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An automated method for the analysis of phenolic acids in plasma based on ion-pairing micro-extraction coupled on-line to gas chromatography/mass spectrometry with in-liner derivatisation

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ABSTRACT

A new method is presented for the analysis of phenolic acids in plasma based on ion-pairing 'Micro-extraction in packed sorbent' (MEPS) coupled on-line to in-liner derivatisation-gas chromatography–mass spectrometry (GC–MS). The ion-pairing reagent served a dual purpose. It was used both to improve extraction yields of the more polar analytes and as the methyl donor in the automated in-liner derivatisation method. In this way, a fully automated procedure for the extraction, derivatisation and injection of a wide range of phenolic acids in plasma samples has been obtained. An extensive optimisation of the extraction and derivatisation procedure has been performed. The entire method showed excellent repeatabilities of under 10% and linearities of 0.99 or better for all phenolic acids. The limits of detection of the optimised method for the majority of phenolic acids were 10 ng/mL or lower with three phenolic acids having less-favourable detection limits of around 100 ng/mL. Finally, the newly developed method has been applied in a human intervention trial in which the bioavailability of polyphenols from wine and tea was studied. Forty plasma samples could be analysed within 24 h in a fully automated method including sample extraction, derivatisation and gas chromatographic analysis.

1. Introduction

Metabolic profiling and metabolomics are rapidly gaining importance in pharmaceutical and nutritional intervention studies. Metabolomics is the comprehensive study of the metabolome, i.e. it involves the comprehensive identification and quantification of all metabolites present in biological systems such as plants, animals or humans. When gas chromatography (GC) is used as the analytical method, the metabolic fingerprint includes small molecules only. These molecules are usually analysed in complex matrices such as plasma, urine or faeces. GC fingerprints offer an unsurpassed peak capacity and sensitivity allowing the analysis of thousands of compounds at good detection limits. Although GC systems are very robust, sample preparation is essential. When body fluids are to be analysed by GC, large-molecular weight compounds need to be removed before analysis and many compounds of interest require a prior derivatisation step. These steps are often offline, labour-intensive and require the use of high amounts of solvents. Key aspects bringing sample preparation forward are therefore automation and miniaturisation.

Automation of sample preparation has been progressed substantially with the introduction of solid-phase microextraction (SPME) [1] and robotic solid-phase extraction (SPE) systems. SPME and GC are nowadays routinely coupled with or without (prior) derivatisation. While SPME possesses some advantages over traditional sample preparation methods such as low or no solvent consumption and the relative ease of online coupling to chromatographic systems, it also has some major disadvantages, mainly related to the lack of coatings that allow the adsorption of polar compounds [2]. Coupling robotic SPE systems with GC is more complicated. The amount and nature of the extraction effluent is usually not compatible with GC and most analytes require a derivatisation step in order to make them amendable for GC analysis. 'Micro-extraction in packed sorbent' (MEPS) [3] is a relatively new miniaturised SPE method that has been shown to be an excellent tool to automate sample preparation protocols. MEPS does not require (expensive) robotic systems as it utilises the 'normal' syringe of the auto-sampler of the chromatographic system. The sorbent material is inserted into the syringe needle and the sample extraction is performed by pulling and pushing the plunger up

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and down. In that way, the analyte solution is taken up and the analytes are adsorbed onto the packing material. The whole extraction procedure can be programmed via the auto-sampler software. Methods coupling MEPS on-line with GC(–MS) have been published, but the focus is on the analysis of compounds that do not require any derivatisation prior to GC analysis [e.g. 4–6].

In this work, the focus was laid on the analysis of phenolic acids in plasma. Phenolic acids can be found at wide concentration levels in the plasma and need to be analysed in the presence of other, highly abundant compounds. For their analysis, we utilised ion-pairing MEPS–GC and introduced an online derivatisation procedure based on 'thermally assisted hydrolysis and methylation' (THM). As shown by Kaal et al. automated on-line derivatisation of organic acids can be performed using tetramethylammonium hydroxide in a packed liner of a PTV injector without any modifications of the GC-system [7]. Realising that typical ion-pairing reagents are the same compounds as those used in THM derivatisation, i.e. tetra-alkylammonium hydroxides, the use of these compounds for the dual purpose of improved extraction of polar analytes by ion-pairing and methyl donation during THM was studied. In this way the whole analysis including the sample preparation could be performed in one single step without any operator intervention. Besides minimising human errors and possible sample losses, automating the extraction and derivatisation procedure significantly increased the analysis speed, thereby reducing analysis time and costs. An additional advantage, especially for large-scale experiments, is that samples were derivatised just before the injection. Because many derivatised compounds were unstable, this reduced the risk of degradation of the derivatives during storage in the auto-sampler.

The optimised MEPS–THM–GC procedure was tested on the analysis of phenolic acids in samples from a human intervention trial that focussed on the bioavailability of polyphenols in wine and tea extracts.

2. Experimental

2.1. Samples and materials

Tetrabutylammonium hydroxide (TBAH), β-glucuronidase containing ~10% sulfatase activity from Helix pomatia Type H-5 as well as all phenolic acid standards as mentioned in Table 1 were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands). Commercial human plasma was purchased from Innovative Research (Novi, Michigan, US). Methanol, sodium acetate, ethylenediaminetetraacetic acid (EDTA) and hydrochloric acid were purchased from Merck (VWR International, Amsterdam, The Netherlands).

Table 1
Phenolic acid standards used for method development, together with the retention times and their target ion.

	Ret. time [min]	Target ion [Da]
2,6-Dimethoxybenzoic acid	15.32	165
3-Hydroxybenzoic acid	16.45	138
3-Hydroxyphenylacetatic acid	16.86	152
4-Hydroxyphenylpropionic acid	18.44	278
Homovanillic acid	18.79	238
m-Coumaric acid	19.72	164
4-Hydroxy-3-methoxy phenylpropionic acid	19.81	308
3,4-Dihydroxybenzoic acid	20.69	154
3,4-Dihydroxyphenylpropionic acid	21.45	350
3-o-Methylgallic acid	21.49	184
Ferulic acid	21.64	194
4-o-Methylgallic acid	21.66	184
Gallic acid	23.05	170

For the human intervention trial, plasma samples of thirty male volunteers were collected at 0 h (baseline) and at nine time points following the intervention (placebo, wine or tea). A full cross-over design was applied and the samples were pooled for each sampling time point. For more details on the study set-up, see van Velzen et al. [8].

2.2. Sample preparation

A phenolic acid stock solution was prepared in methanol at a concentration of approximately 25 μg/mL per compound. The compound list is shown in Table 1. This stock solution was diluted with a 50% methanol/water solution to result in concentrations as described later in the respective paragraphs.

100 μL plasma was stabilised with sodium acetate containing 0.1% EDTA. The sample was then acidified with hydrochloric acid (pH < 2) and milli-Q water containing 3% TBAH was added to result in a final volume of 400 μL. Of this solution, 100 μL was transferred into a GC-vial containing an insert and stored in the auto-sampler. For the spiking experiments, 8 μL of the phenolic acid standard solution were added to the mixture and the amount of water added was decreased in order to maintain a final volume of 400 μL.

The samples of the human intervention trial were prepared in two ways: (i) as described above and (ii) including an enzymatic reaction step. Hereby, 100 μL plasma was stabilised with sodium acetate containing 0.1% EDTA. The sample was then acidified with hydrochloric acid (pH < 2), 8 μL β-glucuronidase (1500 units in 150 mM sodium acetate) was added and milli-Q water containing 3% TBAH was added to result in a final volume of 400 μL. The sample solution was then incubated at 37 °C for 45 min, after which 100 μL was transferred to a GC-vial.

2.3. GC–MS analysis

All GC–MS analyses were performed on a Shimadzu GC–MS-QP2010 (Den Bosch, The Netherlands). The GC system was equipped with an Optic-3 PTV injector (ATAS GL, Eindhoven, The Netherlands) and a "Focus" XYZ robotic auto-sampler (ATAS GL). All analyses were performed using a sintered-bed liner (ATAS GL). The column was a VF-17 ms (30 m × 0.25 mm, df = 0.1 μm) obtained from Varian (Varian, Middelburg, The Netherlands) and the column flow was constant at 1.2 mL/min with helium as the carrier gas. The GC analysis was performed in 35 min with a starting oven-temperature of 70 °C (hold time 5 min) and a single ramp of 10 °C/min to 320 °C (hold time 5 min).

Full-scan mass spectra were recorded in the mass window from 60 to 800 Da in the electron-impact (EI) mode at 70 eV. The MS source and the GC–MS interface were kept at 200 °C and 280 °C, respectively.

2.4. Extraction and injection parameter

MEPS was performed using a 250 μL syringe from SGE Analytical (Victoria, Australia) and a C18 sorbent (ATAS GL). The sorbent bed was conditioned with two times 100 μL methanol and milli-Q water each before every extraction. Details on the MEPS extraction, the derivatisation and the injection method are presented in Section 3.

3. Results and discussion

The first step in the optimisation of analytical methods is the definition of the requirements that the method should meet. In our case, the focus was on the analysis of phenolic acids in plasma. However, to also obtain comprehensive information on the samples, full-scan GC–MS spectra were recorded. The phenolic acids

should be measured at ng/mL-levels and all different kind of phenolic acids should be included, ranging from the very polar to the far less polar extremes. Additionally, due to the limited availability of plasma in human intervention trials, the method should require a maximum of 100 μL sample volume. Finally, the method should be fully automated from extraction to derivatisation and injection to the GC–MS system, allowing the measurement of large sample series without the risk of sample instability and with the benefit of minimising human errors and analyst time.

3.1. Analysis of phenolic acids in plasma

3.1.1. Injection and derivatisation of phenolic acids

The automated derivatisation procedure consisted of several steps: Injection of the sample mixture including the derivatisation reagent at low temperatures into the PTV injector, followed by drying and incubation of the injected sample inside the liner at an elevated temperature; and finally, the actual derivatisation of the sample including the transfer of the derivatives to the GC column at a higher PTV temperature. Automating the entire procedure of derivatisation and injection was possible using the Focus auto-sampler and an OPTIC 3 PTV injector [7,9].

In a series of model experiments, the derivatisation of the phenolic acids was optimised with respect to the derivatisation reagent. N,O-*bis*[trimethylsilyl]trifluoroacetamide (BSTFA) is commonly used as a derivatisation reagent for phenolic acids. However, when using BSTFA, the sample has to be absolutely free of water; something very difficult to achieve in an on-line extraction method. In the on-line derivatisation methods published by Kaal et al., tetramethylammonium hydroxide (TMAH), a reagent forming methylated derivatives from acids and alcohols was used. However, in our case, TMAH cannot be used because some of the phenolic acids occur naturally in their methylated form. Therefore, when using TMAH, no difference can be seen between the naturally methylated derivatives and the methylated products of the derivatisation reaction, resulting in a loss of information. For that reason, we selected tetrabutylammonium hydroxide (TBAH) as the reagent; a comparable derivatisation reagent that will yield butylated derivatives. In literature, TBAH has been used in studies of wood [10] or lignins [11]. To our knowledge, it has so far not been used as a derivatisation reagent for the analysis of phenolic compounds in plasma. One of the disadvantages of using TBAH is the lack of library spectra, which are abundantly recorded for silylated phenolics and, to a lesser extent, methylated phenolics.

A key factor in PTV-automated TMAH or TBAH derivatisation is the temperature at which the sample/reagent mixture is injected. This temperature must be high enough to evaporate the solvent while avoiding extensive reagent loss. The influence of the concentration of TBAH on the derivatisation yield was tested for a test mixture containing five phenolic acids (see Fig. 1). The black bars correspond to a low concentration of TBAH (0.5%) and the striped bars correspond to a high concentration (2%). Clearly, the derivatisation yield was strongly dependent on the concentration of the reagent which hence needed to be optimised. For our work, a concentration of 1% TBAH in methanol was found to be optimum. If the concentration was too high too much reagent enters the GC column resulting in a loss of chromatographic performance (extremely broad reagent peak covering a large range of the chromatograms and the deterioration of peak shapes).

As expected, besides the concentration of the reagent, we have seen that the incubation time also had a strong effect on the derivatisation yield. When the incubation time was selected too short (<60 s) the sample was not completely derivatised. For the phenolic acids, an incubation time of 80 s was found to be optimal. The final PTV temperature did not have a strong impact on the

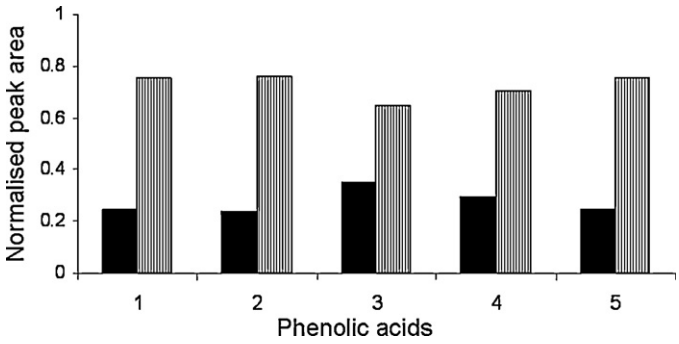

Fig. 1. Peak areas of five phenolic acids, normalised to the total sum: (1) phenylpropionic acid, (2) 4-hydroxybenzoic acid, (3) 2-hydroxy-5-methoxybenzoic acid, (4) dihydroxybenzoic acid and (5) 4-o-methylgallic acid. The black bars correspond to a low concentration of TBAH in methanol (0.5%) and the striped bars to a high concentration of TBAH (2%).

derivatisation yield. When varied between 350 °C and 450 °C, no clear effect could be observed (data not shown).

After optimisation of all parameters the following injector settings were found to be optimal for the compounds listed in Table 1: Initial injection temperature of 40 °C followed by an immediate increase to 70 °C with a ramp rate of 10 °C/s (hold time of 80 s), then an increase to 400 °C at a ramp rate of 30 °C/s (300 s), followed by a decrease to 350 °C until the end of the GC analysis time. The split flow of the injector was high (150 mL/min) during the injection and drying of the sample and low (5 mL/min) during the incubation time. A split of 1:5 was applied during transfer of the derivatives to the GC column.

Fig. 2 gives an example of a mass spectrum and chromatogram of a fully butylated compound, 3-hydroxyphenylacetic acid. The molecular mass of non-butylated 3-hydroxyphenylacetic acid is 152 Da, which is the main fragment in this spectrum (Fig. 2a). The peaks at *m*/*z* 264 and 208 of the butylated compound can be attributed to the addition of butyl groups, each of 56 Da. This pattern is very typical for all spectra and the number of additions can aid in the identification of unknown compounds. When investigating the chromatogram at extracted ions of 152 Da, 208 Da and 264 Da (Fig. 2b) it can be seen that the compound is fully derivatised: only one peak could be found in the chromatogram and this peak contains all three ions. Mono-butylated 3-hydroxyphenylacetic acid would elute earlier than the fully derivatised compound and have an equal spectrum except that it will lack the mass peak at 264 Da. This peak was not present in the chromatograms.

3.1.2. Optimisation of the MEPS extraction procedure

The second step towards a fully automated method was the optimisation of the MEPS-procedure of the analytes from plasma. As with all SPE procedures, a MEPS method consists of several steps: activation of the cartridge, loading of the sample onto the cartridge, washing of the sorbent bed to remove interferences, elution of the compounds of interest and cleaning the sorbent including re-conditioning for the next analysis. All these steps have to be optimised. One of the first difficulties encountered was that the more polar compounds did not have enough interaction with the C18 sorbent bed of the MEPS needle. Because of the wide diversity of the target compounds taking a more polar packing material was not an option. Therefore, we considered improving the interaction of the sorbent with the polar compounds by the use of an ion-pairing reagent. TBAH, our derivatisation reagent, is also known to act as an ion-pairing reagent. It was therefore obvious to use it as both, the ion-pairing reagent during the loading step and as the derivatisation reagent for the THM reaction. For this reason, TBAH

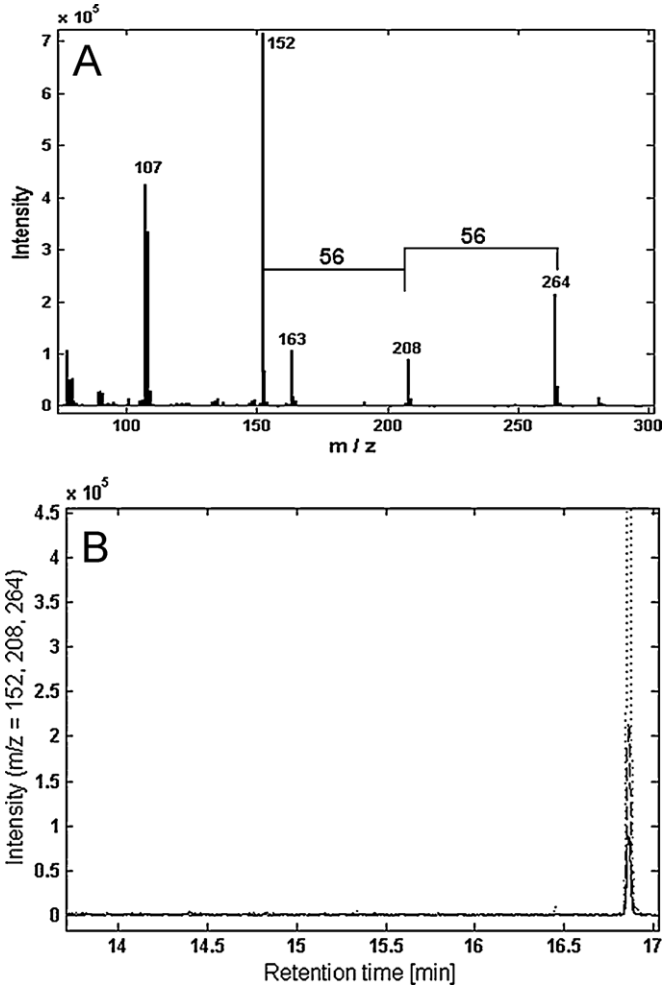

Fig. 2. The mass spectrum obtained for 3-hydroxyphenylacetic acid after butylation (a) and its three extracted ion chromatograms overlaid (b). The solid line corresponds to 152 Da, the dotted line to 264 Da and the dashed line to 208 Da.

was added to the sample solutions prior to the MEPS extraction. Fig. 3 shows the effect of various concentrations of TBAH in the sample on the peak areas of a selected set of compounds. Note that the concentration of TBAH in the elution solvent (methanol) was kept constant.

As expected, the more polar compounds (grey lines in Fig. 3) experienced a larger increase in recoveries with increased TBAH concentration (e.g. the more polar gallic acid experienced an increase of 84%), whereas less polar compounds (black lines in Fig. 3) such as 4-hydroxyphenylpropionic acid only increased with 11%, when comparing no addition of TBAH with the addition of 3% TBAH. Note that not all compounds of comparable polarity benefited in the same way from the addition and the ion-pairing step needed to be adjusted carefully according to the compounds of interest. In our case, we have decided to add 3% TBAH to the samples prior to the MEPS extraction.

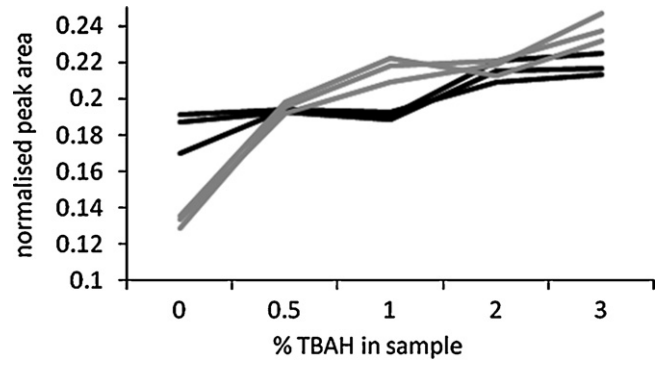

Fig. 3. The peak areas, normalised to the total sum, of six phenolic acids plotted together for various concentrations of TBAH in the sample. Grey lines: 3,4-dihydroxyphenylpropionic acid, gallic acid, 3,4-dihydroxybenzoic acid; black lines: 3-hydroxybenzoic acid, 3-hydroxyphenylacetic acid and 4-hydroxyphenylpropionic acid.

In order to obtain the lowest detection limits, the maximum sample amount of 100 μL plasma was used in all experiments. MEPS sampling was performed by pumping up and down the extract five times. This number was found to be optimal for the extraction of phenolic acids. After loading, the sorbent was washed with 100 μL acidified water (pH < 2), which satisfactorily removed all interferences. A drying step followed by pumping 100 μL air through the sorbent by pushing the plunger up and down.

The maximum injection volume for the in-liner derivatisation method and therefore the maximum elution volume for MEPS has been earlier determined to be 40 μL [11] (without further modifications of the system). When eluting the analytes with 25 μL we did not achieve acceptable recoveries for most compounds (only 5%). Increasing the elution volume to 40 μL also did not result in acceptable recoveries. One explanation for this effect lies in the MEPS-set-up itself. In principle, molecules are eluted from the sorbent by taking up the elution solvent (methanol) with the plunger and then pushing the methanol including the analytes back out of the BIN into the injector. The BIN ("Barrel Insert and Needle assembly") is that part of the MEPS needle that contains the extraction material. However, when taking up the methanol, most molecules are located at the top of the solvent layer and due to the dead volume of the BIN they remain in the syringe when eluting the solvent. This problem could easily be tackled by including an extra air step in the elution process: after taking up the methanol, the syringe was taken out of the solvent reservoir and the plunger was pulled up an additional 20%. This created an extra mixing effect of the methanol and the analytes, resulting in much higher recoveries. In our case, the average increase of response was eight-fold. The dead volume of the BIN, of course, is inherent of the system and cannot be changed. Taking this knowledge into account, we developed a new multiple elution/injection-technique that improved the recoveries significantly. In this multiple elution/injection-step, the analytes were eluted several times using the same volume (25 μL and later 35 μL) of methanol. After the first elution and injection, the injected extract was dried in the PTV liner at low temperature. In the meanwhile, the (remaining) analytes in the sorbent were again eluted with 25 μL (or later 35 μL) methanol and injected at the same temperature. This process was repeated up to three times. Using the combination of the PTV-injector and the XYZ robotic auto-sampler these multiple injections are easy to perform. Fig. 4 shows the recoveries of four phenolic acids when the elution is changed from a single elution step (1× 25 μL), to double elution (2× 25 μL), triple elution (3× 25 μL) and a triple elution of three times 35 μL. It could be seen that the triple elution step with 35 μL elution volume was optimum for all analytes. We did not continue with more elution steps as the recoveries were now increased to an acceptable level of >80% for all compounds.

A beneficial side effect of the multiple elution steps was that it also significantly decreased carry-over effects that were observed after the extraction of plasma spiked with very high concentrations of the phenolic compounds (>2000 ng/mL). Nevertheless, in order to decrease carry-over even further, an extra washing step with iso-propyl alcohol was included after the extraction. The carry-over values were now acceptable for most compounds (lower than 0.7%); however, some of the compounds still showed a very high carry-over (20%). As we did not observe this high carry-over at

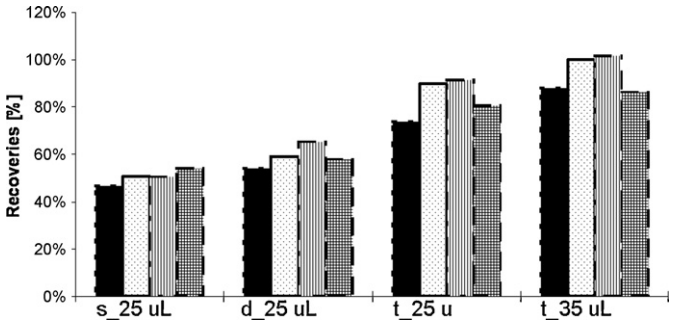

Fig. 4. Comparison of recoveries of four phenolic acids (3-hydroxybenzoic acid -solid-, 3-hydroxy-4-methoxyphenylpropionic acid -dotted-, 3-hydroxyphenylacetic acid -striped- and 4-o-methylgallic acid -squared-) resulting of a single elution/injection (s_25 μL), a double elution/injection (d_25 μL), a triple elution/injection (t_25 μL) and a triple elution/injection of 35 μL (t_35 μL).

expected plasma levels, we did not pursuit further optimisation of the washing steps.

3.1.3. Sensitivity, linearity and repeatability of the new method

One of the method requirements was to achieve a high sensitivity for all phenolic acids in plasma, down to the ng/mL-level. The sensitivity and linearity of the method were tested using the phenolic acid standard mixture spiked to plasma. Most of the phenolic acids gave detection limits of 10 ng/mL or lower. Some compounds, mainly the derivatives of benzoic acid however, had up to 100 ng/mL detection limits. For our experiments, these detection limits met the method requirements. Further optimisation steps can be envisaged if lower detection limits are needed.

The linearity of all compounds was 0.99 or better in the range from the LOD to 5000 ng/mL. This is a very wide linear range, which is especially useful in applications such as metabolomic studies, in which compounds can be present in a wide concentration range.

The repeatability of the whole, fully automated method, including extraction and derivatisation, was excellent with RSD's below 10% for six injections. A detailed table of all detection limits and repeatability's is given in Table 1 of the supplemental material.

With respect to the re-usability of the sorbent material, all optimisation experiments (>300) were performed without any deterioration effects. However, for some compounds, especially when higher concentrations were measured, carry-over can be a problem and must be thoroughly investigated.

3.2. Application of the optimised method to a human intervention trial

The ultimate test of the newly developed fully automated method is its application to a real sample set. In this study, 30 volunteers experienced three single-dose interventions (placebo, wine, tea) and plasma was taken at baseline and at nine time points after the intervention. Afterwards, the samples were pooled per sampling time point and intervention, resulting in a sample set of 30 samples. These samples were prepared as described in Section 2.2 and analysed by the automated ion-pairing MEPS–GC–MS method combined with in-liner derivatisation. The sample preparation time was 20 min and the GC analysis time was 35 min. The whole automated triple extraction/injection procedure was rather long (around 8 min), but it could already be started during the GC analysis of the previous sample. QC samples were used to verify the stability of the instrument throughout the whole series. Commercial plasma was spiked with the phenolic acids mixture (see Table 1) resulting in a plasma concentration of 1000 ng/mL. The average repeatability of absolute peak areas for these phenolic acids was 15%. Note that these values included sample preparation, extraction, derivatisation and injection of the sample, i.e. the whole analytical variability and were obtained without correction with an internal standard. Detailed repeatability's of all phenolic acids can be found in Table 2, supplemental material. The repeatability's found here are slightly higher than those found during method development. Method development did not include the effect of different plasma samples, which may explain the difference found here. The plasma study samples may have contained extra compounds that influenced the analytical procedure. As a repeatability of 15% was still very acceptable we did not investigate this effect further.

Table 2
Tentative assignment of compounds found in the plasma sample at sampling time point zero, following the placebo intervention.

Compound ID[a]	Ret. time [min]
Tributylphosphate	13.81
Reagent	14.43
Lauric acid	15.73
Reagent	16.19
Unknown	16.83
Unknown fatty acid	17.39
Myristic acid	18.03
Unknown fatty acid	19.17
Unknown fatty acid	19.78
Palmitic acid	20.04
Unknown	20.70
9-E-oleic acid	21.25
Stearic acid	21.76
Unknown fatty acid	21.90
Unknown fatty acid	21.99
Unknown fatty acid	22.29
Unknown fatty acid	22.43
Cholesta-3,5-diene	25.44
EDTA	26.51
Cholesterol	26.98
Cholest-4-en-3-one	27.76
Unknown	28.28

[a] Tentative assignment.

In the human intervention study presented here, the bioavailability of tea and wine polyphenols was to be studied. Polyphenols are taken up in the body where they are further metabolised to form smaller phenolic acids. Hence, their presence in plasma is an indicator for the bioavailability of polyphenols in tea and wine. While it has been suggested that phenolic acids in plasma are present in their conjugated forms only [12], we wanted to measure both forms, i.e. measure plasma samples with and without the addition of deconjugation enzymes. This was to additionally investigate changes in the plasma in other than the polyphenolic compounds. As expected, we could not detect any phenolic acids in plasma without the deconjugation step. That means that they are indeed not present in their free form or that their concentration is below the limit of detection of the method. Fig. 5 shows the chromatogram of the deconjugated sample after 24 h (tea intervention). Here, some phenolic acids could be identified. These are marked and numbered in the figure: 3-hydroxybenzoic acid (1), mandelic acid (2), homovanillic acid (3), syringic acid (4), 4-hydroxy-3-methoxyphenylpropionic acid (5), 4-o-methylgallic acid (6), ferulic acid (7) and caffeic acid (8). Clearly, apart from syringic acid, phenolic acids were not the main compounds found in the GC–MS profile. As can be seen in Table 2, the total-ion profile was dominated by fatty acids and cholesterol-derivatives (putative assignments). It is needless to say that much more information is present in the data and advanced data-analytical methods are needed to extract that information from this complex sample set.

As sampling was performed at several time points, the time profiles were further investigated. Fig. 6 shows the concentration

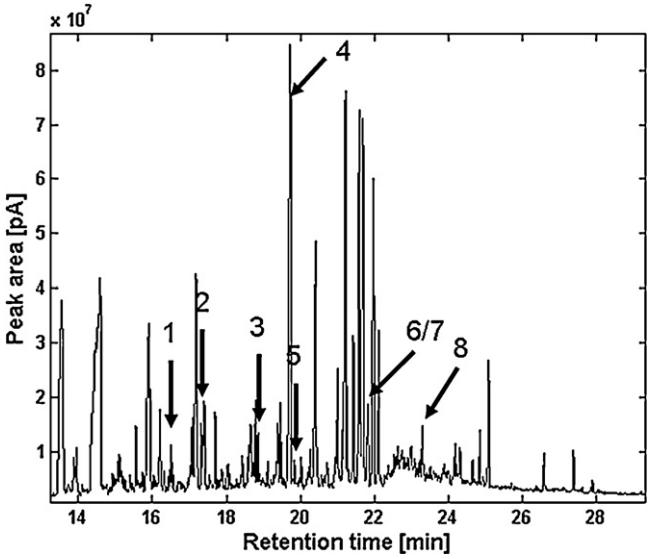

Fig. 5. Total-ion chromatogram of a plasma sample of the tea intervention after enzymatic reaction at time point 24 h.

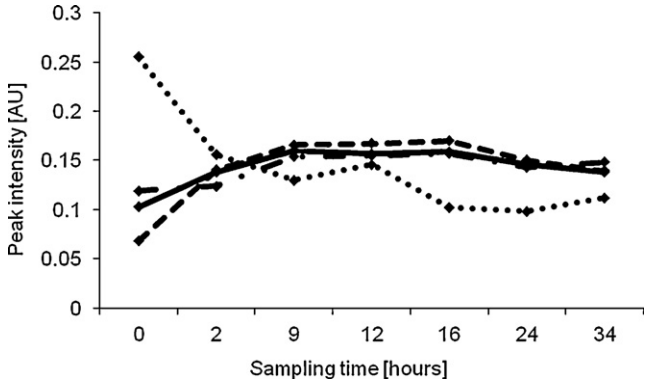

Fig. 6. Concentration profiles obtained for four phenolic acids, following the tea intervention: benzoic acid (dash-dotted line), mandelic acid (solid line), 4-hydroxy-3-methoxyphenylpropionic acid (dashed line) and 4-o-methylgallic acid (dotted line).

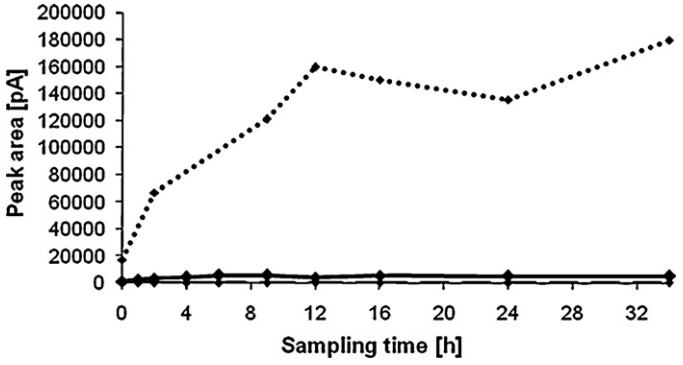

Fig. 7. Concentration profiles of an unknown compound (ret. time = 27.82 min, 514 Da). The solid line corresponds to the concentration profile after placebo intervention, the dotted and dashed line following the tea and wine intervention, respectively.

profiles of four phenolic acids (see caption). While some phenolic acids were formed following the intervention, others decreased, meaning that they were (further) metabolised in the body. Fig. 7 shows an example of the time profiles of all interventions, of an unknown compound eluting at 27.82 min. This compound is increasing with sampling time following the tea intervention, meaning it must be formed by the body throughout metabolism and its source must be the tea itself. No effect can be observed for the wine intervention or the placebo. A further biological interpretation of all effects is not within the scope of this article and was not performed at this point.

4. Conclusions

A new fully automated method for the analysis of plasma has been presented based on 'micro-extraction in a packed sorbent' (MEPS) coupled on-line to in-liner derivatisation-gas chromatography–mass spectrometry (GC–MS). TBAH was used as the combined ion pairing/derivatisation reagent.

The optimised method resulted in a good repeatability and sensitivity and allowed the analysis of all compounds of interest in a very wide linear range (greater than two orders of magnitude). The newly developed method was capable of analysing compounds at low levels even in the presence of other compounds present at high concentrations. This has been shown when applying the method to a human intervention study. 40 samples were analysed using one MEPS BIN without seeing any deterioration of the sorbent material. The samples were analysed within 24 h without any human intervention thereby minimising expensive time of the analyst as well as human errors. After deconjugation, (low-concentrated) phenolic acids could be determined in plasma in the presence of highly abundant compounds of similar chemistry (e.g. fatty acids).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.10.055.

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