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ISOLATION, IDENTIFICATION AND QUANTITATION OF URINARY ORGANIC ACIDS

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SUMMARY

An application of the HISLIB program for the comparison of gas chromatographic—mass spectrometric profiles of urinary organic acids isolated by extraction and ion-exchange methods is described. Ion-exchange methods are clearly superior to solvent extraction in terms of the variety of compounds isolated. However, the former method has practical difficulties which make solvent extraction more attractive for rapid analyses. For the compounds isolated by both methods, the precision of analysis is similar, with standard deviations of relative concentration in the range 10—30% for most compounds.

INTRODUCTION

Experience has shown that only a few inborn errors of metabolism can be diagnosed by clinical criteria alone, and that detection generally depends upon screening patients with suspicious symptoms for a wide range of metabolites. The application of gas chromatography (GC) and combined gas chromatography—mass spectrometry (GC—MS) for the profiling of classes of metabolites has led to the identification of several new disorders [1]. The large numbers of components often seen in such mixtures have led to the application of new techniques such as capillary gas chromatography [2, 3] and advanced computer methods [4—7].

The literature on the use of GC—MS procedures in the analysis of organic acids has been replete with controversy over the choice of isolation method. Over the last several years we have routinely isolated organic acids from urine by acidification followed by solvent extraction. A recent publication [8] directed our attention to the purported superiority of anion-exchange isolation methods. Preliminary experiments with this method were not encouraging [5],

and we found the method considerably more time-consuming. Furthermore, there has been some controversy about the best methods for ion exchange isolation of organic acids [9, 10]. It was the purpose of this study to utilize the data processing techniques described recently [5] to compare critically the two isolation methods. During the course of this investigation we examined a third method for isolation of organic acids and have reported that method in a preliminary communication [11].

EXPERIMENTAL

We have previously described the chromatographs, mass spectrometer and data analysis equipment which are routinely used for our analyses [5]. Standard samples of organic acids were obtained from a variety of commercial sources including Sigma (St. Louis, Mo., U.S.A.), Aldrich (Milwaukee, Wisc., U.S.A.) and Eastman Organic (Rochester, N.Y., U.S.A.). Further samples were obtained from research groups in the Stanford University Department of Chemistry. All solvents were of the highest purity available commercially.

Urine extraction methods

Urine from a 30-year-old male was used to test the different isolation methods. A first morning sample was collected and kept frozen until use. The sample had a creatinine level of 224 mg/dl.

A. Manual extraction. To 3 ml of freshly thawed urine is added the internal standard, 3-chlorophenylacetic acid (0.212 mg), followed by hydroxylamine hydrochloride (30 mg). The pH is adjusted to 12 with 2 *N* NaOH. The sample is heated at 60° for 30 min to form the oximes of keto acids. After cooling, the pH is adjusted to 1 with concentrated HCl, and the acids are extracted with three 6-ml portions of diethyl ether—ethyl acetate (1:1). The combined organic extracts are dried (Na_2SO_4) and evaporated to dryness. The extract is then transferred to a vial with ethyl acetate—methanol (1:1). The solvent is removed with a stream of dry nitrogen, and the sample is sealed with a PTFE-lined cap.

B. Ion exchange. The ion-exchange method is based on the method of Thompson and Markey [8] with some minor variations [12]. Thus, urine (3 ml) and 3-chlorophenylacetic acid (0.212 mg) are treated with barium hydroxide solution (0.1 *M*, 3.0 ml), stirred and centrifuged for 15 sec. The supernatant is removed, treated with hydroxylamine hydrochloride (30 mg) and heated at 60° for 30 min. The cooled solution is adjusted to pH 7—8 with dilute HCl and applied to a short column (5.0 cm × 1.0 cm) of Sephadex A-25. The anion exchange resin was prepared as previously described [8]. Neutral and basic components are eluted with 50 ml distilled water and the acids are eluted with 40 ml of pyridinium acetate buffer (1.5 *M*). The extract is dried by lyophilization at 10—25 mm Hg (normally for 10—15 h) and transferred to a vial with ethyl acetate—methanol as above. We have occasionally found this extract to be highly hygroscopic, and in such cases a further drying period is necessary.

Preparation of aldonic acids.

Certain of the aldonic acids and lactones, which are important components of the ion exchange extract, were prepared by bromine oxidation of the corre-

sponding aldose [13]. The sugar (1 mg) was dissolved in water and treated with bromine (2 drops) and saturated $NaHCO_3$ solution (2 drops). After 30 min at room temperature the solution was evaporated and derivatized. This procedure yielded a mixture of the free aldonic acid and the corresponding 1,4- and 1,5-lactones.

Derivatization and GC

The extract from A, B, or a standard acid sample is derivatized with bis(trimethylsilyl)trifluoroacetamide (100 μl) at 60° for 30 min. The derivatized sample (about 1 μl) and a mixture of hydrocarbon standards (C12, C18, C24, about 1 μg each) for determination of relative retention indices [5] are coinjected onto the GC column at 70—80°. As previously described we use columns of 10% OV-17 on 100—120 mesh Gas-Chrom Q [5]. Starting temperatures are chosen to allow for quantitation of lactic acid. After a wait of 4 min, temperature programming is begun at 4°/min, and the chromatographic effluent is allowed to enter the mass spectrometer. A total of 600 spectra are recorded to a final temperature of 280°.

METHODS

Data analysis

The procedures for analyzing the raw data from our GC—MS computer system are basically the sequence of programs, CLEANUP, TIMSEK, SEARCH and HISLIB, described previously [5]. The following is a brief description of the function of each program:

(i) CLEANUP. The functions of the CLEANUP program include: (1) detection of spectra of components in the raw GC—MS data; (2) resolution of overlapping components; (3) removal of background from column or septum bleed. The current program differs slightly from the one described previously [14] in the following ways: (1) the chemist can specify criteria for component detection; (2) provision is made for mass spectrometric scans of the mass range in either direction and for non-zero dead time between scans; (3) improved multiplet resolution. The output of CLEANUP is a set of representative mass spectra for detected components.

(ii) TIMSEK. The TIMSEK program calculates relative retention indices (RRI's) and relative concentrations of each component detected by CLEANUP. As previously described [5] this requires a hydrocarbon calibration curve for each GC column and each starting temperature, and use of internal standards for quantitation.

(iii) SEARCH. Our mass spectral library search program attempts identification of mixture components by comparison of the unknown mass spectrum with a library of known mass spectra. The acid library began with the TMS components (about 1300) of the Markey library [15]. From this we selected 400 spectra of the acids and neutrals likely to occur in urine extracts. We have added about 70 new spectra to the library from commercially available standards and from literature spectra. A total of 350 of the library spectra have RRI values associated with them. As many of the library spectra are duplicates, there are at present 220 independent RRI values in the library. The SEARCH

program considers only those library components within a retention index window of 20 RRI units. We have also begun to save the spectra of unknown urinary components in a separate library. These spectra are searched during the analysis of new data giving an indication of how often the unknowns are seen.

(iv) HISLIB. The HISLIB program accumulates GC—MS profiles (each profile resulting from the operation of the preceding three programs), while compiling statistics on the occurrence of components (based on both RRI and spectral matching) and their relative concentrations [5]. This program was utilized to compile the data on reproducibility of various methods of extraction of urinary organic acids.

Compilation and use of RRIs

Our procedures for analysis of GC—MS data and comparison of GC—MS profiles do not require the identification of the structure of each component. However, meaningful chemical and biochemical interpretation of results obviously depends on the structural identity of compounds. The HISLIB procedure does require RRIs [16] in conjunction with mass spectra in order to perform comparison of profiles [5]. In addition, the similarity of mass spectra of certain polytrimethylsilyl compounds [17] makes structural identification difficult on the basis of MS data alone. The utility of RRIs in assignment of structure in such cases has been demonstrated by several workers [4, 5, 18]. Because of these considerations we have compiled an extensive list of RRIs of known compounds in order to facilitate component identification and interpretation of results.

At present our list of the RRI values for organic acid TMS derivatives (available from the authors) contains 215 entries. The majority of these are based on authentic samples of the compounds. However, some of the values were obtained from urine components and identified by comparison with library spectra [15, 19—25]. Other values have been adapted from the literature [26—29]. Petersson [27] reports retention data on three liquid phases for over 150 organic acids as TMS derivatives. This listing is based on the determination of retention times and RRI values under isothermal conditions with only one retention time standard present. We had independently determined the RRI values of over 50 of the compounds listed by Petersson. A comparison of our data can be seen in Fig. 1. Interestingly, the two sets of results coincide at an RRI value slightly above 1800, where Petersson's single retention time standard, glucitol-hexa-TMS, elutes. We calculated the least-squares best fit line to the data and used the equation to correct Petersson's RRI values for our use. The differences between our values and the corrected Petersson values for these 50 compounds represent the likely errors we will encounter in using corrected Petersson values with our identification programs. Among the 50 compounds we shared, the corrected values were within 20 RRI units for all but two (oxalic acid and erythrono-1,4-lactone). The standard deviation of the differences between the corrected Petersson RRIs and ours was 7.4 RRI units, while the average absolute difference was 5.5.

The listing of Butts [26] contains retention indices for over 200 biological compounds as TMS derivatives on both OV-1 and OV-17 stationary phases,

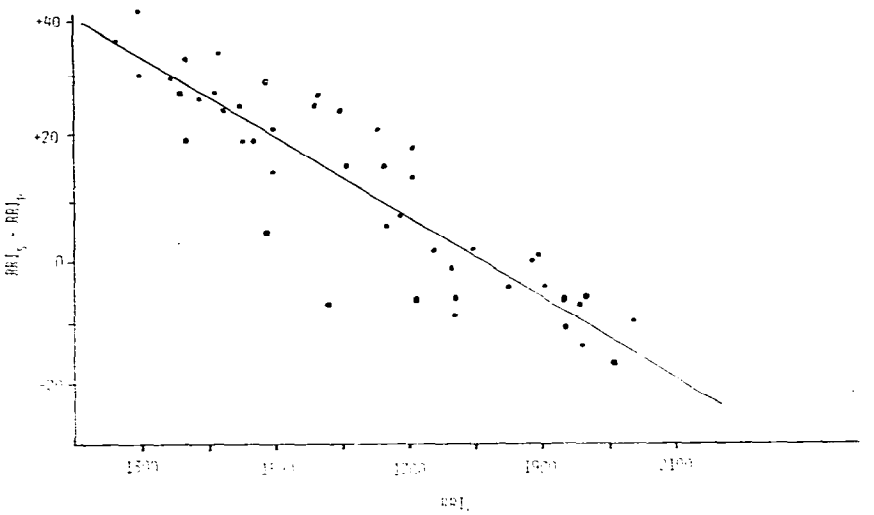

Fig. 1. Comparison between the RRI values obtained by Petersson [27] and our work for selected organic acid TMS derivatives, plotted as the difference between the values against our value. Least squares fit, $RRI_s = 0.938\ RRI_p + 112$.

measured by co-chromatography with a mixture of alkanes and reported as methylene units. Methylene units differ from our RRI values by a factor of 100. A comparison of 50 of the values reported by Butts with those obtained in our laboratory indicate an average difference of 3.0 RRI units with a standard deviation of 7.6. Thus there are no large systematic differences between the two data sets. The average absolute difference between the two sets of RRIs is 6.4 units, which is not much greater than the measured standard deviation of our own values over a long period (see below). Two of the Butts values are significantly different from ours. He reports the retention index of indoleacetic acid as 2093 while we measured 2187 for the di-TMS derivative of indoleacetic acid (Mamer et al. [30] mention the formation of both mono- and di-TMS derivatives of indoleacetic acid). N-Acetylphenylalanine, the mono-TMS derivative of which we recorded at 2051, was reported by Butts at 1933 (this may be a di-TMS derivative).

Over the last 18 months we have used many different GC columns including different batches of OV-17. Changes in operating conditions are taken into account by periodically obtaining a new calibration curve for each column. The standard deviations for the RRI values of 18 common organic acids determined during this period were between 1.8 and 8.4 with an average of 4.1. These standard deviations are based on 25 separate analyses of each component. The two most variable components are lactic (σ, 6.8) and hippuric (σ, 8.4) acids. The former is the first detected component in most runs and is the most sensitive to small changes in GC starting temperature [5]. The latter component often dominates acid profiles and is commonly present in sufficient quantities to overload the GC column or the mass spectrometer, leading to peak broadening and poor RRI reproducibility.

Isolation, identification and profile comparison

We carried out five replicate analyses with the isolation methods discussed in the Experimental section. Aliquots of the same urine were used in all analyses. Each organic acid fraction so obtained was analyzed by GC—MS and the set of computer programs described above. Structural assignments were made on the basis of comparison of RRI values and mass spectra. The set of five GC—MS profiles for a given isolation method was collected in an historical library using HISLIB. The resulting library contains data on the relative concentrations (and precision thereof) for all components detected, whether or not structural identity has been established.

RESULTS AND DISCUSSION

Extraction

The results for the isolation of organic acids by solvent extraction (Table I and Fig. 2, top) indicate satisfactory quantitation of the small aliphatic molecules (e.g. lactic, glycolic) and the aromatic acids (e.g. the hydroxyphenyl-

TABLE I

QUANTITATIVE ANALYSIS OF ORGANIC ACIDS ISOLATED FROM URINE BY TWO METHODS

Quantitative results are expressed in relative concentration units with the internal standard, 3-chlorophenylacetic acid, concentration set at 100. The initial concentration of the standard was 71 μg/ml urine. The relative concentration values have not been corrected for differential extraction or detection coefficients. The coefficient of variation (C.V.) represents five separate analyses.

	Acid*	RRI**	Extraction		Ion exchange	
			Rel. concn.	C.V.	Rel. concn.	C.V.
1	Lactic	1094	78	14	50	9
2		1108	—	—	21	19
3	Glycolic	1132	119	14	100	13
4	Phenol	1133	—	—	34	26
5		1177	—	—	18	28
6	3-Hydroxyisobutyric	1205	51	18	34	11
7	Pyruvic	1222	41	15	26	6
8	Cresol	1239	18	23	91	14
9	3-Hydroxyisovaleric	1243	18	3	—	—
10	?-Hydroxypyridine	1263	—	—	29	12
11		1299	—	—	56	65
12	Phosphate	1353	—	—	346	20
13	Urea	1366	491	35	—	—
14	Glyceric/4-deoxyerythronic	1368	—	—	87	40
15	4-Deoxythreonic/catechol	1383	—	—	170	7
16		1397	43	61	45	27
17	3-Deoxytetronic	1449	36	18	110	21
18	2-Deoxytetronic	1465	84	12	250	11

TABLE I (*continued*)

Acid*		RRI**	Extraction		Ion exchange	
			Rel. concn.	C.V.	Rel. concn.	C.V.
19		1547	33	30	99	11
20	Erythronic	1557	—	—	914	20
21	4-Hydroxycyclohexane-carboxylic	1565	70	11	44	19
22	Threonic	1587	—	—	222	17
23	Adipic	1602	24	18	—	—
24		1648	—	—	18	7
25	2-Deoxyerythropentonic	1663	—	—	165	22
26	5-Hydroxymethylfuroic	1689	305	16	354	6
27	1,4-Arabinolactone	1710	—	—	61	36
28	3-Hydroxyphenylacetic	1733	82	10	116	25
29	1,4-Xylonolactone	1744	—	—	37	20
30	4-Hydroxyphenylacetic	1765	186	6	257	16
31		1839	—	—	1004	34
32	3-Hydroxyphenylpropionic	1846	32	27	—	—
33	Aconitic	1854	65	34	—	—
34	Citric	1886	174	45	—	—
35	Furoylglycine	1891	72	36	48	17
36	3-(3-Hydroxyphenyl) hydracrylic	1935	684	15	1675	12
37	1,4 + 1,5-Gluconolactone	1949	—	—	77	28
38		1966	—	—	84	13
39	Hippuric-di-TMS	1989	650	83	—	—
40		2007	—	—	701	26
41	3,4-Dihydroxyphenyl propionic	2046	—	—	14	15
42	Hippuric-mono-TMS	2111	1788	41	2900	20
43	3-Hydroxyhippuric	2379	841	24	—	—

*An empty space indicates an unknown or mixture of unknowns.

**Relative retention index of the peak.

acetics, 5-hydroxyfuroic acid). Tricarboxylic acids (e.g. aconitic, citric) are isolated but the reproducibility is poor. Hippuric acid tends to give a mixture of the mono- and di-TMS derivatives after solvent extraction, apparently due to variable dryness of the samples prior to derivatization. During the course of this work we developed an improved solvent extraction technique based on the adsorption of urine onto a solid-phase cellulose resin and elution of the hydrophobic acids and neutrals with organic solvents [11]. This technique is qualitatively comparable to the manual extraction method in terms of the type of compound isolated but yields better results in terms of recovery and precision.

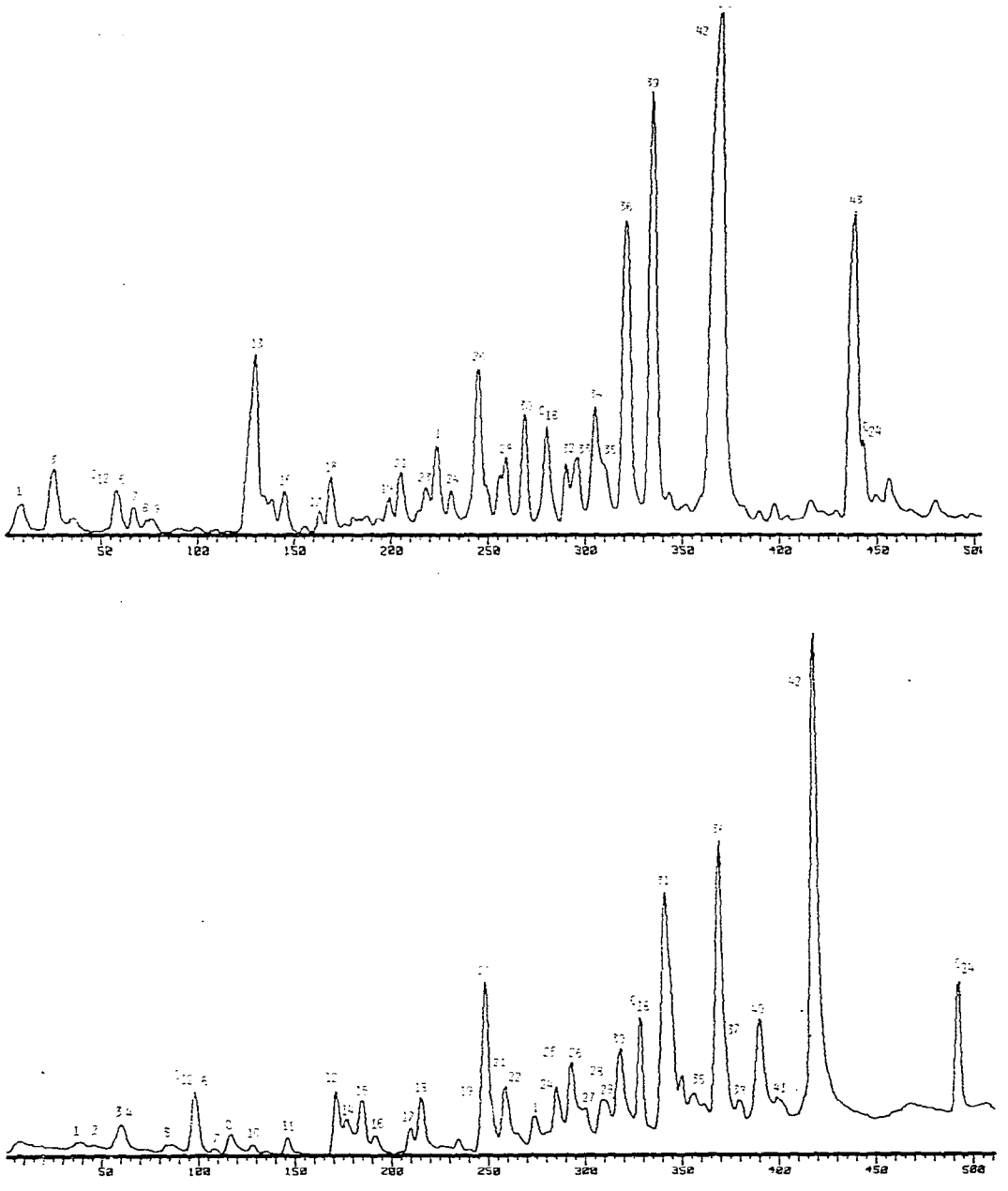

Fig. 2. Top: total ion current trace of the TMS derivatives isolated by manual extraction. Numbers refer to components in Table I. I is the internal standard, 3-chlorophenylacetic acid. Bottom: total ion current trace of the TMS derivatives isolated by ion exchange.

Ion exchange

In our preliminary work with the anion exchange method [5] we had difficulty in obtaining reproducible results for the isolation of organic acids using the procedure of Thompson and Markey [8]. Based on a suggestion of Gates et al. [12], the volume of eluting solvent used in the procedure was increased from 18 ml to 40 ml. With this change the ion exchange method yields reproducible isolation of the majority of the organic acids (Table I and Fig. 2, bottom). This is the only method that isolates carbohydrate derived acids such as glyceric acid. Ion exchange also achieves satisfactory reproducibility for the aliphatic and aromatic acids. Poor results are obtained for the tricarboxylic acids such as citric as these are co-precipitated by the initial barium treatment. A further complication of the ion exchange method is the tendency for aldonic acids to lactonize partially during isolation. For example, the ion-exchange-isolated acids from a child with galactosemia contained a major component identified as galatono-1,4-lactone, but significant quantities of galactono-1,5-lactone and galactonic acid were also observed [31]. Other low level components are not detected in the ion exchange fraction, being obscured beneath the dominant polar acids. We have experienced occassional difficulty in obtaining the ion-exchange-isolated acid fraction in a sufficiently dry state for derivatization. Furthermore, the overnight lyophilization period required to remove 40 ml of water makes this method by far the most time consuming of the methods which we have investigated.

CONCLUSIONS

We have found the use of relative retention indices to be of immense value in the computer assisted identification of organic acids. When measured carefully with co-injected hydrocarbon standards, the RRI values on a particular GC stationary phase are reproducible both with changing conditions in one laboratory and among different laboratories. Furthermore, values determined under less stringent conditions [27] can sometimes be utilized. With the increasing interest in metabolic profiling of body fluids, there is a continuing need for lists of normal components and data repositories [32]. The regular reporting of RRI values of biochemical compounds on OV-17 and other stationary phases along with their mass spectra will be of service to researchers whether established in this field or just entering it.

One of the more critical needs in the evaluation of the diagnostic uses of metabolic profiling is the definition of normal metabolites and their excretion levels [33]. Before the question of normal values can be meaningfully dealt with, methods of isolation and identification must be evaluated as to reproducibility and suite of compounds quantitated. At present none of the available methods is capable of quantitating all organic acids. The ion-exchange method, though clearly more comprehensive than solvent extraction, is limited by the time involved in its application. At present the isolation method of choice will depend on the class of metabolites of interest. For studies with aromatic acids (e.g. catecholamine metabolism), fatty acids or tricarboxylic acid cycle intermediates we recommend solvent extraction techniques [11]. The ion exchange method will continue to be necessary for polar acids. Possible

improvements in the ion exchange method which we hope to pursue are a resolution of the phosphate—citrate problem and a way to avoid the occurrence of both free acid and lactone forms of the aldonic acids. The literature contains some possible solutions to the latter problem, but no application to a complex biological extract [34, 35].

A recent publication from the Michigan State group [12] has addressed several questions on the utility of the data processing techniques which are described in this and previous reports from our laboratory [5, 14]. We have expressed our disagreement with several points raised in the paper of Gates et al. in a separate letter [36], and at this time wish to emphasize an important aspect of our techniques. Our forward library search methods do not require the accumulation of a large data base prior to the analysis of a new compound class as does the MSSMET program [12]. We have successfully applied the methods described here to amino acids, sugars and methyl esters from body fluids [31, 37] and to the analysis of polynuclear hydrocarbons from carbon blacks [38]. Thus we can detect and quantitate (relative concentrations, with absolute values requiring response factors) unknown or previously undetected compounds in these mixtures *in addition* to those observed previously.

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