethylaminonaphthalene-1-sulfonyl-hydrazine	Dns-H	carbonyl	MF/30,	AC	360	500	NP/RP	146
4-Dimethylamino-1-naphthoylnitrile		alcohol	MF/25,	BC	350	530	RP	147
2-Diphenylacetyl-1,3-indandione-1-hydrazine	DIH	aldehyde	VF/25,	AC	425	525	RP	148
		ketone	MF/25,	AC	425	525	RP	148
1,2-diphenylethylenediamine		catechol	F/25		340	480	RP	149
Fluorescein-5-isothiocyanate	FITC	p./s. amine	S/25,	BC	490	520	RP	150
(+)-1-(9-Fluorenyl)ethylchloroformate	FLEC	p./s. amine	VF/25,	pH 8	260	315	RP	151
(9-Fluorenyl)methylchloroformate	FMOC	p./s. amine	VF/25,	pH 8	260	315	RP	152
		alcohol	VF/25,	BC	260	315	RP	152
4-Fluoro-7-(aminosulfonyl)-benz-2-oxa-1,3-diazole	ABD-F	thiol	MF/60,	pH 8	380	520	RP	153
4-Fluoro-7-(N,N-dimethylaminosulfonyl)-benz-2-oxa-1,3-diazole	BDB-F	thiol	F/50,	pH 8	385	515	RP	154
4-Fluoro-7-nitrobenz-2-oxa-1,3-diazole	NBD-F	p./s. amine, thiol phenol	VF/60,	pH 8	470	530	NP/RP	155
4-Fluoro-7-sulfamoylbenz-2-oxa-1,3-diazole	SBD-F	thiol	FS/60,	BC	380	520	RP	156
Glycylglycine	GG	catechol	VF/95		350	500	RP	112
4-Hydrazino-7-nitrobenz-2-oxa-1,3-diazole	NBD-H	aldehyde	F/50		470	570	NP/RP	117
		ketone	MF/50		470	570	NP/RP	158
4′-Hydrazino-2-stilbazole	4H2S	α-keto acid, carbonyl	F/50,	AC	360	430	RP	159
9-(Hydroxymethyl)anthracene	HMA	carboxylic acid	MF/25		365	415	RP	129
Lissamine rhodamine B sulfonyl chloride		p./s. amine	VF/25,	PTC	565	585	RP	160
2-Methoxy-2,4-diphenyl-3(2H)-furanone	MDPF	p./s. amine	MF/25,	BC	390	480	RP	161
2-Methyl-1,1-binaphthalene-2′-carbonylnitrile		alcohol	MF/60,	BC	340	420	NP	162

continued on p. 8.

Table 2
Continued

Label	Abbreviation	Derivatised compounds[b]	Reaction conditions[c]/temp.		Wavelength		NP/RP	Ref.[d]
					λ_{ex}	λ_{em}		
5-Methylphenylaminonaphthalene-1-sulfonyl chloride	see Dns-Cl							163
Monobromobimane	BB	thiol	F/25,	pH 8	380	460	RP	164
Monobromotrimethylaminobimane	BTAB	thiol	F/25,	pH 8	380	460	RP	165
1,2-naphthalenebenzimidazole-6-sulfonyl chloride		p./s. amine	F/40,	BC	365	405	RP	166
Naphthalene-2,3-dicarboxaldehyde	NDA	p. amine	VF/25,	BC	435	490	NP/RP	167
2-Naphthylchloroformate	NCF	t. amine	FS/99,	BC	275	335	RP	168
Phenanthrenequinone	PQ	guanidino	VF/25,	BC	375	460	RP	169
(3-Phenylpyrazoline-1-yl)-4-sulfonylhydrazine		carbonyl	S/25,	AC	365	425	RP	103
4-Phenylspiro[furan-2(3H)-1′-phthalan]-3-3′-dione	Fluram	p. amine	VF/25,	BC	390	475	RP	170
3-(2-Phthalimidyl)benzoyl chloride		alcohol, amine	MF/30,	BC	290	445	RP	171
4-(2-Phthalimidyl)benzoyl chloride		alcohol, amine	MF/30	BC	320	420	RP	171
3-(2-Phthalimidyl)-4-methoxybenzoyl chloride		alcohol, amine	MF/30,	BC	300	415	RP	171
1-Pyrenyldiazomethane		carboxylic acid	FS/25		340	495	RP	172
o-Phthalaldehyde	OPA	p. amine	VF/25,	BC	340	455	RP	173
N-(1-Pyrenyl)maleimide	PYM	thiol	VF/25,	BC	345	375	RP	174
N-Succinimidyl-2-naphthoxy acetate		aminophospholipid	FS/25,	BC	230	340	NP	175

[a] The data in this Table are partly taken from: C. de Ruiter, Pre- and Post-Column Fluorescence Derivatization in HPLC, Dissertation, Free University, Amsterdam, 1989.
[b] p., Primary; s., secondary; t., tertiary.
[c] VF, very fast (0–5 min); F, fast (5–15 min); MF, moderately fast (15–45 min); FS, fairly slow (45 min–2 h); S, slow (>2 h); BC, base catalysed; AC, acid catalysed; PTC, phase-transfer catalysed.
[d] NP, normal phase; RP, reversed-phase.

2. Why perform derivatizations in LC and CE?

Generally chemical derivatization is used to improve detection sensitivity by converting a compound with a poor detector response into a highly detectable product [8–23]. Apart from an increase in detectability, the derivatization step also improves the selectivity of the overall analytical method through the inherent selectivity of the derivatization chemistry employed.

When performed in the pre-column mode, *i.e.* before the analytical or electrophoretic steps/columns, derivatization changes the chromatographic or separation behaviour of the analyte. Thus, in general, peak shape, peak height, plate count, selectivity, resolution, efficiency, alpha value, and other separation performance parameters of the analyte should preferably all be enhanced via suitable, selective derivatization reactions. This may be performed in several ways: (a) a non-UV/FL responding analyte can be converted into one that is, having a different separation pattern and chromatographic properties than the parent molecule; (b) a non-chromatographable analyte can be chromatographed by suitable molecular rearrangements or tagging; (c) an analyte not resolved from other matrix components may be resolved by its conversion into a derivative having a vastly different separation pattern or mechanisms; (d) an analyte having either a poor detector response linearity, a narrow linear response range, or a high detection limit, may be derivatized to a compound improved with respect to all detector response properties; (e) a compound poorly separable in *e.g.* normal-phase LC systems or CE may be assayed in the reversed-phase mode or by a micellar separation procedure by changing its hydrophobic properties through derivatization; (f) using enzymic amplification techniques, it is possible to generate large amounts of different products (derivatives) from the analyte (substrate), all of which will give enhanced detector responses compared to the original analyte itself. This idea of generating several/numerous products from a single analyte molecule via enzyme amplification is an important technique which has been widely employed in HPLC and CE applications [11,13,16].

3. Large *versus* small analyte molecules and their derivatizations

In general, it is easier to derivatize small molecules than large ones. That is, the rates of chemical reactions for very large molecules, such as biomolecules, are usually orders of magnitude slower than for smaller species. The reaction rates are a function of the number of effective chemical collisions, the number of chemical collisions per unit time between reactive sites, the conformational preferences of the biomolecules, and the number of active sites available in a biomolecule [16,17]. This does not mean that biomolecules can not be successfully derivatized–they often are–but the efficiency (percent derivatizations per unit time) is usually much less than for smaller species. Also, the activation energy needed to derivatize a primary amino group in a large molecule is often much larger than that needed for the same derivatization in a very small molecule. This is, of course, a function of the neighboring groups, the conformational preferences, the conformations available, the hydrogen bonding within the biomolecule, and other factors. A considerable problem in the derivatization of large molecules (typically biopolymers) stems from the fact that in most cases, such polymers possess a number of reactive groups, for reasons just specified, which may differ in their reactivity. The result may be the formation of a number of products bearing the same tag in different mole-per-mole ratios. So, while in enzymic amplification techniques formation of multiple products helps identification, in the situation just described, multiple derivatization product formation should be avoided as much as possible. Separation of such mixtures is often difficult, usually resulting in broad peaks and low plate counts. Moreover, it may be difficult to trace which derivative derived from which solute originally present in the sample.

There are numerous chemical reactions that have been used to derivatize different classes of biomolecules in LC and CE, usually with a high degree of success. However, the overall enhancement of the detectability always depends on the particular tags used. That is, derivatization reactions which tag a specific site within the

biomolecule lead to a single, sometimes several, tags incorporated into the derivative. As a function of the tag, there will be improved detector response, but perhaps much smaller chromatographic changes than with small molecules, when performed pre-column, and thus these derivatizations are often performed post-column, where possible. An ideal derivatization scheme would generate many derivatives from the original biomolecule, *e.g.* derivatization via enzyme amplification which is already used to detect intact enzymes, but much less to detect proteins, peptides, nucleic acids, and so forth. Thus, the scheme described by Engelhardt *et al.* [29] using post-column microwave digestion of proteins, followed by a second post-column solution reaction with a FL derivatizing reagent (*e.g.* OPA), has been used for the detection of many amino acids by FL methods. This is, perhaps, a good example of a general approach that greatly improves the detectability of large molecules, such as the enzyme amplification used for enzymes.

4. Off-line *versus* on-line arrangements

We also need to differentiate between off-line and on-line methods (Fig. 2). In the off-line mode, the reactions occur away from the HPLC or CE system, although there are some examples that could be defined as either off- or on-line (*e.g.* reactions occurring in a sample vial in a carousel as part of an automated derivatization–injection system in LC/CE). In the on-line mode, the reactions occur as part of the HPLC or CE systems, integrated into the instrumentation and analysis, being time constrained and controlled. In practice we can imagine four different and distinct types of derivatization approaches or modes for LC and CE: (1) on-line, pre-column; (2) on-line, post-column; (3) off-line, pre-column; and (4) off-line, post-column (Fig. 2).

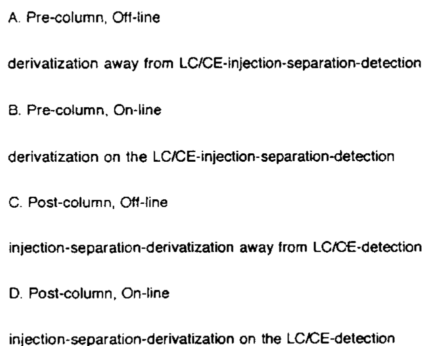

Fig. 2. Pre- *versus* post-column, off- *versus* on-line derivatization modes.

5. Pre-column *versus* post-column arrangements

Derivatization can be carried out in the pre-column or post-column mode, *i.e.* before or after the separation takes place. In the post-column approach, the derivatization reaction does not have to yield a single, stable product, provided that the derivatizations are reproducible. There are several serious disadvantages associated with this technique: (1) excess derivatization reagent may interfere with the detection; (2) reaction kinetics need to be rapid to allow real time detection; (3) additional pumps are needed for non-pulsating supply of derivatization reagent; (4) reaction solvents must be miscible with the mobile phase used for separation; and (5) efficient mixing of the derivatizing reagent with the column effluent is required.

Pre-column derivatization is an alternative for post-column derivatization. One of its advantages is that derivatization is independent of the mobile phase and the reaction kinetics are no limiting factor. Apart from increasing the detectability, pre-column derivatization may also improve the selectivity and chromatographic resolution of the overall method. Excess reagent present in the reaction mixture must be chromatographically resolved from the analyte derivative peaks, and/or be physically or chemically removed from the sample solution prior to injection. If several analytes yield the same derivative(s), these analytes will not be separable, and it will be impossible to determine

which analyte was originally present in the sample. For example, the use of a substrate that can react with several enzymes (in the pre-column mode), would then lead to exactly the same product(s), preventing absolute identification of the enzyme actually present in the sample reaction mixture. For these reasons probably more derivatizations have been performed on-line, post-column, as opposed to on-line, pre-column or even off-line, pre-column, at least in the LC areas. However, in CE applications, because of the difficulties involved in performing on-line reactions, either pre- or post-column, most derivatizations have been performed pre-column off-line. This may change with the introduction of reactions performed on immobilized supports in CE, either pre- or post-column, all on-line [29]. Introduction of so-called "parked reactions" by Bao and Regnier [39] for assaying glucose-6-phosphate dehydrogenase activity, in which both the substrate and the enzyme containing sample are loaded into the capillary electrophoresis column, may serve as an example of how such problems may be solved in the future.

6. Off-line, pre-column derivatizations

Off-line, pre-column derivatizations do not suffer from extra-column loss of efficiency, nor from solvent or kinetic limitations. Derivatization can be conducted under flexible reaction conditions or with harsh reagents. Off-line derivatizations can be optimized for high reaction yields and minimal generation of side-products. Derivatization solvents are preferably miscible with the chromatographic mobile phase. Otherwise, the derivatization solvents have to be evaporated and the derivatives in the residue are reconstituted in a mobile phase compatible solvent. Off-line derivatization does not need to give a 100% theoretical yield, as long as there is good sample-to-sample reproducibility. However, non-automated, off-line, pre-column derivatizations require operator attendance and manual manipulations.

7. On-line, pre-column derivatization

On-line, pre-column derivatization is accomplished by incorporation of a derivatizing reagent into the flow scheme of the liquid chromatograph. All derivatized products are injected onto the HPLC; on-line, pre-column derivatization does not suffer from the solvent dilution problem observed in the off-line derivatization. However, several requirements have to be satisfied to conduct on-line, pre-column derivatization: (1) good chemical and/or pressure stability of the derivatizing reagents in the organic solvent; (2) good solubility of the derivatized products in the mobile phase; (3) no precipitation or gas generated in the derivatization; (4) compatibility of the derivatization solvent with the mobile phase; and (5) a minimum volume of derivatization solvent or use of a well-packed solid-phase derivatization column. In on-line, pre-column derivatization, the extraction and clean-up of complex samples often are integrated in the chromatographic process, and can be automatically (computer/microprocessor interface) performed via switching of valves. Preliminary sample handling is minimized and automated derivatization procedures tend to provide better reproducibility [30].

8. Off-line, post-column derivatizations

This is perhaps the most unwieldy derivatization approach of all imaginable (Fig. 2). It involves separating the analyte of interest from the LC/CE eluent, prior to detection, performing a solution or solid-phase derivatization away from the instrumentation, manually or automated, and then detecting the final derivatized solution. Automation is difficult, at best, reproducibility is less-than-ideal, and even accuracy and precision falter, at times, because of a lack of total automatability. That is probably why this method receives the least emphasis in the literature, and the lowest recommendation of application.

9. On-line, post-column derivatizations

In this approach (Fig. 2) injection–separation steps are followed by on-line derivatization, using automated, fully on-line instrumentation and methods [11–13]. This technique utilizes post-column reactors (low dead-volume mixing tees, knitted open tubular reactors, low dead-volume reaction coils, and so forth), where the chemical reagents are mixed with the LC/CE eluent. A delay time is needed (reaction dependent) to convert the analyte to its product(s), and the entire solution, along with excess reagent(s), is introduced into the detector. This approach can also be applied to on-line liquid–liquid extraction, ion suppression (dual-column ion-chromatography), pH adjustment, organic solvent addition, basic hydrolysis reactions, additional chemical reactions modifying the solutes prior to the derivatization step (*e.g.* oxidation of the imidazole ring in proline and hydroxyproline for their assay by the OPA reaction), enzyme addition, and the use of post-column, immobilized reagents or enzymes. It is perhaps the most widely employed of all techniques for performing derivatizations in LC, but is however much less used in CE applications thus far. A number of chemical reactions have been employed post-column, on-line: sequential reactions, solid-phase/catalytic enhanced reactions (*e.g.* carbamate detection), microwave digestion of proteins, photochemical reactions, and so forth [11–13]. There are, of course, severe constraints or requirements with respect to the nature of the reagent solvent/solution that can be mixed with the LC effluent: detector transparency, prevention of analyte/derivative precipitation before detection, good mixing of reagents with analyte, lack of mixing noise, need for additional instrumentation, mixing tees, connecting joints, and extra tubing connections, and so forth. Nevertheless, at least in LC applications, this particular approach has been the most widely employed. Quite the opposite is the case for CE. For additional information beyond the scope of this volume, see ref. 40.

10. Solid-phase derivatization reagents in HPLC analyses

Chemical derivatizations are performed in either homogeneous solutions or on heterogeneous solid-phase reagents. These two types of derivatization are different in substrate compatibility, derivatization speed, and selectivity. The most commonly used derivatization methods for HPLC detection are homogeneous solution reactions, as above [5–13]. There are several serious disadvantages associated with homogeneous reactions, especially for biofluid-type analysis [13,14,30–32]. Biological samples are a complex mixture of lipids, proteins, and water. Many solution-type derivatization reagents are reactive towards the proteins in biofluids. Derivatization of multiple nucleophilic groups in the sample matrix increases the overall hydrophobicity of the protein molecules and may cause precipitation. Organic derivatization solvents may also cause protein precipitation after mixing with biofluids. These precipitation problems make it impossible to conduct direct derivatization of drugs in biofluids. Thus, extensive sample clean-up is often needed prior to solution derivatization. Sample pretreatments include solvent extraction, removal of excess unreacted reagent, and preconcentration of derivatives. Such purification procedures for biological fluid analysis are labor intensive and difficult to automate. Solid-phase derivatization is a complementary method which overcomes these disadvantageous properties of solution derivatizations [14,33–36]. The following advantages of solid-phase reagents (SPR) have already been noted: (1) no need for an organic reaction solvent; (2) hydrophobic extraction properties of the solid substrate with an increased analyte derivatization selectivity, especially for biological fluids; (3) simple reactions with less contamination and/or background due to excess derivatization reagent; (4) faster, milder, and more efficient reactions; (5) improved chemical stability of the reagent over time; (6) higher reaction capacities due to high concentration of the immobilized reagent; (7)

ease of regenerating the solid-phase reagent; and (8) possibility to use mixed-bed derivatizations with different derivatization tags for analyte confirmation [11–14,30-32,37,38].

Solid-phase reagents can be prepared on many kinds of matrices, such as silica, alumina, and organic polymers. Silica is suitable for supporting solid-phase reagents owing to its well defined pore structure, small particle size, good mechanical stability and large surface area [26]. The excellent pressure stability of silica-based solid-phase reagents in organic solvents, makes them very suitable for on-line derivatization in HPLC. A much more extensive discussion of solid-phase or polymeric reagents for derivatizations in both LC and CE is presented elsewhere in this Special Volume.

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