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COMPARISON OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION AND GAS CHROMATOGRAPHY—MASS FRAGMENTOGRAPHY FOR THE ASSAY OF SALSOLINOL, DOPAMINE AND DOPAMINE METABOLITES IN FOOD AND BEVERAGE SAMPLES

MARK W. DUNCAN*, GEORGE A. SMYTHE and MEGAN V. NICHOLSON

Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney (Australia)

and

PETER S. CLEZY

School of Chemistry, University of New South Wales, Sydney (Australia)

SUMMARY

High-performance liquid chromatography with electrochemical detection (HPLC—ED) and combined gas chromatography—mass spectrometry in the single-ion monitoring mode (GC—MS-SIM) have been used for the determination of salsolinol, dopamine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethanol and norepinephrine in a selection of food and beverage samples. The unique specificity of the SIM mode allows a simple one-step extraction to be used even for complex sample matrices. We have been able to demonstrate the quantitative and qualitative advantages offered by GC—MS over HPLC—ED by direct comparison of the chromatographic data obtained. We demonstrate that the specificity of SIM and the benefits offered by the incorporation of deuterated internal standards make GC—MS-SIM the method of choice for valid identification and precise quantitation of salsolinol, dopamine and dopamine metabolites in a complex sample matrix.

INTRODUCTION

For the analysis of biogenic amines, and biogenic amine derivatives in biological fluids, high-performance liquid chromatography coupled with electrochemical detection (HPLC—ED) has become the method of choice. Under optimal conditions this powerful combination can provide a sensitive and specific assay system.

In most assays a common structural thread, the catechol nucleus, is both the handle for extraction and the electro-oxidizable group. This means that with a combination of alumina extraction and electrochemical detection, overall speci-

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ficity is effectively determined by the complexity of the original sample matrix and the resolving power of the chromatographic step.

In practice, currently employed HPLC—ED procedures for the assay of catecholamines from complex sample matrices (e.g. urine) do not have the resolving power to allow quantitation of the analytes of interest unless there are first multi-step pretreatments to "tidy-up" samples [1]. As well as selectively removing the metabolites of interest such techniques can concentrate these analytes into as small a volume as possible. While these complex clean-up procedures are time-consuming and liable to introduce variations in recovery, particularly in multi-component assay, they are necessary in the analysis of complex sample types.

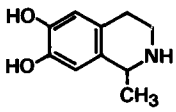

Fig. 1. Chemical structure of salsolinol (SAL).

The tetrahydroisoquinoline alkaloid, salsolinol (SAL, Fig. 1) has a direct structural link with dopamine, in particular retention of the catechol nucleus, and so SAL is also amenable to electrochemical detection. It has been suggested that SAL might be formed in vivo in mammals as a consequence of ethanol consumption; however, it is proving difficult to obtain valid analytical data that might support this proposal [2—5]. The rapid reaction that can take place bebetween dopamine in the sample and free acetaldehyde to form "artifactual" SAL under a wide range of conditions, and the low levels that might be present in biological systems, present particular problems for the detection and quantitation of this compound.

We have recently described a highly sensitive and specific combined gas chromatographic—mass spectrometric (GC—MS) method for the simultaneous measurement of SAL, dopamine (DA), 3,4-dihydroxyphenylethanol (DOPET), 3,4-dihydroxyphenylacetic acid (DOPAC) and norepinephrine (NE) which is suitable for a wide range of sample types [6]. Our continuing investigations in the area of biogenic amine metabolism have led us to examine the suitability of HPLC—ED for this same application. We were particularly concerned about the suitability of HPLC—ED to handle significantly different sample types.

Generally, researchers requiring catecholamine and mammalian alkaloid assays have exclusively used one analytical methodology and they display a very real bias when discussing the suitability of either GC/MS or HPLC—ED. This bias is sometimes evident in the views expressed about the benefits and limitations of alternative methodologies [4, 7, 8]. In this paper we directly compare the results obtained using both HPLC—ED and GC—MS assay systems when applied to catecholamine, catecholamine metabolite and salsolinol assay in a selection of food and beverage samples. Single-step alumina extraction giving high recoveries was used throughout. The results provide an opportunity for qualitative and quantitative comparison of these two techniques and discussion of their respective merits and limitations. We make particular reference to the suitability of each technique for the identification and quantitation of trace levels of salsolinol.

MATERIALS AND METHODS

Chemicals and reagents

Salsolinol·HCl was prepared via the method of King et al. [9] and recrystallized to a constant melting point. The final sample was shown to be free of impurities by GC—MS and HPLC analysis. Satisfactory elemental analysis was also obtained. The results reported in this study are uncorrected and refer to free acids and bases. Each reference compound was of the highest grade obtainable; dopamine·HCl, L-norepinephrine bitartrate and dihydroxyphenylacetic acid were all obtained from Calbiochem-Behring (Carlingford, Australia). 3,4-Dihydroxyphenylethanol was obtained from Regis (Morton Grove, IL, U.S.A.). 3,4-Dihydroxybenzylamine·HBr (DHBA), Tris and EDTA (disodium salt) were obtained from Sigma (St Louis, MO, U.S.A.). Alumina was prepared by essentially the same procedure described by Anton and Sayre [10]. Trifluoroacetic anhydride (TFAA) was obtained from Pierce (Rockford, Il, U.S.A.) and trifluoroethanol (TFE) from Sigma. All other reagents were of the highest grade obtainable.

HPLC instrumentation and instrumental conditions

HPLC determinations were performed with a Hewlett-Packard 1081 isocratic liquid chromatograph fitted with an auto-injector and auto sample changer. A BioAnalytical Systems Model LC-4 amperometric detector coupled to a Hewlett-Packard 3390A recording integrator was used for data acquisition. A glassy carbon electrode set at 0.72V vs. Ag/AgCl reference electrode was used directly after the column for detection. Detector sensitivity was set at either 5 or 10 nA full scale during these studies. Chromatography was achieved on a 250 × 4.6 mm I.D. Beckman ultrasphere ODS reversed-phase column (5 μm; Beckman, Sydney, Australia). All elutions were isocratic.

Mobile phase

The mobile phase (pH 3.0) was monochloroacetic acid buffer (0.15 *M*) containing EDTA (2.0 m*M*) and sodium octyl sulphate (75 mg/l). The mobile phase was filtered through a Millipore HA filter (0.45 μm) and degassed ultrasonically prior to use. A flow-rate of 1.5 ml/min at a temperature of 35°C was employed in all studies. The mobile phase was continuously recycled.

Extraction procedure

The extraction procedure used was alumina-based and is a slight modification of that described by BioAnalytical Systems for plasma extraction. Formic acid (0.4 *M*) was used in place of perchloric acid in the final elution step. Absolute recoveries were >70% for each analyte.

Preparation of food samples. Samples (0.2—1.0 g) were mechanically homogenized (Ultra-Turrax) in 0.1 *M* hydrochloric acid (10 ml). Samples were then centrifuged (3000 *g*, 30 min, 4°C), filtered (0.4 μm; Millipore) and portions (generally 50 μl) were extracted as described above. Sample volumes extracted were, however, adjusted to give "on-scale" chromatograms with fixed instrument parameters to allow a direct comparison.

Preparation of beverage samples. Carbonated beverages were freshly opened

and ultrasonically degassed before analysis. A portion (1—2 ml) of each beverage was extracted as previously described.

Standard curves for HPLC—ED analysis

Standard solutions containing varying known amounts of NE, DA, DOPET, DOPAC and SAL in 0.25 *M* formic acid were prepared, and a fixed (and known) concentration of DHBA was added to each of these. Samples were then extracted as described and injected into the HPLC system (10—25 μl). Standard curves were constructed by plotting the NE/DHBA, DA/DHBA, DOPET/DHBA, DOPAC/DHBA and SAL/DHBA peak height ratios against the concentration of the appropriate analyte. The standard curves were prepared by unweighted least-squares linear regression analysis from single samples and single estimations of peak heights. The lowest point on each standard curve corresponded to 5—30 pmol per sample (i.e. approx. 1—5 ng) extracted but varied slightly. Coefficients of variation (i.e. *r* values) were greater than 0.999.

Sample concentrations were calculated by standard techniques incorporating adjustment for varying recoveries using DHBA as internal standard.

GC—MS-SIM

Full details of the method used have been described elsewhere [6]. Samples were treated with a deuterated standard mixture and then extracted via the alumina technique. Formic acid in methanol (5 *M*, 1:4) was used to elute the catechols. Samples were then evaporated to dryness under nitrogen and derivatized with TFAA (200 μl) and TFE (50 μl) at 60°C for 20 min. After evaporating off the excess reagent under nitrogen the samples were reconstituted with ethyl acetate (10 μl) and a portion (1—2 μl) was injected into the GC—MS instrument.

TABLE I

RETENTION TIMES AND MAJOR IONS FOR EACH ANALYTE UNDER THE CHROMATOGRAPHIC CONDITIONS DESCRIBED

Column Type 3% OV-17; 1-m glass column. For chromatographic conditions see Materials and methods.

Analyte	Retention time* (min)	*m/z* monitored	Percentage of base peak	Group in which ion monitored
DA	2.85	328	(100%)	3
d_3-DA	2.85	331	(100%)	3
DOPAC	1.3	442	(54%)	1
d_5-DOPAC	1.3	447	(54%)	1
DOPET	1.2	328	(100%)	1
d_3-DOPET	1.2	331	(100%)	1
NE	2.1	440	(100%)	2
d_3-NE	2.1	442	(100%)	2
SAL	3.5	452	(100%)	4
d_4-SAL	3.5	456	(100%)	4
		467	(21%)	4
		471	(21%)	4

*Retention times in samples may vary because of changes in column length and temperature profile used.

A Hewlett-Packard 5993 A combined gas chromatograph—mass spectrometer was used. Glass columns, either 0.7 or 1.0 m, packed with 3% OV-17 were used. The carrier gas flow-rate (helium) was 30 ml/min. Temperatures were: column 146—200°C; injection port 222°C [6]. The ions selected for each analyte and their appropriate deuterated analogues are shown in Table I.

Peaks obtained during the GC—MS run have generally been normalized on the Y-axis to give full scale display. This facilitates precise area calculation. The appropriate area is indicated (arbitrary units) adjacent to each peak. Two traces, (top; deuterated internal standard; bottom; endogenous) are required for each analyte. Four ions, base peak and molecular ion for deuterated standard and endogenous were monitored for SAL to obtain maximum specificity. Ions within each group are displayed in decreasing order of m/z (top to bottom).

RESULTS

Fig. 2 compares direct chromatographic data obtained via HPLC—ED and GC—MS-SIM assay for the three sample types banana pulp, beer and soy sauce.

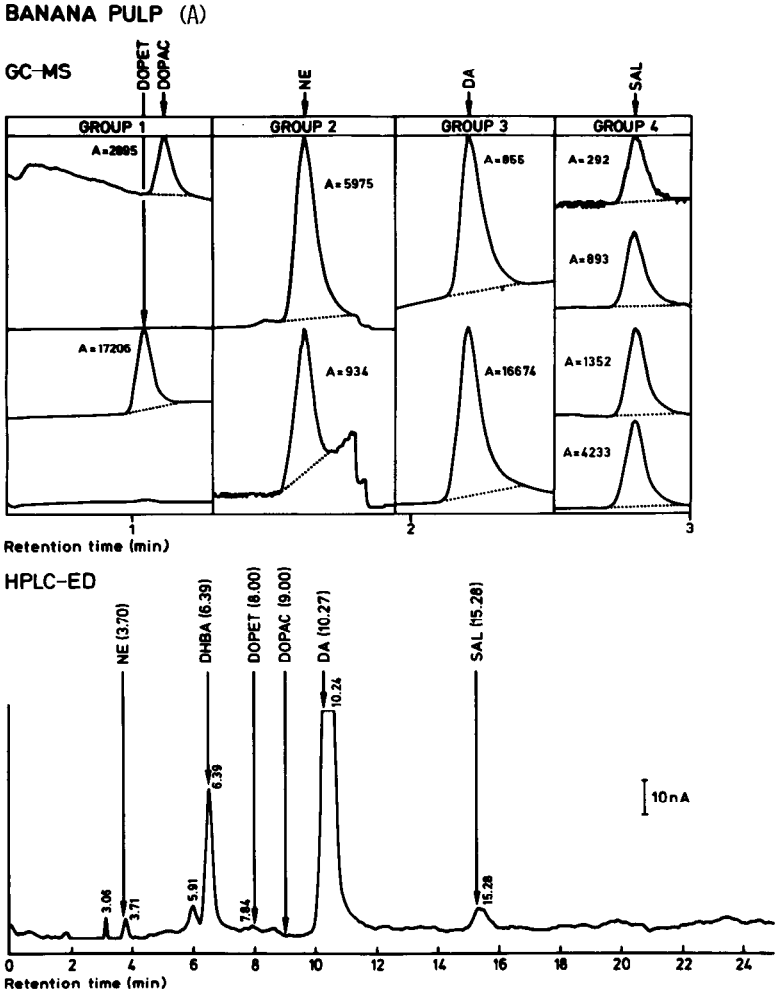

Fig. 2.

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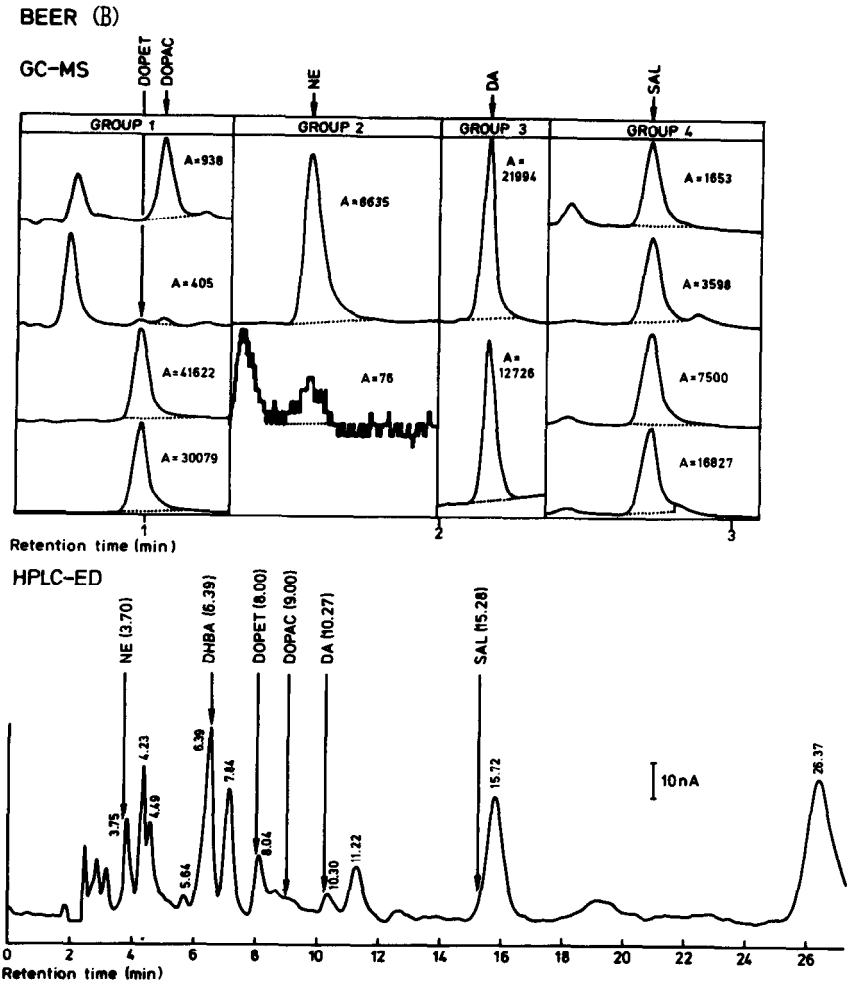
BEER (B)
GC-MS
DOPET
DOPAC
NE
DA
SAL
GROUP 1
GROUP 2
GROUP 3
GROUP 4
A=938
A=405
A=41622
A=30079
A=6635
A=76
A= 21994
A= 12726
A=1653
A=3598
A=7500
A=16827
Retention time (min)
HPLC-ED
NE (3.70)
DHBA (6.39)
DOPET (8.00)
DOPAC (9.00)
DA (10.27)
SAL (15.28)
3.75
4.23
4.49
5.64
6.39
7.84
8.04
10.30
11.22
15.72
26.37
10nA
Retention time (min)

Fig. 2.

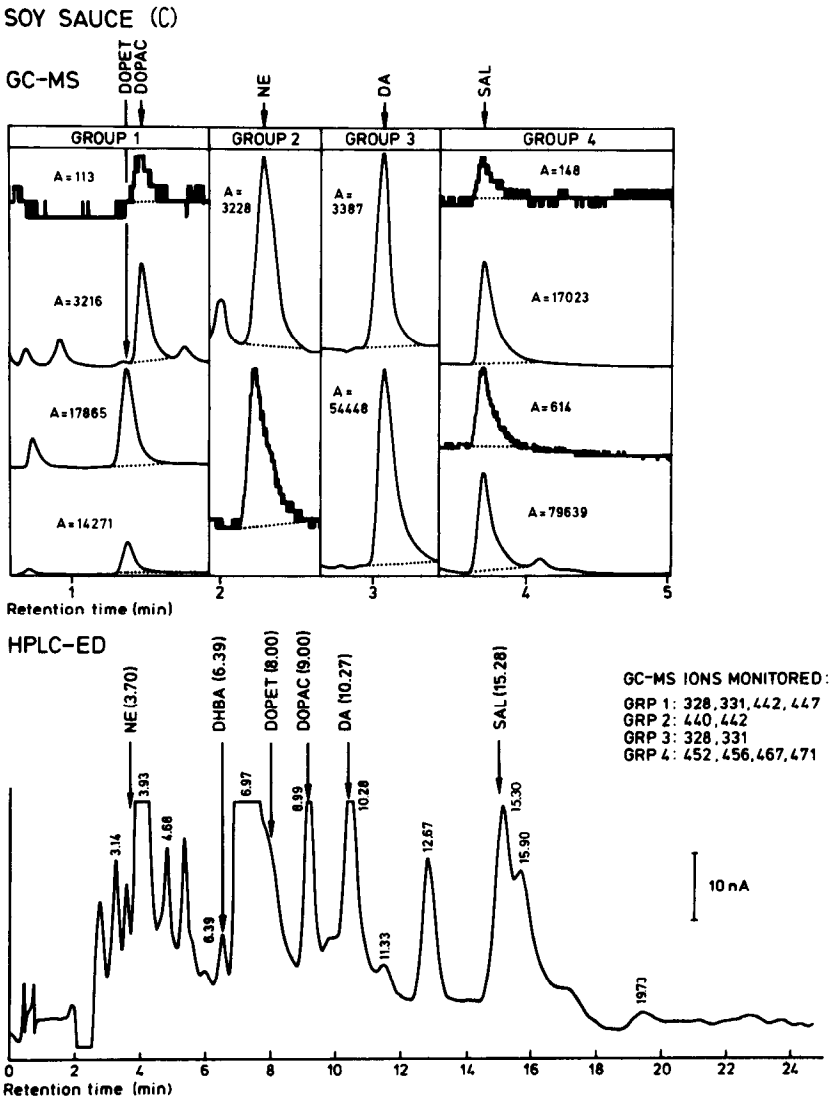

Fig. 2. Direct comparison of chromatographic data for three alumina-extracted samples. A, Banana pulp; B, beer; C, soy sauce.

The HPLC—ED trace is clearly marked in each instance with arrows indicating the retention times corresponding to the analytes of interest (based on standard samples run previously). For each sample run in the GC—MS-SIM mode arrows indicate the deuterated internal standard peaks; individual retention times may sometimes vary with minor changes in chromatographic conditions and column length. Wherever possible peaks corresponding to the retention time of the analyte of interest in the HPLC—ED trace were used to calculate original sample concentrations by reference to extracted standard samples and these results have been compared with previously obtained GC—MS results in Table II.

In only one instance (i.e. soy sauce NE) did an interfering peak in the GC—MS-SIM trace prevent accurate quantitation at the limits of sensitivity for an analyte.

TABLE II

QUANTITATIVE RESULTS VIA GC—MS AND HPLC—ED ASSAY (pmol/g)

Sample	Method	DOPET	DOPAC	NA	DA	SAL
Beer	(GC—MS)	140—210	38—58	ND*—1.5	2.5—31.8	28—75
	(HPLC—ED)	108	QI**	QI	33	QI
Soy sauce	(GC—MS)	138	$1.87 \cdot 10^3$	QI	796	$2.66 \cdot 10^3$
	(HPLC—ED)	QI	$4.45 \cdot 10^3$	QI	$2.1 \cdot 10^3$	$4.89 \cdot 10^3$
Banana pulp	(GC—MS)	$1.17 \cdot 10^3$	–	$1.02 \cdot 10^4$	$2.13 \cdot 10^5$	352***
	(HPLC—ED)	ND	–	$1.81 \cdot 10^4$	$6.63 \cdot 10^5$	$2.2 \cdot 10^5$ §
Dried banana	(GC—MS)	–	–	–	–	$1.6 \cdot 10^5$—$1.7 \cdot 10^6$
	(HPLC—ED)	–	–	–	–	$1.9 \cdot 10^5$—$1.6 \cdot 10^6$

*ND = not detected.
**QI = identification and quantitation impossible due to interfering peaks.
***Climateric pulp.
§Post-climateric pulp.

In this sample it was, however, established that NE levels were < 10 pmol/ml by direct comparison with the internal standard.

DISCUSSION

Low hardware costs, ease of automation, high sensitivity and the success of HPLC—ED in catecholamine assay to date make it an obvious choice for tetrahydroisoquinoline assay. Since the first reported application of HPLC—ED to the determination of salsolinol in biological materials by Riggin and Kissinger in 1977 [11] relatively little further work has appeared. More recent studies have dealt with the chromatographic problems associated with resolving mixtures of synthetic alkaloids or reaction mixtures into their components [4, 12, 13]. For samples of biological origin the problems are considerably more complex. The low concentrations involved, complexity of the sample matrix and, in particular, the possibility of artifactual SAL formation during sample work-up have slowed progress in this area. The alumina-based catechol extraction that forms the foundation of our GC—MS assay has proven to be a reliable, selective and efficient procedure. As well as recovery of the amines NE, DA and SAL the alumina extraction also recovers the catechol acid and neutral metabolites (e.g. DOPET and DOPAC) in a relatively "clean" medium. We found a single alumina extraction sufficed to give samples suitable for derivatization and direct GC—MS analysis in every instance. Using essentially the same extraction procedure, we found samples were not generally suitable for direct HPLC—ED analysis. The comparative chromatograms (Fig. 2) illustrate the complexity of the HPLC trace. Samples such as beer and soy sauce give complex chromatograms with an abundance of unknown peaks often with retention times corresponding to, or overlapping with, the less abundant peaks of interest. Late eluting peaks in the HPLC—ED trace in some samples (up to 2 h after injection) significantly increased the minimum time required between injections. The maximum time required for any GC—MS-SIM run was 5 min.

In the case of urinary catecholamine assay, or previously reported HPLC—ED assays for salsolinol, additional extraction or pre-treatment steps have been used to clean samples. For example cation exchange to isolate amines, or a solvent extraction step can be added to remove interfering components. However, there are disadvantages with this approach. Further sample clean-up will also reduce recoveries (and overall sensitivity) as well as resulting in a loss of some of the metabolites present that might otherwise have been measured concurrently. Complex extraction procedures also reduce the suitability of a single internal standard for multi-component analysis and therefore reduce precision.

Alternatively, attempts can be made to optimize the chromatographic conditions for each sample. However, although a set of parameters (e.g. temperature, pH, type and concentrations of ion-pairing reagent) might yield a satisfactory solution to one analytical problem, these conditions may not be optimal for a different sample type.

There is currently no simple, universally applicable extraction procedure and set of chromatographic conditions that allows complex and varied sample types to be assayed via HPLC—ED. For a fixed sample matrix (e.g. urine) a combination of extraction procedures and chromatographic conditions can be developed to assay any particular analyte. Once developed the procedure is cheap and easily automated. An important role exists for HPLC—ED in detecting and quantitating analytes via this approach in essentially "same" sample types. However, when considering the assay of salsolinol the highest priority must be given to avoiding the possibility of artifactual formation. Time-consuming and elaborate extraction procedures are far more susceptible to introducing errors of this type and must be used with caution.

The specificity associated with SIM is responsible for a significant reduction in the complexity of the data obtained when using a GC—MS system. The comparative chromatograms for each sample type aptly illustrate the advantages of this approach to complex and varied sample analysis. GC—MS-SIM allows an efficient single-step alumina extraction to be used for sample preparation. The incorporation of appropriate deuterated standards facilitates identification, adjusts for variable recovery of each analyte and allows precise quantitation. Where it is possible to use a single-extraction procedure a wider variety of analytes can be simultaneously quantitated in the one sample, recoveries are improved and most importantly, minimized sample manipulation significantly reduces the possibility of artifactual SAL formation. Judicious use of appropriate deuterated standards in GC—MS can provide the researcher with additional information not otherwise available. Suitable "tagged" dopamine can be added to samples during the extraction procedure and its conversion to tagged SAL monitored, thus providing a "built-in" check against artifactual formation for each sample type assayed.

The chromatograms included in Fig. 2 display the problems associated with determining a working limit of sensitivity in this type of study. In practice, it is the stability and level of background interference in the region of the analyte of interest that dictates the lowest level that can be detected. This is a function of the complexity of the original sample matrix, the extraction procedure used and the detection method. Sample type variations can therefore account for considerable changes in the level of sensitivity obtained. The unique specificity

of the SIM mode of detection ensures that practical sensitivity limits for samples closely approach estimates based on standard samples runs. Practical HPLC—ED detection limits are significantly higher and more variable than studies with standard samples indicate. It is only after elaborate pre-treatment steps are taken that a sufficiently stable baseline can be obtained to allow quantitation of trace components.

The same sample was extracted and used for all quantitative comparisons with the exception of banana flesh. The remarkable increase in SAL levels reported in the HPLC—ED result (post-climateric, or over-ripe flesh) compared to the GC—MS results (climateric, ripe flesh) is real and is a consequence of fruit aging. Riggin et al. [14] have observed similar increases in SAL content as the banana ripens. The poor comparison between HPLC—ED and GC—MS-SIM results for the soy sauce sample is associated with noise in the chromatogram in the region of the internal standard DHBA. Since precise quantitation of each analyte requires an accurate assessment of the DHBA peak height, background interferences in the region adversely affects measurement of all components in the sample.

The data presented illustrates that for an uncomplicated sample matrix (e.g. banana) a single-step alumina extraction combined with reversed-phase ion-pairing chromatography can provide quantitative data for a range of catechol derivatives. It was essentially this approach that was used by Riggin and co-workers to identify and quantitate levels of salsolinol in banana [14] and cocoa [15]. More complicated sample types are not amenable to this analysis unless elaborate sample pre-treatment steps are taken. For any constant sample matrix (e.g. urine, plasma or beer) it is possible to devise an extraction protocol and a set of chromatographic conditions suited to the analyte(s) of interest. In many instances considerable manipulation may be required to obtain a workable chromatographic system. However, once developed the procedure is cheap to run and easily automated. An important role exists for HPLC—ED in the analysis of samples of essentially similar or identical matrix type.

The comparative chromatograms presented illustrate the versatility and power of a GC—MS-SIM assay system for trace analysis of this type. The availability of specific ion detectors that can be directly coupled to capillary gas chromatographs is helping to offset the cost advantages that HPLC—ED has previously offered over conventional GC—MS systems. GC—MS-SIM combined with appropriate deuterated internal standards eliminates elaborate sample pre-treatment even with a complex sample matrix as well as allowing precise multi-component quantitation at low levels. The unique problems associated with salsolinol assay require elaborate precautions be taken against artifactual formation, and therefore necessitate fast, uncomplicated extraction procedures. These requirements are also best met by a combined GC—MS technique.

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