JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

HPLC Analysis of Rosmarinic Acid in Feed Enriched with Aerial Parts of *Prunella vulgaris* and Its Metabolites in Pig Plasma Using Dual-Channel Coulometric Detection

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This paper describes a sensitive isocratic HPLC/ECD method developed for the determination of rosmarinic acid (RA) in plant material, animal feed, and pig plasma. The plasma sample preparation only includes protein precipitation and adjustment of the pH. The applicability of the method was tested on plasma samples of pigs that were exposed to a 91-day oral intake of RA via feed enriched by aerial parts of *Prunella vulgaris*. The plasma was directly analyzed using the method described as well as after enzymatic hydrolysis. When no hydrolysis step was included, RA and caffeic acid (CA) were quantified in the plasma. In hydrolyzed plasma samples, several other metabolites were determined, including dihydrocaffeic, ferulic, and dihydroferulic acid. The dual-channel coulometric detection employed, as an alternative to mass spectrometry, offers good selectivity and sensitivity owing to the electrochemical properties of the phenolic constituents.

KEYWORDS: HPLC/ECD; coulometric detection; rosmarinic acid; pigs; metabolism; feed

INTRODUCTION

Phenolic antioxidants are attracting more and more interest among scientists in various research areas for their protective role in processes connected with oxidative stress and related cell damage (*1*). Phenolic acids, a large group of secondary plant metabolites which belong in a subclass of phenolics, widely spread in the plant kingdom, are regularly ingested through plant-based food in relatively high quantities: it is estimated that the daily human intake of phenolic acids in food ranges from 25 mg to 1 g depending on diet (*2*). Phenolic acids are also assumed to contribute to the color and other sensory qualities of food. Owing to their ubiquity, they could be the natural constituents of regular animal feed as well.

Although some metabolic pathways of common phenolic acids (such as caffeic (CA), chlorogenic, ferulic (FA)) are already known (*3*), many others remain unclear and unexplored. This is mainly because many more in vitro studies have been done than in vivo ones. In recent years in vivo studies have appeared in the literature as well as for rosmarinic acid (RA) (*4*–*7*). However, these experiments for the most part used single administrations of compounds. In reality, the normal intake of phenolics via food consumption is a long-term process.

RA is an ester of caffeic and 3,4-dihydrophenyllactic acid (for structures see **Figure 1**), commonly found in plants of the families Boraginaceae, Nepetoideae, and Lamiaceae, was isolated for the first time from *Rosmarinus officinalis*, and is also present in *Mentha* or *Salvia* plants. It has also been found in relatively high quantities in *Prunella vulgaris* (*8, 9*).

RA is an interesting compound from the medical point of view, as it possesses a number of pharmacologically important properties: astringent, antiviral, antibacterial (*10*), anti-inflammatory, antiallergic (*11*), antimutagen, and cytoprotective (*12*). Hence, it may be considered that rosmarinic acid and related compounds can also act as protective agents against cancer as well (*13*).

HPLC coupled with electrochemical detection (HPLC-ECD) has become widely accepted as a powerful analytical technique offering high sensitivity and selectivity provided that electroactive compounds are analyzed. Phenolic acids are good candidates, and simple amperometric detection is very suitable for the determination of this class of compounds in various plant materials (*14*).

Using the amperometric detection mode, only about 3–10% of the analyte reacts at the electrode surface (a diffusion-driven process), and the rest remains analytically unused, unlike the coulometric mode, where a conversion efficiency of up to 100%

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10.1021/jf070883e CCC: $37.00
Published on Web 08/21/2007

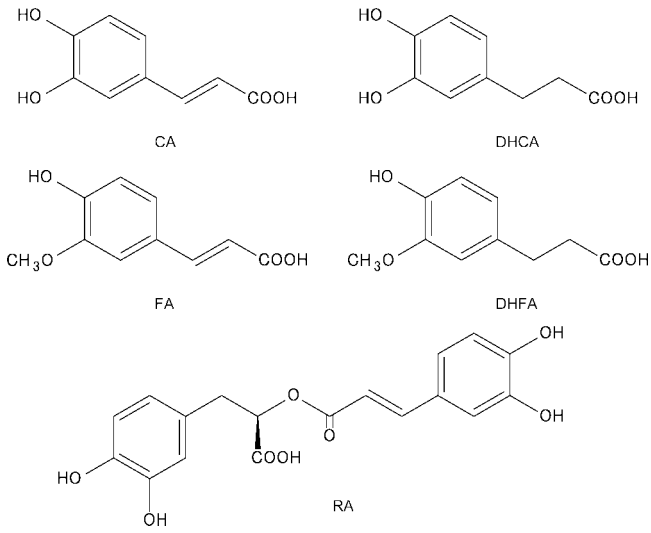

Figure 1. Structures of the compounds of interest. Description: CA = caffeic acid, DHCA = dihydrocaffeic acid, FA = ferulic acid, DHFA = dihydroferulic acid, and RA = rosmarinic acid.

can be achieved. Therefore, the latter often provides much greater sensitivity than amperometry. Further, the signal stability is considerably greater and the detector response much more stable owing to a large electrode surface; only a small fraction is needed for the completion of the redox reaction, in which case partial electrode surface deactivation does not affect the overall response significantly. As a result, under certain conditions, the electroactive component can be completely "removed" (e.g., oxidized) at the first electrode. For this reason, at the second coulometric sensor it does not interfere or it can be restored by the appropriate reversed redox process. This also contributes to the greater selectivity of coulometric detection. These benefits are invaluable, especially when labile compounds or samples with complex biological matrices are handled. Employing common detection methods generally requires time-consuming and complex preconcentration and/or preseparation steps. Since these can significantly influence the validity of the analytical data, especially in the case of labile system analysis, only minimum sample processing is desirable.

Coulometric detection has been successfully used in the analysis of a wide range of phenolic acids from various sources, such as fruits and vegetables (*15*), drinks (*16*), plants (*16–19*), urine, and plasma or serum (*7, 20*).

Despite the fact that RA is a strong antioxidant, we found few articles on its determination in biological matrices using a combination of HPLC and any type of electrochemical detection, amperometric (*21*) or coulometric (*6*). A number of authors used LC-MS (*5, 6, 22, 23*).

The aim of this work was to develop a sufficiently sensitive and selective method for the determination of RA and its metabolites in biological matrix-intact plant material, feed, and plasma within concentration ranges normal for long-term natural food intake and with minimum preseparation steps.

EXPERIMENTAL PROCEDURES

Chromatographic System. The HPLC system consisted of an ESA isocratic pump (model 582) (ESA Inc., Chelmsford, MA) with a pulse damper, manual injector (Rheodyne, Cotati, CA) equipped with a 5 μL loop, and an ESA coulometric detector Coulochem III with a dual-electrode standard analytical cell (model 5010A) combined with a guard cell (model 5020) prior to the injector (all ESA Inc., Chelmsford, MA).

The chromatographic station Clarity (DataApex, Prague, Czech Republic) was used for simultaneous dual-channel chromatogram recording.

The samples were introduced into the system via a glass 25 μL syringe (Hamilton, Reno, NV). All fittings, ferules, and tubings were of PEEK. The HPLC column Purospher Star RP-18 (5 μm), 125 × 4 mm i.d., end-capped, with a guard column (Purospher Star RP-18, 4 × 4 mm i.d.) in a ManuCart cartridge holder (all Merck, Darmstadt, Germany) was thermostated at 25.0 °C during the analysis using a Techlab K5 (Techlab GmbH, Erkerode, Germany) thermostatic HPLC oven.

Mobile phase composition: 20 mM sodium dihydrogen phosphate, pH 3 (set up with phosphoric acid)/(acetonitrile (82/18, v/v)). The mobile phase was filtered through a 0.2 μm porous nylon syringe filter and degassed under vacuum prior to use. The flow rate was 1.6 mL/min.

The first cell working potential was +250 mV, and the second was +0 mV vs. Pd. The polarity of the second signal was reversed to obtain positive peaks in the case of cathodic current. The setup gain ranged from 500 nA/V to 2 μA/V, according to a particular sample type. The guard cell potential was set to +300 mV vs Pd. All analyses were carried out in triplicates.

Chemicals. The standards of rosmarinic, caffeic, ferulic, dihydrocaffeic (DHCA), and dihydroferulic acid (DHFA) were purchased from Aldrich (Milwaukee, WI) (**Figure 1**). Work standard solutions (dissolved in methanol and further diluted by the mobile phase) were prepared fresh daily. For the mobile phase preparation, sodium dihydrogen phospate and phosphoric acid purchased from Fluka (Fluka, Buchs, Switzerland) of trace select purity and gradient grade acetonitrile and methanol (Merck, Darmstadt, Germany) were used.

Citric acid, TCA, and sodium chloride (all p.a. grade) were obtained from a local dealer, and β-glucuronidase/arylsulfatase from *Helix pomatia* (40/20 U/mL) was purchased from Merck (Merck, Darmstadt, Germany).

Extraction Procedures for Plant Material and Feed. Ground aerial part of *P. vulgaris* and feed for the control group, standard feed (Biostan A1, Biostan Ltd., Blučina-Kolperky, Czech Republic), as well as feed for experimental group supplemented with ground aerial part of *P. vulgaris* were extracted with methanol in a Soxhlet extractor for 16 h, and the methanol extracts were subjected to HPLC analysis.

Accuracy, Precision, and Recovery. Statistical evaluation of phenolic acid assay was carried out in spiked blank plasma samples at the concentration levels expected in real samples (10 and 100 μg/L for each compound). Intraday precision was evaluated for each concentration level (three replicates), and interday precision was determined by analyzing spiked plasma samples during a 6-day period (six replicates).

Plasma Samples from in Vivo Experiment. The design of the in vivo study carried out on pigs has been described previously (*24*). This study was conducted in accordance with the *Regulations for the Care and Use of Laboratory Animals* (311/1997, Ministry of Agriculture, Czech Republic) and the *Guiding Principles in the Use of Animals in Toxicology*. Control group (pigs, $n = 3$) and experimental group (pigs, $n = 3$) were fed a diet, supplemented with ground *P. vulgaris* to achieve a RA of concentration 450 mg/kg of feed for a period of 91 days. The supplement and the feed were stored for later analyses. At the end of the experiment, determination of RA content was taken from the *vena cava cranialis*. Plasma from sodium heparin-treated blood was isolated by centrifugation (2500*g*, 10 min) and stored at −80 °C.

Enzymatic Hydrolysis of Phenolic Acid Conjugates. Plasma samples were treated with a mixture of β-glucuronidase/arylsulfatase from *Helix pomatia* in 1 mM NaCl and 0.1 M citrate buffer with pH 3.8 as pH optimum for β-glucuronidase or pH 6.2 for arylsulfatase, respectively. After 10-fold dilution of the plasma, the final activity of β-glucuronidase/arylsulfatase in the reaction mixture was 0.2/0.1 U/mL. The final volume was set to 1 mL by an appropriate buffer. The mixture was incubated 1 and 5 h at 37 °C with continual shaking.

Standards of RA and CA were dissolved in water to achieve concentrations of 1 mg/mL for incubation. Final concentrations of 1 μg/mL were used for the acid stability studies under our experimental conditions.

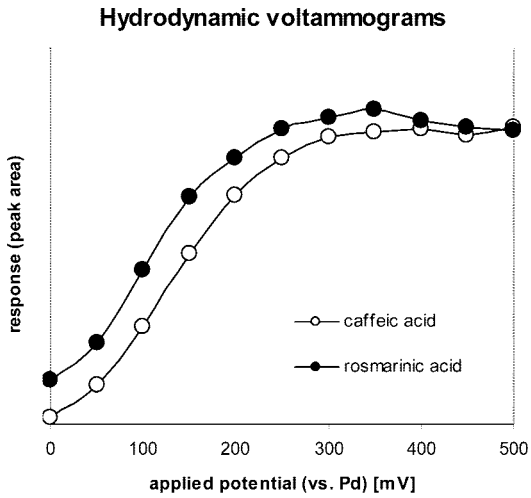

Figure 2. Hydrodynamic voltammograms of caffeic and rosmarinic acid.

After incubation, hydrolysates were stored at −80 °C until HPLC analysis. Further hydrolysate processing was done in the same manner as for plasma samples.

Plasma Treatment for HPLC Analysis. To reduce the risk of labile compound decomposition owing to sample handling, only a minimum of elementary and rapid sample preparation steps were carried out on the pig plasma, as follows:

Plasma samples (100 μL, frozen at −80 °C) were allowed to thaw in a water bath at 37 °C and then vortexed. 100 μL of acetonitrile was then added to the sample in order to precipitate proteins. The sample was then vortexed and centrifuged at 7200*g* for 4 min. The supernatant (50 μL) was withdrawn and mixed with 50 μL of 2% (w/w) phosphoric acid. This sample was directly injected into a HPLC system and analyzed as described. Feed and plant extracts (extracted by MeOH) were treated in the same manner as plasma samples using the above-described procedure and diluted by the mobile phase as required.

RESULTS AND DISCUSSION

Optimizing Detection Conditions Using Dual-Channel Coulometric Detection. Both RA and CA, as main target molecules of interest (*5*, *6*), are known to show strong antioxidant activity (*25*, *26*), and a direct relationship between this and the electrochemical behavior of phenolic acids has been discovered.

Owing to very low oxidation potential, based on corresponding hydrodynamic voltammograms, it was possible to work at low positive potential, and this contributed to the excellent detection selectivity even in biological matrices, as there are fewer compounds oxidizable under these conditions (**Figure 2**)

Unlike CoulArray detectors (which employ coulometric electrode array technology, providing simultaneous multipotential recording), dual-channel coulometric detectors do not allow the use of gradient elution. This can of course become a limiting factor particularly when separating a large number of compounds of dissimilar polarity. It was necessary therefore to find the optimal combination of both chromatographic and electrochemical conditions.

The electrochemical conditions were set up in order to effectively exploit the selectivity of the coulometric detection while maintaining reasonable chromatographic separation and analysis time: At the first electrode, both caffeic and rosmarinic acid are oxidized, while the second analytical electrode potential selectively reduces caffeic acid. In this manner, eventual coeluting species do not interfere. Lowering the second channel potential even more into the negative potential range results in a better response for the oxidation product of caffeic acid, though the selectivity decreases as the number of reduction peaks increases.

Using the guard cell reduces the background noise level because the oxidizable species eventually present in the mobile phase are removed before they enter the analytical cell. It is recommended that the guard cell potential be slightly higher than the highest potential applied at the one of the analytical electrodes. The use of chemicals and water of the highest possible purity is essential, however.

Table 1. Parameters of Calibration Curves in Pig Plasma[a]

compound	retention time [min]	correlation coefficient R^2	LOQ [μg/L]	slope	intercept
DHCA	1.8	0.9986	1	7.7 ± 1.3	1.9 ± 1.3
CA	2.0	0.9983	2	11.3 ± 0.7	2.5 ± 1.7
DHFA	3.5	0.9978	2	18.5 ± 1.8	0.9 ± 1.4
FA	4.3	0.9974	2	5.4 ± 1.1	1.2 ± 0.5
RA	7.1	0.9969	5	2.9 ± 1.2	1.4 ± 1.0

[a] Results are presented as mean ± SD, $n = 3$.

Calibration Curves, Linearity, LOQ, Accuracy, Precision, and Recovery. Calibration curves were measured and constructed with a minimal six concentrations in the range 1–100 μg/L for CA, DHCA, RA, FA, and DHFA acid by adding a known amount of the desired standard into the blank plasma, processed according to the plasma sample preparation described previously. Calibration curves were found to be linear over the measured range for all studied substances. Calculated correlation coefficients (R^2) are summarized in **Table 1**, and model HPLC separation of possible metabolites is shown in **Figure 3**.

The limits of quantification (LOQ) were determined as the lowest concentration of calibration curve that could be determined with acceptable precision and accuracy. This value is typically significantly higher than theoretical LOQ's calculated from the calibration curves because of the low-frequency character of the electrochemical detector noise profile. Peak areas (i.e., Coulombic charge required for electrochemical conversion) were used for all quantitative data as well as for measuring HDV's (**Table 1**). Accuracy, precision, and recovery for studied compounds are given in **Table 2**.

Determination of Rosmarinic Acid in *Prunella vulgaris* and Feed. In order to confirm the amount of administered RA in the long-term in vivo experiment on pigs, the ingoing material (*P. vulgaris* as well as feed) was subject to HPLC analysis. For this purpose, calibration curves were constructed in the range 100–1000 μg/L by the procedure used for the feed sample preparation described above. Linear fits were obtained over all concentration ranges for both CA and RA, with correlation coefficients R^2 0.9997 (CA) and 0.9998 (RA).

A large amount of RA was found in *P. vulgaris*. The total content of RA determined in dried plant was relatively high, over 3% (w/w); CA was present in much less quantity. Complete feed mixtures were analyzed to control the accuracy of the feed preparation (**Table 3**). Small quantities of RA and CA were found in the standard diet of the control group as well owing to the ubiquity of RA and mainly CA in the plant kingdom.

Analysis of Unconjugated RA Metabolites in Plasma. The recovery of compounds of interest from pig plasma was tested by comparing several common methods of plasma sample treatment: (a) liquid–liquid microextraction into diethyl ether; (b) overall protein precipitation using different protein precipitation agents—trifluoroacetic acid (TFA), methanol, and acetonitrile (data not shown). As a result of these experiments, acetonitrile was chosen as the most convenient precipitant for

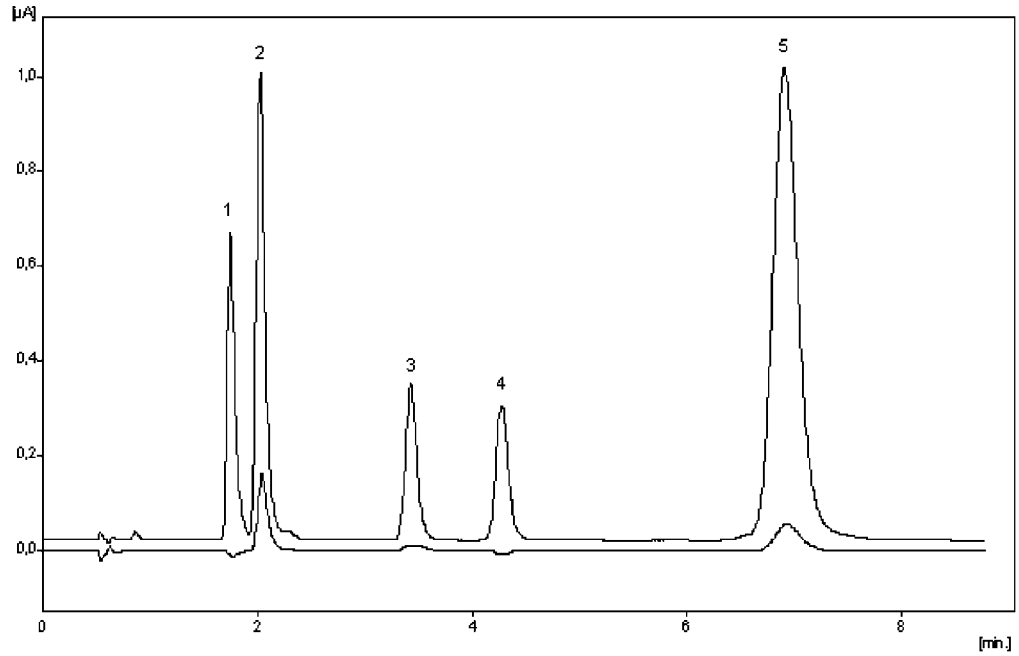

Figure 3. HPLC analysis of standard mixture. Description: 1 = dihydrocaffeic acid, 2 = caffeic acid, 3 = dihydroferulic acid, 4 = ferulic acid, 5 = rosmarinic acid. Working electrode potentials: first channel E1 = +250 mV (vs Pd), second channel E2 = 0 mV (vs Pd, output polarity reversed). For other conditions see Experimental Procedures.

Table 2. Precision and Recovery of Phenolic Acid Assay in Spiked Blank Plasma Samples

compound		spiked analyte c (μg/L)	found analyte c (μg/L)	recovery (%)
DHCA	intraday ($n = 3$)	10	9.73 ± 0.43	97.33
		100	98.49 ± 2.60	98.49
	interday ($n = 6$)	10	10.62 ± 0.40	106.24
		100	103.58 ± 3.20	103.58
CA	intraday ($n = 3$)	10	9.53 ± 0.57	95.30
		100	98.42 ± 3.91	98.42
	interday ($n = 6$)	10	11.35 ± 0.82	113.48
		100	105.47 ± 5.93	105.47
DHFA	intraday ($n = 3$)	10	10.80 ± 0.52	108.04
		100	103.84 ± 3.43	103.84
	interday ($n = 6$)	10	9.98 ± 0.84	99.80
		100	102.41 ± 3.13	102.41
FA	intraday ($n = 3$)	10	9.36 ± 0.80	93.57
		100	98.64 ± 1.53	98.64
	interday ($n = 6$)	10	9.54 ± 0.96	95.42
		100	102.25 ± 3.77	102.25
RA	intraday ($n = 3$)	10	8.95 ± 1.12	89.51
		100	100.58 ± 1.89	100.58
	interday ($n = 6$)	10	9.42 ± 1.26	94.24
		100	99.25 ± 2.17	99.25

Table 3. Analysis of the Source Material and Feeds and Content of Caffeic and Rosmarinic Acid[a]

sample	RA (mg/kg)	CA (mg/kg)
aerial part of *P. vulgaris*	35700 ± 1500	816 ± 52
feed	1.78 ± 0.42	3.34 ± 0.42
feed supplemented with *P. vulgaris* (450 ppm of RA)	283.78 ± 17.65	8.23 ± 0.65

[a] Results are presented as mean ± SD, $n = 3$.

our purposes as it worked effectively (*27*) while maintaining conditions very similar to those in the mobile phase. Similarity of the sample and the mobile phase is of major importance when using electrochemical detection techniques.

According to data published by Nakazawa (*4*) and Baba (*5*, *6*), it can be anticipated that, apart from RA, some other phenolic acid products of RA metabolic transformation (e.g., CA, FA) can also be found in plasma. It is reported that the spectrum of RA metabolites differs not only in diverse clinical sample types (urine, plasma) but also in ratios of free/conjugated acid. The majority of the ingested RA has been described as conjugates in plasma, while urine contained considerably higher quantities of free (intact) metabolites (*5*).

Differences in the metabolism of RA were found between rat and human (*4*, *5*). Hence, further metabolic variability in the case of pigs can be expected. These differences can be explained by the interspecies diversity of biotransforming enzymes battery. During our extended experiment, experimental animals ingested phenolics continuously via feed. This may also affect and/or modify the metabolic profile, as we assume a steady-state in enzymatic kinetics has been reached.

Pig plasma samples, taken from test animals and treated as described in the Experimental Procedures, were analyzed under the described experimental conditions by HPLC with dual-channel coulometric detection. After 91-day oral intake of

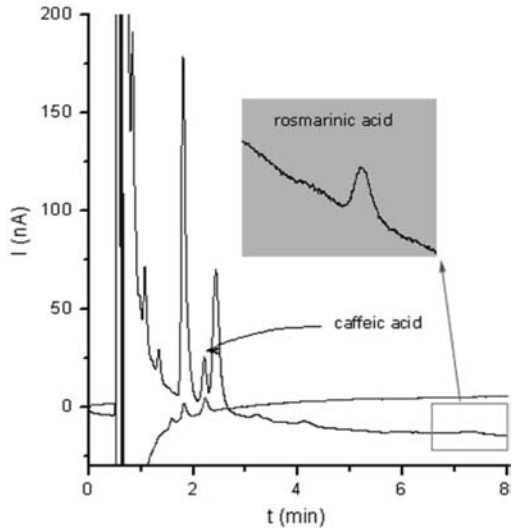

Figure 4. HPLC analysis of pig plasma (representative sample) without enzymatic cleavage, experimental group. For conditions see Experimental Procedures.

enriched feed, RA and its major metabolite, CA, were found in pig plasma (**Figure 4**).

The concentrations reached for CA and RA were 21.43 ± 1.68 and 11.07 ± 2.19 μg/L, respectively. This reflects the steady state of the pig metabolism after long-term administration. Other assumed metabolites were under the LOQ of the method used, or we were not able to identify them.

As predicted, the amounts of RA and CA found in pig plasma differ slightly in each sample, representing the variation of individual metabolism.

Analysis of Conjugated RA Metabolites in Plasma. The original RA and its metabolites however—on the basis of published data—are assumed to be predominantly present as conjugates with glucuronic/sulfuric acid (*5*). Therefore, HPLC analysis of both acids as well as their conjugates is necessary.

In order to release phenolic acids from conjugated forms, enzymatic hydrolysis by the mixture of β-glucuronidase/arylsulfatase from *Helix pomatia* was carried out. On the basis of pH optimum for the activity of the enzymes used, in a citrate buffer pH 3.8, β-glucuronides should preferentially be cleaved while in a citrate buffer pH 6.2, it is assumed that the hydrolysis of sulfates dominates.

The stability of RA and CA in buffers, in the system with buffers and β-glucuronidase/arylsulfatase solution, and finally in a complete system with buffer, β-glucuronidase/arylsulfatase solution and blank plasma was examined. As a control analysis, the determination of free/conjugated RA and CA in blank plasma samples was performed. For these purposes, we used the blood plasma of animals not fed by the *P. vulgaris*-enriched feed. In these blank plasma samples, no detectable amounts of either CA or RA were found.

Two different incubation times (1 and 5 h) were used for the sample hydrolysis. As expected, CA remained stable in all studied systems, while variable degradation of RA was observed: In the systems containing RA in buffer (at both pH values), no degradation was found. On the other hand, in the presence of the enzyme mixture, degradation of RA occurred. Also, at a pH 6.2, there was a noticeable difference in the degradation rate between systems with/without blank plasma: in the presence of pig plasma, the degradation of RA proceeded more slowly than in the system with the enzyme mixture/buffer only. At pH a 3.8, there was no significant hydrolysis progress evident,

Table 4. RA Stability toward Enzymatic Hydrolysis[a]

	recovery of RA (%)			
	without plasma		with plasma	
incubation time (h)	pH 3.8	pH 6.2	pH 3.8	pH 6.2
1	92.0 ± 2.1	63.3 ± 1.8	95.0 ± 2.4	80.3 ± 1.9
5	65.0 ± 1.6	17.8 ± 0.5	81.3 ± 2.3	36.5 ± 1.0

[a] Content of remaining RA in buffer/enzyme systems with and without plasma of control group after 1 or 5 h of incubation, respectively. Results are presented as mean ± SD, $n = 3$.

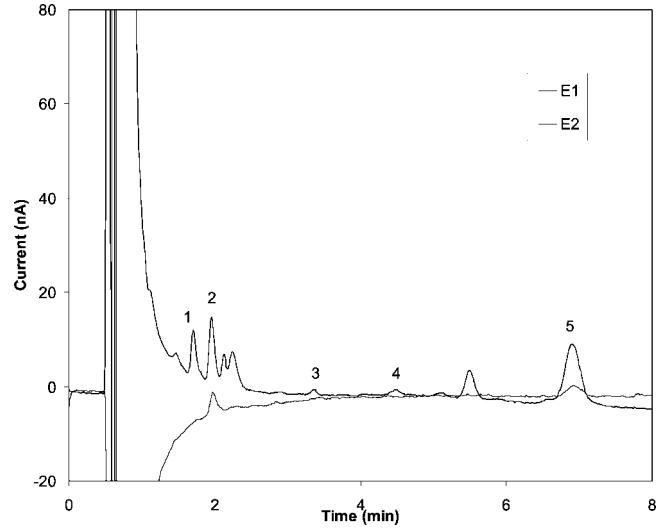

Figure 5. HPLC analysis of pig plasma (representative sample) after enzymatic hydrolysis (pH 6.2). Description: 1 = dihydrocaffeic acid, 2 = caffeic acid, 3 = dihydroferulic acid, 4 = ferulic acid, 5 = rosmarinic acid. Working electrode potentials: first channel E1 = +250 mV (vs Pd), second channel E2 = 0 mV (vs Pd, output polarity reversed). For other conditions see Experimental Procedures.

regardless of plasma content. Nonetheless, it was obvious that longer reaction times resulted in extensive degradation of RA, namely in the system at pH 6.2 (**Table 4**).

To minimize the observed influence of enzymatic hydrolysis on acids, the plasma samples were then treated 1 h with the enzyme mixture.

Pig plasma samples were incubated and treated with enzymes as described (see Experimental Procedures) and then routinely processed prior to HPLC analysis in the same manner as described above. Using this procedure, cleavage of the bound acids is assumed. Apart from RA and CA, other significant peaks appeared in the record (**Figures 5** and **6**). Some of these peaks (1, 3, and 4) were identified by spiking with standards and, additionally, comparing their electrochemical behavior with standards of possible metabolites. As reported by Baba et al., among other RA metabolites also FA was identified in hydrolyzed rat plasma samples (*6*). Furthermore, we identified a derivative of FA, dihydroferulic acid (DHFA), which also seems to play a role in the RA metabolic pathway of pigs. The concentrations found in the samples are summarized in **Table 5**.

Enzymatic cleavage at pH 6.2, which is an optimum for arylsulfatase, produced considerably higher amounts of RA than the cleavage at pH 3.8. After enzymatic cleavage of the conjugates higher amounts of CA and mainly RA were found. The enzymatic cleavage of the conjugates allows the determination of DHCA, DHFA, and FA, which concentrations were under LOQ in plasma without the treatment with β-glucuronidase/arylsulfatase cocktail.

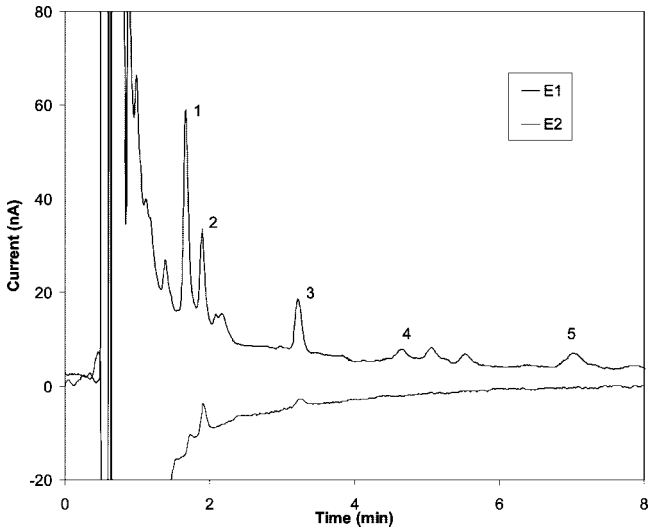

Figure 6. HPLC analysis of pig plasma (representative sample) after enzymatic hydrolysis (pH 3.8). Description: 1 = dihydrocaffeic acid, 2 = caffeic acid, 3 = dihydroferulic acid, 4 = ferulic acid, 5 = rosmarinic acid. Working electrode potentials: first channel E1 = +250 mV (vs Pd), second channel E2 = 0 mV (vs Pd, output polarity reversed). For other conditions see Experimental Procedures.

Table 5. Phenolic Acids Found in Pig Plasma after 1 h Enzymatic Hydrolysis[a]

	c (μg/L)	
compound	enzymatic hydrolysis at pH 3.8	enzymatic hydrolysis at pH 6.2
DHCA	47.4 ± 6.1	16.8 ± 1.7
CA	29.0 ± 2.7	19.6 ± 2.0
DHFA	18.6 ± 2.2	3.7 ± 0.8
FA	7.0 ± 1.2	4.1 ± 1.1
RA	32.0 ± 3.3	124.7 ± 19.3

[a] Results are presented as mean ± SD; all analyses were carried out in triplicates of each animal of experimental group.

On the basis of the above data, the following conclusions can be drawn: A sensitive isocratic HPLC/ECD method suitable for the determination of RA in plant material, feed, and pig plasma was developed. Detection limits of individual studied phenolic acids are in a nanomolar concentration range. The preseparation steps carried out with plasma samples consist only in protein precipitation and adjustment of the pH.

The applicability of the method was tested on plasma samples of pigs exposed to a 3-month oral intake of RA via enriched feed. When no hydrolysis step was included, RA and CA were found and quantified in plasma. In hydrolyzed plasma samples, several other metabolites were determined, including DHCA, FA, and DHFA. The employed dual-channel coulometric detection, as an alternative to mass spectrometry, offers good selectivity and sensitivity owing to the electrochemical properties of phenolic constituents.

A minimum number of preseparation steps and relatively short analyses result in only negligible impact of the whole treatment procedure on the real state in the sample.

The method allows determination of phenolic acids in standard feed. Even if phenolic acids in plasma were below the LOQ of the method for the control pig group fed normal (nonsupplemented) feed, the method can be applied also for the more detailed studies of phenolic acid metabolism.

ABBREVIATIONS USED

ECD, electrochemical detection; HDV, hydrodynamic voltammogram; HPLC-ECD, high-performance liquid chromatography coupled with electrochemical detection; LC-MS, high-performance liquid chromatography coupled with mass spectrometry; MeOH, methanol; Pd, palladium reference electrode; RA, rosmarinic acid; CA, caffeic acid; FA, ferulic acid; DHCA, dihydrocaffeic acid; DHFA, dihydroferulic acid; 9 TCA, trichloroacetic acid; TFA, trifluoroacetic acid; LOD, limit of detection; LOQ, limit of quantification.

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Received for review March 27, 2007. Revised manuscript received July 2, 2007. Accepted July 13, 2007. This work was supported by the Ministry of Education Youth and Sports of the Czech Republic, Grant MSM 6198959216.

JF070883E