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Identification and determination of phenolic constituents in natural beverages and plant extracts by means of a coulometric electrode array system

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ABSTRACT

A general method for the evaluation of phenolic compounds in fermented beverages, fruit juices and plant extracts was developed using gradient HPLC and coulometric detection. In a single injection (10 μl) it was possible to identify and determine 36 different molecules (flavonoids and simple and complex phenols), without sample extraction, purification or concentration, in several kinds of beers, red and white wines, lemon juice and soya, forsythia and tobacco extracts. The analytical performance of the method is reported. In addition to components already identified and described in the literature, a large number of other phenolic constituents were resolved. These other components may also be useful for the characterization of these beverages and extracts,

INTRODUCTION

Phenolic compounds are present in both plants and fruits and in their derived products such as wines, beers, juices and plant extracts. These compounds are very important in wines and beers as their bitterness and astringency contribute to taste. They also play a fundamental role in the ageing of wine as they act as natural preservatives. Some phenolic components are transferred from the barrel to the wine. This improves the taste or, as with distilled beverages such as brandy and whisky, generates the fundamental characteristics of the product.

Many researchers have made detailed studies of the characterization and determination of phenolic compounds. However, the great complexity of natural beverages has been a major obstacle to the identification of their constituents. In the last few years, in most of the work published on this topic high-performance liquid chromatography (HPLC) was used to separate the compounds, with different detection systems for determination of the separated compounds, e.g., UV [1–5], electrochemical [6–8], photodiode array [9,10] and atomic absorption spectrometry [8]. Even though there have been numerous publications on this subject, only a relatively small number of phenolic compounds have been investigated at one time in each individual study.

This paper reports the separation and determination of 36 different compounds in a single run without sample extraction, purification or concentration. This was achieved using gradient reversed-phase HPLC and an array of sixteen coulometric electrodes for electrochemical detection.

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EXPERIMENTAL

Chemicals

The mobile phase used in the gradient runs was purchased from ESA (Bedford, MA, USA). Mobile phase A was 34.7 μM sodium dodecyl sulphate (SDS)-0.1 *M* monobasic sodium phosphate-50 n*M* nitrilotriacetic acid-50% aqueous methanol (pH 3.45) and mobile phase B was 173 μM SDS-O.1 *M* monobasic sodium phosphate-50 n*M* nitrilotriacetic acid-50% aqueous methanol (pH 3.45). Solutions A and B were filtered through 0.2-μm PTFE lyophilic filters (Millipore, Bedford, MA, USA) and degassed by sonication under vacuum for 10 min prior to use.

The water used for the dilution of the strandards and of the samples was purified with a Milli-Q R/O water purification system (Milipore).

Apparatus

A Coulochem electrode array system (CEAS) from ESA was used. The instrument consisted of a refrigerated autosampler capable of variable-volume injections with a 100-μl loop. A circulating bath was used to maintain sample vials between 0 and 4°C prior to analysis. Gradient operation was provided by two HPLC pumps capable of operating from 0.05 to 10 ml/min. The output of the pumps was connected to a dynamic gradient mixer. Solutes were separated on an HR 80 column (ESA) containing PTFE-lined ODS of 3-μm particle size (80 mm × 4.6 mm I.D. The detection system consisted of four cell packs in series, each pack containing four porous graphite working electrodes with associated palladium reference electrodes and platinum counter electrodes. The detector, column and a pulse damper were housed in a temperature-controlled compartment. Two additional pulse dampers were placed before the column and cell compartment. The autosampler, pumps, detectors, temperature-controlled box and all associated electronic circuitry were monitored and controlled by the CEAS software installed on a Model 386 computer equipped with a 80 Mbyte hard disk and a 1.2 Mbyte floppy disk drive. The computer was coupled to a high-resolution colour monitor with a "touch screen" interface and to a matrix graphic printer. The computer system also performed data storage. analysis **and report generation. An appropriate** software package was used for summary reports of the final data (Lotus 123).

Chromutogruphic method

A method capable of completely separating the 36 compounds chosen was developed. It consisted of a gradient where the organic modifier, pH and counter ion were altered during the run. The time line showing the gradient used in separation is presented in Fig. 1. The total flow-rate was 1 .OO ml/min and the temperature compartment was maintained at 37°C. The sixteen detector potentials constituted a symmetric array: − 250 mV at electrode 1 (to perform negative screening), 60 mV at electrode 2 with increments of 60 mV at each subsequent electrode until a value of 840 mV is reached at electrode 15, and -250 mV at electrode 16 (to verify possible generation of reversible components). The indicated potentials are referred to the solid-state palladium reference electrode built in the coulometric cells; their absolute value is about 250 mV lower than the corresponding potential measured by using an Ag/AgCl reference electrode.

Standard and sample preparation

All standards (Table I) were purchased from Sigma (St. Louis, MO, USA). The primary stock standard solutions were made by dissolving 10 mg of the component in 50% aqueous methanol. Individual secondary stock standard solutions were made by diluting each component of the primary solutions with deionized water in order to give a concentration of 1 μg/ml. These concentrates were then subdivided into l-ml portions. They were stored at -30°C and thawed when necessary at 4°C. A 36-

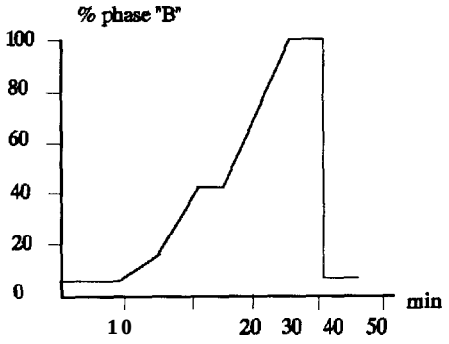

Fig. I. Time line showing the gradient profile used in the method.

TABLE I

CHROMATOGRAPHIC AND ELECTROCHEMICAL CHARACTERISTICS OF THE EXTERNAL STANDARDS

Identification No.	Name	Retention time (min)	Concentration (ng per 10 μl)	Dominant potential (mV)	Within-run R.S.D. (%)	Between-run R.S.D. (%)
1	Gallic acid	3.25	3.15	60	1.20	2.20
2	Tyrosine	4.88	3.75	540	2.50	1.80
3	Gentisic acid	5.13	3.75	60	1.60	1.90
4	Protochatechuic acid	6.12	3.75	120	3.70	4.20
5	3,4-Dihydroxyphenylacetic acid (DOPAC)	1.94	3.75	60	0.80	3.50
6	Vanillyl alcohol	10.54	3.75	300	0.70	2.50
1	4-Hydroxybenzoic acid (4-HBAC)	10.77	1.87	720	1.60	5.20
8	4-Hydroxyphenylacetic acid (4-HPAC)	13.32	3.75	540	1.80	3.40
9	Salsolinol	14.52	3.75	120	3.20	3.40
10	Tyrosol	15.27	3.15	600	2.80	2.60
11	Esculin	15.97	3.75	540	1.50	2.70
12	Vanillic acid	15.93	3.75	480	2.60	5.20
13	Salicylic acid	15.97	3.75	720	0.80	3.50
14	Caffeic acid	16.51	3.75	120	1.10	3.80
15	Catechin	16.58	3.75	480	2.50	2.20
16	Esculetin	17.14	3.75	180	2.40	4.40
17	Tannic acid (1)	17.47	56.20	120	3.20	4.80
18	Chlorogenic acid	17.98	3.75	120	2.50	3.80
19	Vanillin	18.17	3.75	480	1.10	4.30
20	Syringic acid	18.77	3.15	360	4.30	5.10
21	Coniferyl alcohol	20.78	3.75	300	3.80	3.70
22	Syringaldehyde	21.14	3.75	420	2.80	4.30
23	p-Coumaric acid	21.66	3.75	540	4.20	5.80
24	o-Vanillin	22.44	3.75	420	1.30	3.20
25	Tanic acid (2)	22.68	56.20	120	1.50	4.20
26	Ferulic acid	24.69	3.75	360	2.90	4.80
27	Tannic acid (3)	25.11	56.20	120	2.10	3.40
28	Narigin	28.71	7.50	660	3.80	5.50
29	Hesperidin	29.39	15.00	420	2.80	3.80
30	Rutin (1)	29.44	7.50	180	1.80	2.50
31	Rutin (2)	29.68	7.50	780	3.20	5.90
32	Myricetin	30.78	15.00	60	0.70	2.50
33	Quercitrin (1)	30.84	15.00	180	1.20	2.60
34	Quercitrin (2)	31.06	15.00	780	3.20	4.40
35	Naringenin	32.47	7.50	660	2.70	3.20
36	Quercetin	33.15	15.00	120	1.60	3.40
37	Hesperetin (1)	33.49	7.50	360	3.80	2.70
38	Hesperetin (2)	33.57	7.50	780	2.80	5.20
39	Kaempferol	35.71	11.20	180	1.90	3.40

component working standard solution was prepared by combining and diluting the aliquots of each of the secondary stock standard solutions to the concentratiosn given in Table I. The concentrations of the various components making up the working standard solutions were chosen in order to have a good compromise with those found in the samples. Prior to injection all standard solutions were filtered through a 0.22-μm membrane (Millipore). This method was used to measure the phenols present in four wines (Orvieto, Gewurtztraminer, Barbera, Barolo), three beers (Becks,

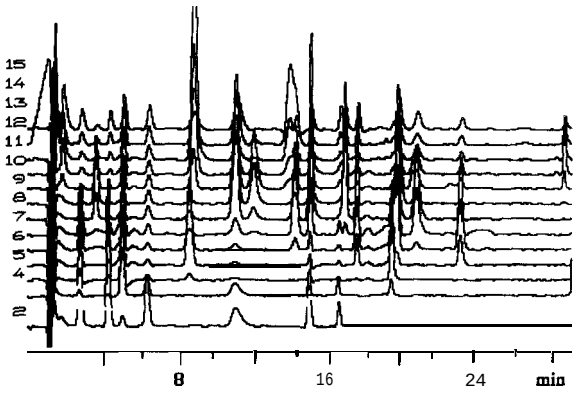

Fig. 2. Chromatogram of a 10-μl sample containing the 36 standard components as external standard at the concentrations indicated in the Table I. Sensitivity full-scale, 0.5 μA.

Groschs, Splugen), several lemon juices and three plant extracts. Soya , forsythia and tobacco extracts were obtained by digesting their respective beans (soya) or leaves (forsythia and tobacco) in methanol for 1 week. Prior to injection all samples were filtered through a 0.22-μm membrane (Millipore) and immediately diluted 1: 100 with filtered, deionized water.

Precision

To investigate the within-run precision, twenty sets of pure standards (at the concentrations given in Table II) were injected and analysed under the conditions described previously. Analysis of the same sample over a 10-day span (the sample was stored in aliquots at -20°C between assays) was used to measure the between-run precision.

RESULTS

The 36 standards are listed in Table I according to their retention times. Their injection concentrations, the within- and between-run precisions and their dominant potentials are also reported (the dominant potential is that electrode potential where the maximum signal occurs).

Confirmation of peak identification was carried out by spiking samples with the relevant standard. The peak identity confirmation was finally achieved by comparing the matching ratio (R) between a standard and the actual sample (R is the dominant channel/subdominant channel ratio [11]).

Fig. 2 shows the chromatogram of a 10-μl sample containing the 36 components as external standard at the concentrations reported in Table I. The total analysis time was 36 min. Retention time reproducibility reported during the precision studies (carried out over a IO-day span) of each individual standard was found to be excellent (R.S.D. $<1.6\%$). This was due to the strict control of both the gradient profile and the column temperature. As a result, a double injection (with and without standard added) as suggested by Cartoni *et al.* [4] was avoided.

The reproducibility of the method was tested by repeated injections (twenty times) of a standard solution containing the concentration reported in Table I. The within-run concentration variability (R.S.D.) ranged from 0.70 to 4.30%. The between-run reproducibility was investigated by analysing the same standard solution over a 10-day span. The R.S.D. ranged from 1.80 to 5.90%. All but three standards were easily characterized as they had only one peak. The tannic acid standard, one of the three exceptions, produced three distinct peaks. All three peaks showed the same oxidation potential maximum but different absolute amounts. The components were identified as tannic acid 1, 2 and 3 according to their increasing retention times. The tannic acid 1–tanic acid 2–tannic acid 3 concentration ratio found was 1:17.5:36.5. No attempt was made to identify which structure corresponded to which peak. Similarly, hesperetin generated two peaks at two different retention times. In this instance the hesperetin 1–hesperetin 2 concentration ratio was 1: 1.7. Quercitrin, the glucoside of the flavonol quercetin, contained two components in the standard. They had similar retention times but very distinct oxidation potentials with quercitrin 1 at 180 mV and quercitrin 2 at 780 mV. Although this can be interpreted as a single structure with two different oxidation maxima, the observation of a single peak with only one oxidation maximum in the samples suggests that the standard contained two distinct forms. The quercitrin 1–quercitrin 2 concentration ratio was 1: 1.22.

The standard mixture was used to examine phenol levels in different kinds of wines, beers, lemon juices and plant extracts. Four distinct Italian wines were analysed: Orvieto, a white, dry, thin-bodied wine from Umbria; Gewürtztraminer, a white, aromatic, rich-bodied wine from Alto Adige; Barbera,

TABLE II

CONCENTRATIONS OF THE COMPOUNDS IDENTIFIED IN THE DIFFERENT BEVERAGES AND EXTRACTS

Compound	Concentration (mg/l of sample)						Concentration (mg per 100 g of dry material): plant extracts		
	Wines				Beer: Becks (Germany)	Fruit juice: lemon			
	Orvieto	Gewürz-traminer	Barbera	Barolo			Forsythia	Soya	Tobacco
4-HBAC			1.8				1.7		
4-HPAC	1.7			3.4	1.2				4.5
Caffeic acid	17.6	6.5	2.1	45.7			4.4		
Catechin	16.4	1.2		47.1	5.4		5.9		
Chlorogenic acid	1.7	1.1	1.6	3.6		5.8	1708.9		211.3
Coniferyl alcohol			5.2				3.1		
DOPAC									
Esculetin		1.4	5.6	1.1			20.3		
Esculin							5.4		
Ferulic acid	1.2	5.1	6.5	1.2	6.5		2.5		
Gallic acid	52.6	188.9	480.9	231.2					
Gentisic acid	5.0	14.6	7.5	2.9			0.6		
Hesperetin (1)	3.2	3.1		2.1			4095.1		
Hesperetin (2)									
Hesperidin		3.4	5.5	15.8		1891.1	130.4	275.3	
Kaempferol					16.4		4.5		
Myricetin								7.0	
Naringenin							48.5		
Naringin		16.4	22.0	53.5			11.8		
o-Vanillin				2.1	1.6		0.7		
p-Coumaric acid			2.0				1.9		7.5
Protochatechuic acid	14.2	14.1	61.1	26.7			4.4		10.7
Quercetin							5.0		
Quercitrin (1)		6.1	5.9	3.2			6.0		
Quercitrin (2)									
Rutin (1)			3.3		1.8	24.5	1591.4		506.0
Rutin (2)			17.3						
Salicylic acid			22.6						
Salsolinol			32.2						
Syringaldehyde	6.1	23.8	86.7	35.3	0.7		*11.7*		
Syringic acid	1.6	1.2	0.7	25.2	0.5		*13.8*		
Tannic acid (3)	28.9		138.5	62.4			*211.6*		
Tannic acid (2)			327.0	145.5			*102.0*		
Tannic acid (1)	4682.2	9332.2	4135.6	34 213.1			*25.3*		
Tyrosine	73.5	237.3	64.5	3.6	54.8	36.2		*15.3*	*14.5*
Tyrosol		19.3	2.0						
Vanillic acid	3.9	4.4	133.1	20.5	3.6				
Vanillin	1.5	2.0	8.4	2.0			2.4		
Vanillyl alcohol							1.3		
Sum of the measured phenolic compounds	5911.3	10 888.1	7179.6	35 947.8	1093	2958	8121	398	137

a red, fresh, acidic, low tannin, medium-bodied wine from Piemonte; and Barolo, a red, tannic, high-bodied wine, aged 6 years in an oak barrel, from Piemonte.

After injecting the wine samples, many hundreds of peaks are obtained in addition to those generated by the compounds contained in the extrernal standard. Such complexity of the natural matrix may

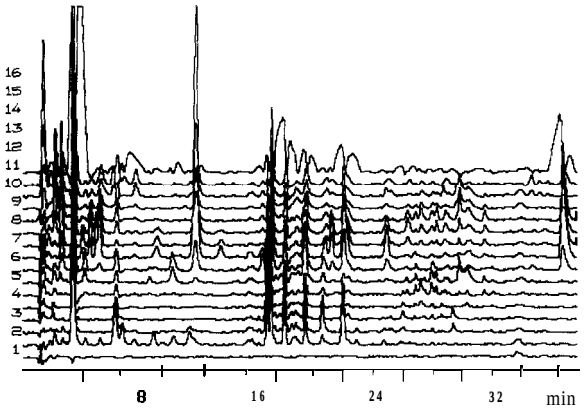

Fig. 3. Chromatogram of a 10-μl Barolo wine sample showing the components found with the results presented in Table II. Dilution 1:100, filtered with a 0.22-μm membrane. Sensitivity full-scale, 1 μA.

cause problems in identifying and measuring the peaks of the external standard, which may be very close to or even overlap some peaks of the natural product. To investigate better the validity of the present separation for natural wines, we chose well differentiated varieties of wines. In no instance did the matrix effect represent a problem. We also focused our attention on three types of plants extracts which contain in high concentrations certain compounds. All three plants extracts and their major compounds are described in the Merck Index [12]. Hesperidin, a major component of lemon juices, and rutin, a major constituent of forsytia and tobacco leaves, are two examples.

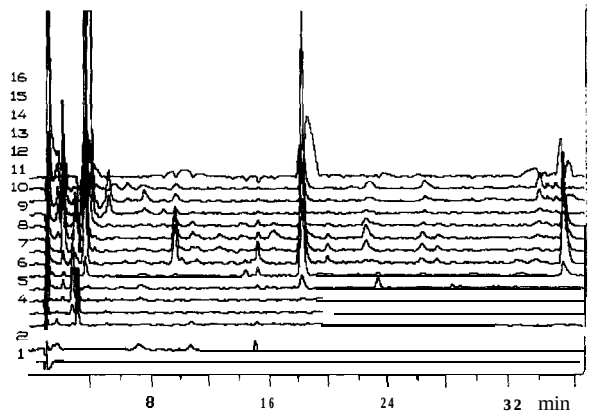

Fig. 4. Chromatogram of a 10-μl beer sample (Becks) showing the components found with the results presented in Table II. Dilution I: 100, filtered with a 0.22-μm membrane. Sensitivity full-scale, 1 μA.

Three different beers were also examined: German (Becks), Dutch (Groschs) and Italian (Splugen Double Malt). As they all presented similar data. only one is reported here. Lemon juices from different sources were also tested. The juice was produced by squeezing the fruit. The suspension asfter filtration was used. They also presented homogeneous data, hence only one is reported here. Lastly, methanol extracts of soya beans. forsythia leaves and tobacco leaves were analysed. The concentrations of the various components found in these samples are reported in Table II in alphabetical order.

The sixteen-channel chromatogram for one of the wine samples (Barolo) is shown in Fig. 3 and that for one of the beer samples (Becks) in Fig. 4.

DISCUSSION AND CONCLUSION

The use of the CEAS for the determination of neurochemicals in tissues and biological fluids has already been reported [13–15]. This type of separation and determination has been studied recently in the determination of 33 neurochemicals in the cerebrospinal fluid [11]. The coulometric efficiency of each element of the array allows a complete voltammetric resolution of analytes as a function of their reaction potential. Some peaks may be resolved by the detector even if they are unresolved when they leave the chromatographic column. In this study we have demonstrated that this technique can also be applied to phenolic compound in various natural beverages and plant extracts.

We separated 36 phenolic compounds in less than 36 min with this method. For the samples analysed here, we were able to measure 16-19 compounds in the white wine, 22226 in the red wines, 10 in the beers, 4 in the lemon juices, 27 in the forsythia leaves, 3 in the soya beans and 6 in the tobacco leaves. The complexity of the matrix, in most instances showing hundreds of peaks, never interfered with the identification and determination of the compounds contained in the external standard.

In addition to those peaks which were measured, other peaks of electroactive molecules were found to be present in all the wine and beer samples. An

electrogenic components profile could therefore give a sort of fingerprint useful for characterizing the product.

In conclusion, we have demonstrated that by combining RP-HPLC with a highly selective array electrochemical detector it is possible to determine simultaneously large numbers of phenolic compounds in very different beverages and extracts. The reproducibility of the retention time coupled with the selectivity inherent to this detector allows measurements with high precision of a variety of different compound families in a single sample. The characterization among the various types of wines and their ageing in wooden barrels are possible subjects of future study.

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